CHITOSAN COAGULATION FOR HOUSEHOLD WATER TREATMENT
IN DEVELOPING COUNTRIES

Ampai Soros

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Approved by:
Mark Sobsey
Lisa Casanova
Louise Ball
Jill Stewart
Orlando Coronell
ABSTRACT

Ampai Soros: Chitosan Coagulation for Household Water Treatment in Developing Countries
(Under the direction of Mark Sobsey and Lisa Casanova)

Providing safe drinking water for underserved populations is still a challenge. Almost 800 million people around the world consume untreated or improperly treated water. Household water treatment is a critical and immediate intervention to provide safe drinking water where there are no improved sources of drinking water or water treatment systems do not function properly. The purpose of this research was to determine the efficacy of chitosan as a novel coagulant for water treatment in the home and determine whether characteristics of chitosan, especially molecular weight and degree of deacetylation, influence the performance of chitosan for removal of turbidity and microbes (bacteria and viruses) from water. Effects of water quality parameters such as pH, turbidity and salinity on chitosan coagulation efficacy were also evaluated. Zeta potential during coagulation and antimicrobial effects of chitosan in water were examined to give insight into potential mechanisms of chitosan action. The jar test method was used to evaluate the effects of chitosan characteristics on removal of turbidity and representative microbes (E. coli and bacteriophage MS2) from water.

Overall, this research suggests that chitosan is an effective natural coagulant to use for household water treatment. Chitosan efficiently removed kaolinite or bentonite turbidity at low optimum chitosan dose of 3 mg/L. It also exhibited 3 - 5 log_{10} removal of E. coli bacteria and bacteriophage MS2 at chitosan doses of 3-10 mg/L. The chief mechanisms of turbidity and
microbial removal by chitosan include interparticle bridging and charge neutralization. The antimicrobial activity of chitosan appeared to play a minor role in microbial removal. Raw water qualities such as pH, salinity and turbidity had minimal effects on chitosan’s efficacy. Chitosans with intermediate molecular weight and high degree of deacetylation are the best candidates for turbidity and microbial removal, and there is a range of effective doses; chitosans exhibit high removal of turbidity and microbes, including bacteria and viruses, across a range of raw water qualities.
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CFU/ml  Colony forming unit/milliliter
CLSI  Clinical and Laboratory Standards Institute
Da  Dalton unit
DD  Degree of deacetylation
DOC  Dissolved organic carbon
EPA  Environmental Protection Agency
meq/g  Milliequivalents per gram (meq/g)
mg/L  Milligram per liter
mPa·s  Millipascal-second
MW  Molecular weight
NTU  Nephelometric Turbidity Unit
PFU/ml  Plague forming unit/milliliter
ppt  Part per thousand
rpm  Revolutions per minute
<table>
<thead>
<tr>
<th>Acronym</th>
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<tbody>
<tr>
<td>TDS</td>
<td>Total dissolved solid</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic Soy Broth</td>
</tr>
<tr>
<td>UNICEF</td>
<td>United Nations International Children’s Emergency Fund</td>
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1.1 Background and Significance

Access to safe drinking water

Safe drinking water is a major concern in developing countries. Almost 800 million people around the world lack access to improved sources of drinking water and 84% of this population lives in developing countries. Each year, there are nearly 2 million diarrheal deaths related to unsafe water and sanitation; the majority of them are children age under 5 years in the developing regions. Currently, 91% of the world’s population, approximately 6.6 billion people, uses improved sources of drinking water (WHO, UNICEF, 2015). Improved sources of drinking water are defined as types of technology and levels of services that are more likely to provide safe water than unimproved technologies. Improved sources include household connections, public standpipes, boreholes, protected dug wells, protected springs, and rainwater harvesting. Other sources, including unprotected wells, unprotected springs, vendor-provided water, bottled water (unless water for other uses is available from an improved source) and tanker truck-provided water are considered unimproved water sources (WHO, 2011). The proportion of people with access to improved water sources is large in developed regions with 99% of the population using improved water resources (96% piped on premises, 3% other improved sources). In developing regions, 89% of the population uses improved sources of drinking water but only 49% are using piped water: 72% in urban and only 28% in rural areas. There is a large gap in improved water access between urban and rural areas in developing regions. While 95%
of the urban population of developing countries uses improved sources of drinking water, only 83% of the rural population does. The rural population without access to an improved source of drinking water is 4 times greater than the urban population (WHO, UNICEF, 2015). However, a large proportion of the improved water sources are microbiologically contaminated. Studies in Cambodia and Vietnam illustrated contamination of *Escherichia coli* in the improved water sources such as piped water and stored rain water and *E. coli* found in these drinking water sources ranged from 1 to > $10^3$ CFU/100 ml (Shaheed et al, 2014). Similarly, Onda et al (2012) evaluated the safety of drinking water in 5 counties namely Ethiopia, Jordan, Nicaragua, Nigeria and Tajikistan and the authors reported contamination of thermotolerant coliform bacteria in drinking water from the improved water sourced. The author also estimated that of 5.8 billion people with the improved sources of drinking water in 2010; there were approximately 1 billion people with the improved sources that were possibly microbiologically unsafe.

**Water status of Thailand**

In Thailand, the WHO/UNICEF Joint Monitoring Programme for water supply and sanitation (JMP) (2013) reported that 96% of the population uses improved sources of drinking water: 48% use piped water and 48% use other improved sources such as protected dug wells and rainwater. The proportions of urban and rural populations that use improved sources of drinking water are similar, 97% and 95% respectively. However, the use of piped water in urban areas is over 2 times the use in rural areas, with 80% of urban residents and only 31% of rural residents using piped water. The rural population in Thailand, last reported in 2010, was 66% of total population (World Bank, 2013). Although many rural households have piped water on their premises (at least one tap connected into user’s dwelling, ground or plot), the quality of water and level of water treatment vary according to water technicians’ skill and budget for
construction and maintenance. In rural Thailand, 64% of the population uses other improved water resources including rainwater. Rainwater harvesting in Thailand has been commonly practiced for thousands of years and Thai people widely use rainwater for drinking purposes without any treatment. A survey by the Ministry of Public Health in 1996 showed that rainwater was still an important source of drinking water, used by about 44% of people around the country, 55% in regional and rural area, but only 7% in Bangkok (capital of Thailand). Only limited studies on quality of rainwater have been carried out in Thailand. Pinfold et al (1993) reported that rainwater collected from roofs and stored in household storage containers was a low risk source, with approximately 5 fecal coliforms (FC) per 100 ml among the samples tested compared to water from shallow wells which was at intermediate risk with approximately 30 fecal coliforms (FC) per 100 ml, according to WHO guidelines for *E. coli* in drinking water. The study also showed contamination from enterococci in both rainwater and in shallow wells. Wirojanagud et al (1989) studied stored rainwater quality in Northeast Thailand and found that rainwater was contaminated with bacteria; only 40% of 189 rainwater samples from household storage containers met the WHO drinking water standards. Bacterial species found in this study included *Salmonella* group E, *Aeromonas sp.*, *Salmonella* group C, and *Vibrio parahaemolyticus*. Appan (1997) studied rainwater quality in Southeast Asia including Thailand, and concluded that more than 76% of samples exceeded the WHO standards for *E. coli* (see Appendix 1), and approximately 79-82% of microbial contamination could come from animal droppings on roof catchments that were washed into rain jars. Quality of drinking water in rural areas around Thailand had been surveyed by the Department of Health and Provincial Offices of Public Health during 1997-8 (see Appendix 2). The survey compared quality of different sources of drinking water (i.e. groundwater from deep wells and shallow wells, village pipe water, and stored
rainwater) to the WHO drinking water guidelines. The overall result showed that fecal coliform was one of the major problems; chemical contamination with iron, chloride, nitrate and hardness was also high throughout the country.

By the JMP (Joint Monitoring Program) definition, piped water on premises and rainwater collection are considered improved sources of drinking water. However, studies conducted in rural areas of Thailand have shown that improved sources of drinking water do not always mean safe drinking water, in terms of microbial contamination (Wirojanagud et al, 1989; Pinfold et al, 1993; Appan, 1997). Such microbially contaminated improved drinking waters require further treatment to make them safe. Water treatment and distribution systems are expensive investments and take time, and the systems also require skilled technicians for operation and maintenance. For developing countries and especially in many rural areas, these types of water treatment facilities are unfeasible and unaffordable in the near future. Technologies that are simple, accessible and cost-effective are needed.

**Household water treatment**

Household water treatment (HWT) is a critical intervention now available to help people achieve safe drinking water. There are several technologies, both physical and chemical treatments, to improve the microbial quality of drinking water in developing regions. With point-of-use (POU) technologies, people can treat their drinking water at home, leading to improved quality of household water where it is used. However, there are many POU technologies that cannot produce water at quality that meets the recommended high performance levels for microbial removal. USEPA requires a POU technology to be capable of a 6 log$_{10}$ (99.9999%), 4 log$_{10}$ (99.99%), and 3 log$_{10}$ (99.9%) reduction for bacteria, virus, and parasite removal, respectively (EPA, 1989). WHO has reported that microbial reduction in actual field practice
achieved by a POU technology is generally only half of its maximum log$_{10}$ reduction capability. For example, free chlorine disinfection of bacteria and virus achieves 3 log$_{10}$ reductions in actual field practice, whereas its maximum capacity in well controlled condition is 6 log$_{10}$ reductions. Solar disinfection of bacteria gives 3 log$_{10}$ reductions in actual field practice instead of 5+ log$_{10}$ reduction of its maximum capacity (WHO, 2011). Recently, WHO introduced performance targets for HWT technologies which have been categorized based on log$_{10}$ reduction of bacteria, viruses and protozoa into 3 levels: interim (now called minimum protection), protective and highly protective (WHO, 2011). The interim performance target needs to achieve the protective target for 2 classes of pathogens and results in health gain. The protective target should produce ≥2, ≥3 and ≥2 log$_{10}$ reduction for bacteria, viruses and protozoa, respectively. The highly protective target requires achieving ≥4, ≥5 and ≥4 log$_{10}$ reduction for the same 3 classes of pathogens, respectively. As a single treatment technology may not be able to meet the higher WHO performance targets of highly protective or protective, there are needs for add-on or supplemental technology that can work with and improve the efficacy of existing POU treatments. Ease of use, accessibility and affordable cost are important factors for household water treatment, in order to achieve effective, consistent and long term practice and reduce mishandling or misuse of the technology. One particularly promising POU technology for household water treatment involves coagulation where suspended particles including microbes can be coagulated or precipitated to separate them from water intended for use in the homes.

**Coagulation: a water treatment technology**

Coagulation is a chemical treatment that is widely used in developed country drinking water treatment plants, and has potential to be adapted as a household water treatment. It is an important water treatment process which has been used as early as 2000 B.C. when the
Egyptians used almonds smeared around the interior surfaces of water vessels to clarify river water. Around 77 AD, alum was used as a coagulant by the Romans and by 1757 a municipal water treatment plant in England was using alum for coagulation (IWA, 2011). The coagulation process uses chemical coagulants to destabilize particles in water and stimulate binding of those particles to form clumps that are then easy to remove from the treated water. The conventional coagulants used in water treatment are aluminum sulfate, ferric sulfate, and ferric chloride. These coagulants are widely accepted for their capability in reducing turbidity and particles at low doses. However, these metal salt coagulants depend on the pH of the water and precise doses to produce consistently high microbial removal. These strict performance requirements are limitations of conventional coagulants that make them less suitable for household water treatment, where people need simple but robust and safe methods to treat their water at home.

**Chitosan as a coagulant for water treatment**

Chitosan is a derivative of chitin which naturally occurs in shells of crustaceans, fungi and insects. It is a long chain carbohydrate that is non-soluble in water but dissolves in most acids, and contains positively charged moieties. Possessing properties such as non-toxicity, biocompatibility, and biodegradability, chitosan has been studied for its application in many sectors such as industrial wastewater treatment, pharmaceuticals, cosmetics, agroindustry, agriculture, and biomedical use. Chitosan has been granted Generally Recognized as Safe (GRAS) status from the US-FDA. It is approved for dietary application in many countries around the world such as Japan, Italy, and Finland. US-FDA also approved chitosan as safe for wound healing. Kean and Thanou (2010) reviewed the toxicity of chitosan from current studies and concluded that, in general, chitosan is relatively non-toxic to mammalian cells, biocompatible
and biodegradable. However, the purity of chitosan must be assured to prevent negative effects from impurities (i.e. proteins, metals or ash, including residues of reactants used in chemical modifications). The impurities, especially metals or heavy metals, should be thoroughly removed or should not be present over the allowable safety limit. All these impurities are present in crustaceans by natural uptake from their living environment, so chitosan produced from crustaceans living in highly contaminated environments will be likely to have high levels of impurities. However, there are standards that limit the amount of impurities; Thai industrial standards for chitosan limit the maximum concentration of lead (Pb) at 1 mg/kg, mercury (Hg) at 0.5 mg/kg, and arsenic (As) at 2.0 mg/kg (TISI, 2007).

Chitosan has never been carefully studied as a coagulant for water treatment to remove both microbes (especially bacteria and viruses) and turbidity. Existing literature also has not explored the separate or joint effects of coagulant use and antimicrobial activity for water treatment; coagulant activity research has traditionally focused on water, wastewater and beverage sectors whereas antimicrobial activity research has commonly focused on food, agricultural and medical applications. Research on microbial coagulation using chitosan is limited particularly on virus removal from water; important characteristics of chitosans, and conditions which are critical for microbial removal have not yet been well defined. Chitosan coagulation has the potential to provide a simple, accessible and affordable method for household water treatment especially in developing countries.
1.2 Objectives

1. Evaluate the efficacy of chitosans for the removal of water turbidity by coagulation using the jar test method

   a. Examine the effects of different molecular weights of acid-soluble chitosans on turbidity removal from water,
   b. Examine the effects of degree of deacetylation of acid-soluble chitosans on turbidity removal,
   c. Evaluate chitosan efficacy for turbidity removal at different levels of water pH, salinity, and turbidity.
   d. Compare turbidity removal by acid-soluble chitosans with the removal by water-soluble modified chitosans.

2. Evaluate the efficacy of chitosans for microbial removal by coagulation using the jar test method

   a. Examine the effects of different molecular weights of acid-soluble chitosans on removal of bacteria (*E. coli*) and virus (coliphage MS2),
   b. Examine the effects of degree of deacetylation of acid-soluble chitosans on removal of bacteria (*E. coli*) and virus (coliphage MS2),
   c. Evaluate chitosan efficacy for microbial removal in different levels of water pH, salinity, and turbidity.
   d. Compare microbial removal by acid-soluble chitosans with the removal by water-soluble modified chitosans.
3. Evaluate the antimicrobial activity of chitosan candidates
   
   a. Determine the minimum inhibitory concentrations (MIC) of chitosans against bacteria and viruses using the broth macro-dilution method.

   b. Determine infectivity of bacteria (*E. coli*) and virus (coliphage MS2) in treated water and settled flocs after jar test coagulation
2.1 Chitosan

Chitosan or poly \([\beta(1-4)-2\text{-acetamido-2-deoxy-D-glucopyranos}]\)-poly \([\beta(1-4)-2\text{-amino-2deoxy-D-glucopyranos}]\), is a linear biopolymer originally from shells of crustaceans and cell walls of insects and fungi. It is polysaccharide and a derivative of chitin. There are two types of monomers randomly distributed on chitosan’s chain, namely \(N\text{-acetyl glucosamine (GlcNAc)}\) and glucosamine (GlcN), as shown in Figure 2.1.

![Molecular structure of N-acetyl glucosamine (GlcNAc), glucosamine (GlcN) and chitosan.](image)
Chitosan is obtained from partial deacetylation of chitin which is removal of acetyl groups (-CH₃CO) on N-acetyl glucosamine (GlcNAc) units of chitin polymer to reveal amino groups (-NH₂), as shown in Figure 2.2.

Figure 2.2 Deacetylation of chitin to obtain chitosan, acetyl group (-CH₃CO) on acetyl glucosamine monomer on chitin chain is removed to reveal amino group (-NH₂) becoming glucosamine monomer making chitosan.

Chitosan is a non-toxic polymer and has the characteristics of coagulants: it is non-soluble in water but dissolves in most of acids, contains positively charged moieties, and is safe to ingest. Chitosan is defined by the US-FDA as GRAS (Generally Recognized as Safe) which primarily refers to food additives. Chitosan is commercially available as food supplements and weight control products in many countries.

**Production of chitosan**

Chitosan can occur naturally with deacetylation of chitin by many natural enzymes (e.g. chitinase, hydrolase, pectinase, etc.) (Mourya and Inamdar, 2008; Wrona et al, 2007). Commercially available chitosans are produced from crustacean shells as waste of the seafood industry. After being washed and dried, crustacean shells are treated by dilute hydrochloric acid
to dissolve calcium carbonate followed by alkaline extraction with dilute sodium hydroxide to solubilize protein; the result of these 2 steps is chitin. These two steps can be employed in reverse order. After those chemical treatments, chitin will go through the deacetylation process by boiling with concentrated sodium hydroxide to obtain chitosan. A diagram of the commercial chitosan production process is shown in Figure 2.3.

**Figure 2.3** Chitin-chitosan production from crustacean shell.

**Characteristics of chitosan**

Two important characteristics of chitosan are molecular weight (MW) and degree of deacetylation (DD). These two parameters influence the physical-chemical characteristics of chitosan, such as solubility, viscosity and electrical charge properties, which determine the potential applications of this polymer.

**I. Molecular Weight (MW) of chitosan**

Molecular weight (MW) of chitosan indicates the length of the chitosan chain and MW increases with the chain length. MW is expressed as dalton or gram per mole of chitosan. Chitosan MW ranges approximately from 10,000 daltons (Da) to 1,000,000 Da (Pillai et al,
but smaller MW down to < 5,000 Da or higher MW up to 5,000,000 Da can be produced. It was found that viscosity of chitosan is often used to express chitosan MW. Theoretically, MW relates to viscosity with the Mark–Houwink equation, as shown below:

\[
[\eta] = KM^a
\]

Where \(\eta\) = intrinsic viscosity,

\(M\) = molecular weight

\(K\) and \(a\) are constants dependent on the particular polymer-solvent system.

High MW chitosan when dissolved will be sticky and appear to have a gel-like texture because it also has high viscosity. Also, chitosan is a semi-crystalline polymer so it can form a gel-like appearance when dissolved (Rinaudo, 2006).

### II. Degree of Deacetylation (DD) of chitosan

Degree of deacetylation (DD) indicates the amount of amino groups (-NH\(_2\)) along the chitosan chain or it refers to replacement of acetyl (-CH\(_3\)CO) groups with amino (-NH\(_2\)) groups, as shown in Figure 2.2. DD of chitosan shows the proportion of the acetyl glucosamine monomer on the chitosan chain that is chemically modified or turned into glucosamine monomer. DD then expresses the percentage of the total number of acetyl groups replaced with an amine group. There is no standard DD value to determine chitin or chitosan. However, chitosan usually has a DD from 40% to 98% and chitin has a DD below 40% (Mourya and Inamdar, 2008). Chitosan has a nitrogen content higher than 7% in its molecular constituents (Muzzarelli&Muzzarelli, 2005). Chitosan usually becomes soluble at a neutral pH at a DD of 50% and higher DD results in greater solubility. When dissolved, the amino groups on the glucosamine units will protonate
along the chitosan chain, making chitosan readily available for many reactions and applications, unlike chitin. Chitosan also dissolves in diluted acids. The pKa of the amino group of the glucosamine residue is about 6.3 (Renault et al. 2009, Muzzarelli&Muzzarelli, 2005) so pH of medium can influence its solubility. The positive charge of chitosan comes from the protonation of the amine group in the glucosamine unit. Therefore, the positive charges of chitosan are also closely related to its DD. Higher DD chitosan is expected to exhibit more positive charges.

**Modification of chitosan**

Chitosan is usually modified to give properties which are desired for the intended applications. The advantage of modification of chitosan is that its main structure does not change, still being a long chain polymer that basically retains its original physical and chemical properties. There are 3 functional groups on chitosan monomer that are amendable to modification as shown in Figure 2.4, without disturbing the main structure of polymer chain. Chitosan can be modified via adding functional groups onto the chains or cross-linking to other polymers.

**Figure 2.4** Functional groups on chitosan monomer for modification

The poor solubility of chitosan and chitin is related to their crystallinity, which is supported by hydrogen bonds mainly through amine and hydroxyl groups. Presence of glucosamine units (GlcN) in chitosan helps increase its solubility and also allows it to form salts and become a soluble polymer (Muzzarelli & Muzzarelli, 2005). Chitosan salts are the simplest
form of modified chitosans for achieving higher solubility in water. They are produced by treating chitosan polymers with selected acids such as acetic, lactic, hydrochloric, formic, ascorbic acids etc. The products are chitosan acetate, -lactate, -hydrochloride, -formate, and - ascorbate. The common commercially available modified chitosans are chitosan acetate, chitosan lactate, and chitosan HCl. These chitosan salts are easily dissolved in water and can be used in many applications.

Having qualities such as non-toxicity to mammalian cells, biodegradability and, biocompatibility, chitosan has been studied for its application in many sectors such as pharmaceuticals and biomedical use (Aranaz et al, 2009; Sashiwa & Aiba, 2004), cosmetics (Kumar, 2000), food industry (Arora et al, 2011; Aranaz et al, 2009), agriculture (Rabea et al, 2005; Meng et al, 2008; Romanazzi et al, 2012), textiles (Tseng et al, 2009), and water and wastewater treatment (Gerente et al, 2007; Crini & Badot, 2007; Bhatnagar & Sillanpaa, 2009).

This research will focus on the possibility of using chitosan for drinking water treatment at point-of-use in homes.

2.2 Chitosan and coagulation-flocculation

Coagulants in water treatment are chemicals that are used to destabilize particles, colloids, or organic matter in water. They are added to water mainly for turbidity removal and also for microbial removal. Coagulants generally should have the following characteristics: nontoxic and without adverse effects on human health, form insoluble or low solubility compounds in pH ranges of common water treatment processes, usually between pH 4.0-11, depending on coagulant used (Eckenfelder, 1989), easy handling, ready availability, and effectiveness to work as a coagulant, e.g. trivalent metallic cations or polymers (Shammas,
The ability of a coagulant to produce coagulation-flocculation normally depends on its electrical charge and molecular size. Theoretically, the larger the charge and the greater the size, the more effective the coagulant will be.

Possessing characteristics such as positive charges from protonation of \(-\text{NH}_2\) and a long-chain, linear polymeric structure supports the use of chitosan as a candidate coagulant of interest. These are positive attributes because most of the colloids in water (i.e. small particle usually less than 10 µm such as clays, bacteria and viruses) have negative surface charges so that they should quickly react with positively charged coagulants. These physical-chemical characteristics of chitosan suggest that coagulation using chitosan likely involves two coagulation mechanisms, namely interparticle bridging and charge neutralization.

Interparticle bridging is a mechanism whereby the polymer coagulant can form colloid-polymer-colloid structures, and the coagulant adsorbs onto the surface of more than one colloidal particle to form large flocs which then settle out, leading to effective removal of colloids. When attaching to a colloidal particle, polymer still has more sites on its remaining long chain to attach to other colloidal particles. Once this remaining portion extends away into water and attaches to other particles, bridges are formed between particles and polymer. This will result in coagulation and particle agglomeration (Shammas, 2005). Larger polymer size is expected to provide a higher removal of colloids. Chitosan is a long chain polymer having \(-\text{NH}_2\) as reactive groups randomly distributed along the chain so it can attach and bridge with many negatively charged colloidal particles, e.g. turbidity or microbes in water. Therefore, chitosan can destabilize those colloidal particles and cause them to settle out of water.

Charge neutralization is a mechanism where electrical charges of a coagulant molecule interact with the opposite electrical surface charges of colloids resulting in zero or near zero net
charge at the surface of the colloids. Positive charge and solubility of the coagulant themselves are important for charge neutralization of negatively charged colloids in water. Positively charged coagulants can attach to the negatively charged colloids (e.g., cell membranes of bacteria or surface of clay particles and viruses) resulting in neutralization of the surface charge of the colloids to a zero or near zero net charge. Then colloidal particles can come close and interact with each other forming flocs and can settle out of the water (Binnie and Kimber, 2009). Chitosan exhibits positive charges from protonation of –NH₂ groups on its molecules when dissolved. Therefore chitosan with more –NH₂ groups on its molecule will express high positive charge. This characteristic helps chitosan to attach to negatively charged colloids in water and neutralize them through this attachment. Destabilization of colloids is a result and colloids will agglomerate and can be physically removed from water. Chitosan usually dissolves in water at acidic and neutral pH and its solubility decreases as the pH increase. The pKa of amine group of the glucosamine unit is approximately 6.3-6.4 (Guibal & Roussy, 2007). Charge neutralization by chitosan is likely influenced by pH of water. The role of water pH on performance of chitosan coagulation will be determined in this study.

2.3 Chitosan on removal of pollutants

In the water sector, chitosan is of interest as a coagulant-flocculant or adsorbent to clarify water, to remove suspended particles, metal ions, synthetic polymers or dyes.

*Turbidity removal using chitosans*

Existing work in the literature reports various results on the ability of chitosan to coagulate particles such as bentonite, kaolinite, and natural organic matter and the efficiencies of
chitosan coagulation were dependent on types of colloidal particles and types of chitosans used in the studies. Domard et al (1989) studied kaolinite removal by three chitosans with different MW (characterized by viscosity $\eta$: 604, 404, and 280 ml/g) and found that higher molecular weight chitosan adsorbed more kaolin than lower molecular weight. According to the results, at an equilibrium dose of 0.2 g/L, the higher MW (604 ml/g) chitosan absorbed kaolin from the suspension at 20 mg/g whereas the lower MW (280 ml/g) chitosan absorbed kaolin at 12 mg/g.

Chen et al (2003) studied bentonite coagulation-flocculation by four chitosans with different MWs (300,000, 130,000, 78,000 and 27,900 Da) and found that higher MW chitosans gave better bentonite removal. The highest coagulation efficiency was obtained from the highest MW (300,000 Da) chitosan, with dose 2.5 mg/l giving approximately 90.9% removal (110 NTU to <10 NTU), whereas the lowest MW (27,900 Da) almost had no effect at the same chitosan dose. In the same research the authors also studied bentonite removals based on DD of chitosan. Four chitosans with different DD: 69%, 75%, 84% and 92% (same MW of 78,000 Da) were evaluated for their coagulation efficiencies and according to the results, the higher DD chitosan was more effective than lower DD. Less chitosan was needed for bentonite removal by coagulation when chitosan DD increased.

*Microbial removal using chitosans*

Like most of natural colloids, microbes such as bacteria and viruses have negatively charged outer surfaces so that they can be removed from water by coagulation-flocculation using chitosan coagulant. However, there were limited studies on water treatment focusing on microbial coagulation-flocculation.
Strand et al (2001) studied bacterial flocculation using chitosan hydrochloride (chitosan-HCl) of different DDs and reported that lower DD (38%) required 10 times less amount of chitosan-HCl than higher DD (99%) to flocculate *E. coli* in bacterial suspension. The authors concluded that flocculation efficiency increases with the decreasing DD.

*Other pollutant removals using chitosans*

Bhatnagar & Sillanpaa (2009) reviewed studies that utilized chitin and chitosans for water and wastewater treatment in the past 20 years. They reported that chitin and chitosan have been focused on for removal of water pollutants such as metal ions, radionuclides dyes, phenols and other anionic pollutants (e.g. pesticides and fungicides). The authors also reported adsorption of humic acid and fulvic acid by chitosan. Ngah et al (1998) studied adsorption of humic acid by chitosan and reported that the adsorption capacity of humic acid at 27°C was 28.88 mg/g while Zhang et al (2005) used chitosan hydrogel beads to adsorb fulvic acid and reported the adsorption capacity of fulvic acid was 1.67mg/g at pH 6. These results suggest that chitosan can be used for removal of natural organic matter.

Guibal & Roussy (2007) use chitosans of MW 80,000 and 300,000 Da with the same DD of 90% for coagulation-flocculation of anionic dye at acidic conditions. Results from jar test experiments showed that chitosan was effective for removal of anionic dye by > 80% and high MW chitosan required a higher dose than low MW chitosan for comparable dye removal. The authors suggested that charge neutralization was a main mechanism of chitosan coagulation at acidic conditions.
Yang & Zall (1984) studied adsorption of metals by chitosan and reported a strong chelating capacity, with selectivity in metals uptake as follows: copper (Cu) > chromium (Cr) = cadmium (Cd) > lead (Pb) > zinc (Zn).

Gerente et al (2007) reported that adsorption of Cu by chitosan was greater than that of other metals such as silver (Ag), or platinum (Pt). Absorption capacities of Cu from literatures varied and had wide ranges of 5-500 mg Cu/g for chitosan flake, 60-1100 mg Cu/g for chitosan bead, and ≈ 170 mg Cu/g for chitosan membrane. Differences in adsorption capacities were possibly related to factors such as types of chitosan, chemical groups added onto chitosan, experimental model, and theory used for calculation. However, the authors suggested that chitosan had potential for metal removal and could help improve the quality of industrial wastewaters containing metals.

2.4 Other coagulants on turbidity and microbial removal

Metal salt coagulants such as aluminum and iron salts are common coagulants used in conventional water treatment plants. Effectiveness of these salts is widely accepted. Aluminum salt such as aluminum sulphate or alum can effectively remove kaolinite turbidity up to 95% at dose of 12 mg/L at pH 7 (Yang et al, 2012) and can produce approximately 2 log_{10} reductions of *E. coli* bacteria or 3.4 log_{10} reductions of bacteriophage MS2 (WHO, 2004). Iron salt such as iron chloride can remove *E. coli* bacteria at 2 log_{10} and can remove bacteriophage MS2 at 2.9 log_{10} (WHO, 2004). However, the effectiveness of alum and iron salts is highly dependent on coagulant dose and pH of water, which make them difficult for people to use at homes and in developing countries. Performances of metal salts coagulants relate to hydrolysis products (e.g. Al^{3+}, Al(OH)^{2+}, Al_{3}(OH)_{4}^{5+}, and Al_{13}O_{4}(OH)_{24}^{7+}) which will occur after adding the coagulants.
into water. Amounts and types of hydrolysis products affect coagulation mechanism such as charge neutralization and sweep flocculation. In a coagulation system, transformations of these hydrolysis products are closely related to pH or alkalinity of water and coagulant doses; therefore the effectiveness of metal salt coagulants also depends on water pH and coagulant dose (Ye et al, 2007; Duan & Gregory, 2003; Licsko, 1997; Van Benschoten & Edzwald, 1990)). Moreover, metal salt coagulant, such as alum, influences the treated water quality which makes it more difficult to apply at home by non-skilled people. Arnoldsson et al (2008) reported changes of pH, alkalinity and conductivity in treated water using alum coagulant because of reaction between alum and alkalinity in water. Adjustment of water pH after coagulation will be necessary and this pH adjustment will also increase cost of treatment.

In recent years, there is growing interest in natural origin coagulants; extracts from plants such as red bean, sugar maize, red maize, Strychnos potatorum and particularly Moringa oleifera have been studied for their coagulation properties (Gunaratna et al, 2007; Babu & Chaudhuri, 2005). Much of the interest is on M. oleifera, a tropical tree, which has been studied as an alternative coagulant source particularly for household and small size water supplies (Ghebremichael, 2004). The active agent for coagulation-flocculation of M. oleifera is reported to be protein extracted from the seed (Gunaratna et al, 2007). Arnoldsson et al (2008) studied efficiency of M. oleifera on turbidity removal and reported optimum doses between 17 mg/L and 67 mg/L to obtain residual turbidity < 2 NTU from initial turbidity of 15 – 50 NTU. Sutherland et al (1994) reported that seed extract of M. oleifera at dose 75 mg/L could effectively remove 98 – 99% turbidity. For microbial removal, M. oleifera exhibited approximately 2 log₁₀ reduction of fecal coliforms and heterotrophic bacteria and 3 – 4 log₁₀ reduction of poliovirus when used at dose 200 mg/L followed by direct filtration (Babu & Chaudhuri, 2005). Problem of using
*Moringa Oleifera* seed is that treatment efficiency of the seeds decrease over storage time. Moreover, the extract solution of the seed which contains coagulant protein also has short storage duration and it is recommended to use within 24 hours to prevent degradation of the extract (Katayon et al, 2006). Thereby the seed extract should be prepared on site to obtain the best coagulation results. These drawbacks will cause difficulty in producing of ready to use seed extract of *M. oleifera* as a commercial product for people to purchase and use at homes.

Other natural plants tested for their coagulation efficiency by jar test method such as cactus (*Cactaceous opuntia*) exhibited up to 95% turbidity removal in surface water (Shiong, 2007). Similar to *M. Oleifera*, cactus extract have to be prepared on-site by users and it is still not commercially available in chemically well-characterized and stable forms.

At the moment, there is no commercial coagulant that is recognized as a simple and effective coagulant for turbidity and microbial removal at household level in developing countries by the Household Water Treatment and Safe Storage Program of WHO.
CHAPTER 3
TURBIDITY REMOVAL BY CHITOSAN COAGULATION

3.1 Introduction

Turbidity is a physical water quality characteristic describing the cloudiness or clarity of water. Causes of water turbidity can be clay, silt, microorganisms or other finely dissolved organic and inorganic materials. Turbidity is a water quality issue because it can interfere with chemical disinfection by creating oxidant demand and providing protection to microbes in aggregates, as well as interfere with physical disinfection methods such as UV light by blocking light transmission. Turbidity also causes negative impacts on consumer acceptance of water, since cloudiness may create the impression that water may not be clean or safe to drink.

Although different constituents can cause turbidity, turbidity levels themselves can be used as a surrogate measure of water safety. One study found associations between water turbidity and hospital admission for gastrointestinal illness within the elderly population in Philadelphia (aged 65 and older) and found a stronger association in population aged over 75 than aged 65-74 (Schwartz et al, 2000). Another study over a 12-year period (1993-2004) in Atlanta found that water turbidity (in both filtered and raw water) contributed minimally to endemic gastrointestinal illness in the general population, but for children aged ≤ 5 years, the association between raw water turbidity and gastrointestinal illness were somewhat stronger than other age groups (Tinker et al, 2010).
Although turbidity is not necessarily a direct measure of microbial contamination, microbes are often associated with particles in water and removing turbidity in water is a crucial step for providing safe drinking water. Nephelometric measurement is a method to determine turbidity; it evaluates light scattering at an angle of 90° that relates this angle-scattered light to actual turbidity of the sample (Sadar, 2014).

The World Health Organization has suggested < 1 nephelometric turbidity unit (NTU) for water that will undergo disinfection and < 4 NTU for water to be acceptable to the naked eye (WHO, 2011). The US Environmental Protection Agency sets the maximum level of turbidity in finished drinking water at 1 NTU and at no time > 5 NTU (EPA, 2014). Regulations for drinking water turbidity vary from country to country ranging from 0.3 – 25 NTU (WHO, 2014). For example, Chile set turbidity for drinking water at 20 NTU but requires a monthly average ≤ 2 NTU (WHO, 2014). Thailand set the maximum acceptable turbidity at 5 NTU and set maximum allowable turbidity at 20 NTU (PCD, 2014).

In areas without water treatment systems or with impaired sources of drinking water, turbidity level varies depending on the source water, and water may need treatment at the household level to remove turbidity, prepare water for further treatment, and render it safe to drink. Coagulation-flocculation, a treatment process commonly used to remove water turbidity at centralized water treatment plants, can also be used at the household level to treat water. Conventional coagulants used in large-scale water treatment are largely metal salts such as aluminum sulfate, ferric sulfate and ferric chloride. These coagulants depend on the pH of water and precise dosing to produce consistently high coagulation efficiency. When coagulation using metal salts is done, the resulting sludge also contains residual metals that must be properly disposed of so that they do not pollute. These limitations of conventional coagulants make them
less suitable for household level water treatment, where people need simple but robust and safe methods to treat their water at home.

Chitosan, a biopolymer, is a candidate coagulant-flocculant to remove turbidity from water. It possesses properties of a promising household-level water coagulant, such as positive charge when dissolved, non-toxicity, and biodegradability. Based on its positive moieties in molecular structure, chitosan can be predicted to coagulate negatively charged particles, such as bacteria, viruses, and clays, in water. Chitosan has been reported previously as an effective coagulant for reductions of contaminants in aqueous solutions, including suspended solids, turbidity, algae, oil, and grease (Renault et al, 2009). It has been shown to be effective for turbidity removal in surface water (Rizzo et al, 2008). Chitosan was shown to effectively remove kaolinite clay turbidity in tap water at low chitosan doses (0.75 – 2 mg/L) in the presence of turbidity levels ranging from 10 – 160 NTU (Divakaran & Sivasankara, 2001). Several other studies also reported that chitosan could efficiently coagulate-flocculate colloidal particles in water (Chen et al, 2003; Roussy et al, 2004; Fabris et al, 2010; Chen & Chung, 2011).

Chitosan as a coagulant for household water treatment should effectively remove turbidity from source waters that are normally used for drinking water. In this study, acid-soluble and water-soluble chitosans were tested for turbidity removal from artificial surface water used as a model for household drinking water. Efficacies of acid-soluble chitosans with different molecular weights and degrees of deacetylation were compared with water-soluble chitosans for removal of bentonite and kaolinite turbidity using the jar test method.
3.2 Materials and Methods

**Chitosans and preparation of their stock solutions**

A total of 17 commercially available chitosans belonging to two groups: acid-soluble and water-soluble, were tested, of which 11 were acid-soluble chitosan polymers and 6 were water-soluble modified chitosans. The information regarding MW and DD of all chitosans were obtained from the chitosan vendors. To study the effects of molecular weight on turbidity reduction, five chitosan polymers with different molecular weight and similar DD (≈ 90%) were compared: 5000, 50,000, 100,000, 600,000 and 1,000,000 daltons. These chitosans with different MW were bought from Heppe Medical Chitosan Gmbh (Halle, Germany) and Acros Organics (Bridgewater, NJ, USA). To test the effects of degree of deacetylation (DD) on turbidity reduction, another set of chitosan polymers with different DD and approximately the same MW (≈ < 50,000 Da), viscosity of 5 millipascal-second (mPa·S), were used: 70, 75, 80, 85, 90, and 95% DD. These chitosans with different DD were bought from Heppe Medical Chitosan Gmbh (Halle, Germany). Six commercially available modified (water-soluble) chitosans—chitosan acetate, chitosan lactate, chitosan HCl, carboxymethyl chitosan, and two commercial coagulants made of chitosan acetate and chitosan lactate, were also bought from Heppe Medical Chitosan Gmbh (Halle, Germany). The two commercial chitosan coagulants for turbidity removal, which were obtained from HaloSource (Bothell, WA), were chitosan acetate-StormKlear (SK) and chitosan lactate-SK.

Stock solutions of chitosan were made for all polymers at 10,000 mg/L. Chitosan powder was dissolved in 0.5% acetic acid and stirred at room temperature until totally dissolved. Stock solutions were stored at room temperature (25°C). The stock solutions of modified (water-soluble) chitosans were prepared similarly, using deionized water instead of acetic acid.
Characteristics of the tested chitosans (MW, DD, and viscosity) including zeta potential values obtained from measurement in this study were summarized in Appendix 11.

**Turbidity material**

Two mineral clays, kaolinite and bentonite, were used to create turbidity in test waters. These clays were selected because they cause turbidity in natural water and are commonly used to create turbidity in test waters for the evaluation of water treatment methods (Barany et al, 2009; Chen & Chung, 2011; Huang & Chen, 1996). Kaolinite and bentonite have different structures that may react differently with chitosan polymers. Bentonite has 2 silica tetrahedral sheets connected to one aluminum octahedral sheet while kaolinite has one silica tetrahedral sheet connected to one aluminum octahedral sheet (Figure 3.1). Bentonite has higher cationic exchange capacity (CEC) than kaolinite (0.8-1.2 versus 0.03-0.15 meq/g) and bentonite also has larger surface area than kaolinite (40-800 versus 5-40 m²/g) (Grim and Kodama, 2014). The CEC of clays may influence coagulation when using positively charged coagulants. It is expected that clay with higher CEC can receive more cations from coagulant and may increase contact between coagulant and clay, resulting in better coagulation.
Test water

The test water was artificial surface water prepared based on the recommended parameters from USEPA, NSF and WHO for efficacy testing of POU technology as listed in Appendix 3 (EPA, 1987; NSF, 2008; WHO, 2011). For testing of turbidity removal, the test water was created by spiking dechlorinated tap water with: 300 mg/L of total dissolved solids (TDS), 3 mg/L of total organic carbon (TOC) and ≥ 30 NTU of turbidity. Sodium chloride (NaCl) was used as an adjustment material for TDS and tannic acid was used as an adjustment material for TOC. Water pH was not adjusted after adding clay, NaCl, and tannic acid and water pH ranged pH 7 – 7.5.

Jar test experiment

A jar test method of coagulation testing was used. The mixing conditions were: rapid mixing at 100 revolutions per minute (rpm) for 1 minute followed by slow mixing at 25 rpm for
15 minutes and settling for 30 minutes. All experiments were conducted at 25°C. Supernatant was recovered at 2 centimeters from the top of the beakers for turbidity analysis using a pipette and without disturbing the floc. Turbidity was measured by a turbidity meter (Hach 2100AN Turbidimeter, Hach, Loveland CO). Water pH was also measured before and after the jar test experiment using a pH meter.

Doses of chitosans were: 1, 3, 10 and 30 mg/L. These doses were selected because they are in the same ranges as optimum doses of conventional coagulants (2-5 mg/l for aluminum and 4-10 mg/L iron salt coagulants) (WHO, 2011) and in the ranges of chitosan effective doses for turbidity removal in our preliminary study. Three replicates, plus one control (no chitosan) for natural settling, were performed for each set of experimental conditions.

**Data analysis**

Turbidity removal was calculated as percent turbidity removal relative to the natural settling control as shown below:

\[
\text{Percent removal} = \left[ 1 - \frac{\text{sample turbidity}}{\text{control turbidity}} \right] \times 100
\]

Statistical comparison of the effects of different chitosans on turbidity removal was performed using GraphPad Prism (GraphPad, San Diego, CA). Two-way ANOVA and Tukey posttest analysis was employed for comparing effects of water-soluble and acid-soluble chitosans and chitosan doses, and one-way ANOVA was used for comparing effects of chitosan doses of each chitosan tested.

**Zeta potential measurement**

Zeta potential of all 17 chitosan stocks (in 0.5% v/v acetic acid) was measured. One candidate chitosan with a MW of 100,000 daltons and that demonstrated high removal of
turbidity, bacteria, and virus in jar testing was tested to observe changes of the surface charge through the coagulation-flocculation process. Zeta potentials of prepared test water containing bentonite or kaolinite with turbidity of 5, 30 and 300 NTU were also measured.

A Malvern Zetasizer nano ZS (Malvern, Worcestershire, UK) was used to study zeta potentials in this research. Zeta potential in this research was measured in 2 different ways. First, zeta potentials of chitosan stock solutions and water samples were analyzed for their background electrical charges. Secondly, using a titration method, zeta potential was measured during the titration of water samples with chitosans. For this measurement, water samples containing 5 NTU kaolinite or bentonite at neutral pH were titrated with chitosan stock solution at doses between 0 and 50 mg/L. Water pH was automatically measured during the titration. Titration graphs showing changes in zeta potential while chitosan was being added into the water sample were generated showing chitosan dose and pH at which the isoelectric point (zero point of charge) occurred.

3.3 Results

3.3.1 Turbidity removal by chitosans

**I. Effects of chitosan molecular weight (MW) on turbidity removal**

The effects of chitosan polymer MW on reduction of bentonite turbidity at varying chitosan doses are shown in Figure 3.2. Overall, bentonite removal differed significantly by MW ($p < 0.0001$). In general, higher MW chitosans produced greater turbidity removals, with poor reductions at the lowest MW and a very low reduction by 100,000 MW chitosan at a dose of 30 mg/L. The highest reductions in bentonite turbidity were achieved at doses of 1 and 3 mg/L and
to some extent at 10 mg/L; a higher dose of 30 mg/L produced consistently lower turbidity removal.

Figure 3.2 Effects of chitosan polymer MW (DD ≈ 90%) on bentonite reduction at varying doses

The effects of MW on reduction of kaolinite turbidity at varying chitosan doses are shown in Figure 3.3. Overall, kaolinite removal differed significantly by MW (p < 0.0001). The highest reductions in kaolinite turbidity were achieved at doses of 1 and 3 mg/L, and higher doses did not produce higher turbidity removal, with the exception of MW 5,000 Da at dose 30 mg/L. Higher MW chitosans produced greater turbidity reductions, with poor reductions at the lowest MW. Removal of kaolinite turbidity was generally poorer at higher doses (10 and 30 mg/L) than reduction of bentonite turbidity (p>0.05).
Chitosans between 50,000 and 1,000,000 daltons exhibited bentonite reduction > 90% at dose 1 and 3 mg/L as shown in Figure 3.4. At chitosan dose 1 mg/L, bentonite removal differed significantly by molecular weight (one-way ANOVA, p < 0.0001). Bentonite removal by a low MW chitosan (5,000 Da) was poor (<30%). At the 1 mg/L dose, 50,000 Da had significantly lower bentonite removal (92.6%) compared to the larger MW chitosans: 100,000 (98.1%), 600,000 (98.2%) and 1,000,000 Da (96.2%) (p < 0.05). There were no statistically significant differences between bentonite removal by 100,000, 600,000 and 1,000,000 MW chitosans (p > 0.05) at dose 1 mg/L. MW 100,000, 600,000, and 1,000,000 Da chitosans showed similar bentonite reduction between 1 and 3 mg/L doses. When chitosan dose was increased from 1 to 3 mg/L, bentonite removal by the lower MW chitosans of 5,000 and 50,000 Da was improved somewhat. For example, MW 5,000 Da chitosan improved removal from 10.45% at 1 mg/l dose
to 24.66% at 3 mg/L dose. Also, MW 50,000 Da improved removal from 92.62% at 1 mg/L dose to 98.82% at 3 mg/L dose, a difference that was not statistically significantly different from the removals by higher MW chitosans (p >0.05). At a chitosan dose of 3 mg/L, bentonite removals were no longer statistically significant different for chitosans MW 50,000 Da to 1,000,000 Da.

Figure 3.4 Reduction of bentonite by chitosans with different molecular weight (DD ≈ 90%) at dose 1 and 3 mg/L

MW 50,000, 100,000 and 1,000,000 Da chitosans produced kaolinite reduction ranging from 87 – 90 % at 1 and 3 mg/L doses (Figure 3.5). At doses of 1 and 3 mg/L, there was no significant difference in kaolinite reduction between MW 50,000,100,000, and 1,000,000 Da chitosans (p >0.05). MW 600,000 Da chitosan was an aberration and was less effective than the other MWs, with <25% reduction. The lowest MW chitosan, 5,000 Da, performed very poorly (<1% turbidity reduction).
Figure 3.5 Reduction of kaolinite by chitosans with different molecular weight (DD ≈ 90%) at dose 1 and 3 mg/L

For the same MW chitosan and at the same dose, removal of bentonite was significantly better than removal of kaolinite at dose 1 and 3 mg/L (one-way ANOVA, Tukey’s Posttest, p <0.05). The exception was MW 50,000 Da chitosan at dose 1 mg/L which showed similar removals of bentonite and kaolinite (p>0.05).

Chitosan Dose 10 mg/L

The smallest MW chitosan of 5,000 daltons had somewhat better bentonite turbidity reduction (46%) at dose 10 mg/L (Figure 3.6) than at lower chitosan doses of 1 and 3 mg/L, but still not as good as higher MW chitosans. For higher MW chitosans, bentonite reductions did not improve compared to the removals at dose 3 mg/L but actually decreased significantly with an increase in dose to 10 mg/L (p< 0.05). Nevertheless, bentonite reductions by a 10 mg/l dose of chitosans of 50,000 daltons MW or higher were in the range of about 80-90%, which were significantly greater than reductions by 5,000 daltons MW chitosan, which were only 46%.
Effects of molecular weight of chitosans at dose 10 mg/L on bentonite reduction

![Graph showing turbidity reduction as a function of chitosan molecular weight.](image)

**Figure 3.6** Reduction of bentonite by chitosans with different molecular weight (DD ≈ 90%) at dose 10 mg/L

All MW chitosans, even those that showed removal at doses of 1-3 mg/L, performed poorly for kaolinite reduction at dose 10 mg/L (Figure 3.7), with <5% reduction for all MWs.

![Graph showing turbidity reduction as a function of chitosan molecular weight.](image)

**Figure 3.7** Reduction of kaolinite by chitosans with different molecular weight (DD ≈ 90%) at dose 10 mg/L

At dose 10 mg/L for the same MW chitosans, removals of bentonite were significantly better than removals of kaolinite (one-way ANOVA, Tukey’s Posttest, p <0.05).
**Chitosan Dose 30 mg/L**

Bentonite removal was similar between 10 and 30 mg/L doses for MW 5,000 Da chitosan. However, for the higher MW chitosans of 50,000 Daltons or more, bentonite reduction by this highest chitosan dose of 30 mg/L was 30-90% less when compared to lower doses and ranged 2 to 64% as shown in Figure 3.8.

![Figure 3.8](image)

**Figure 3.8** Reduction of bentonite by chitosans with different molecular weight (DD ≈ 90%) at dose 30 mg/L

Most MW chitosans, even those that showed effective turbidity removals at doses of 1-3 mg/L, performed poorly for kaolinite at dose 30 mg/L, with <5% reduction (Figure 3.9). The exception was MW 5,000 Da chitosan, which increased from <1% at lower doses to ~80% reduction at dose 30 mg/L.

Removal of bentonite was significantly higher than removal of kaolinite for the same MW chitosan at 30 mg/L dose (one-way ANOVA, Tukey’s Posttest, p <0.05). The exception was MW 5,000 Da which showed higher kaolinite removal (~80%) than bentonite removal (~41%).
Figure 3.9 Reduction of kaolinite by chitosans with different molecular weight (DD ≈ 90%) at dose 30 mg/L

There were 3 MW chitosans that effectively removed both kaolinite and bentonite at dose 1 and 3 mg/L: MW 50,000 Da, 100,000 Da, and 1,000,000 Da. These MW chitosans could achieve >90% turbidity removal, and bring residual turbidity from 30 -70 NTU to under 3 NTU for bentonite and 5 NTU for kaolinite (Table 3.1).

Table 3.1 Residual turbidity (NTU) as a function of chitosan molecular weight (MW) and dose in test water with kaolinite or bentonite clay

<table>
<thead>
<tr>
<th>Chitosan MW (Da)</th>
<th>Turbidity</th>
<th>Initial turbidity</th>
<th>Residual turbidity (NTU) at each chitosan dose (± 95%CI, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 mg/L</td>
</tr>
<tr>
<td>5000</td>
<td>kaolinite</td>
<td>80.8</td>
<td>73.3 (±7.12)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>34.4</td>
<td>28.4 (±0.39)</td>
</tr>
<tr>
<td>50,000</td>
<td>kaolinite</td>
<td>47.5</td>
<td>43.3 (±3.41)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>36.7</td>
<td>34.4 (±2.74)</td>
</tr>
<tr>
<td>100,000</td>
<td>kaolinite</td>
<td>34.2</td>
<td>32.5 (±14.52)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>35.6</td>
<td>20.0 (±2.57)</td>
</tr>
<tr>
<td></td>
<td>kaolinite</td>
<td>bentonite</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>600,000</td>
<td>49.1</td>
<td>37.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(±9.56)</td>
<td>(±6.01)</td>
<td></td>
</tr>
<tr>
<td>1,000,000</td>
<td>55.4</td>
<td>37.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(±1.17)</td>
<td>(±1.17)</td>
<td></td>
</tr>
</tbody>
</table>

**II. Effects of chitosan degree of deacetylation (DD) on turbidity removal**

Chitosans with six DDs and comparable MWs (approximately 5 mPa·s viscosity, < 50,000 Da) were tested: 70%, 75%, 80%, 85%, 90%, and 95%. Chitosans differing in degrees of deacetylation and dose were significantly different in turbidity reduction (p < 0.0001 for both different chitosan doses and different DDs). Optimal chitosan dose for all DD values was 3 mg/L with kaolinite removals ranging from 87.6% to 92.7% and bentonite removals ranging from 98.84% to 99.20%. The effects of chitosan DD and dose on bentonite reduction are shown in Figure 3. The highest reduction for all DD values was at 3 and 10 mg/L doses, with lowest reduction at the highest dose of 30 mg/L.
Figure 3.10 Effects of DD (5 mPa·s viscosity) and doses of chitosans on bentonite reduction

As seen in Figure 3.11, overall turbidity reduction was lower for kaolinite than bentonite, but highest reductions of 88 to 93% were seen at the chitosan dose 3 mg/L for all 6 DDs.

Figure 3.11 Effects of DD (5 mPa·s viscosity) and doses of chitosans on kaolinite reduction
**Dose 1-3 mg/L**

All DDs of chitosans exhibited bentonite reductions >80% at dose 1 and 3 mg/L (range from 84.5 – 99.2 % reduction, residual turbidity < 5 NTU), (Table 3.2 and Figure 3.12). At dose 1 mg/L, the DD 70% and 80% chitosans showed significantly higher mean bentonite reductions than other DD chitosans (p < 0.05). These two DD values had similar bentonite reductions of 90.50% (p > 0.05). All tested DDs of chitosans showed the best bentonite reduction at dose 3 mg/L, with approximately 99% reduction and residual turbidity < 1 NTU. The 85%DD chitosan exhibited significantly higher reduction (99.20%) than other DD at this dose, but the magnitude of difference was very small, with the lowest achieving 98.84 % reduction by chitosan 90% DD.

**Figure 3.12** Reduction of bentonite by chitosans with different degrees of deacetylation (similar MW of viscosity 5 mPa·s) at dose 1 and 3 mg/L

Kaolinite removal differed significantly across the DD values tested (p < 0.0001) and effects of DD on kaolinite reduction were significantly influenced by chitosan doses (p < 0.0001) (Table 3.2 and Figure 3.13). All DD chitosans performed best at dose 3 mg/L, with kaolinite reductions of ~88% - 93%. At dose 3 mg/L, 70% and 75% DD chitosans exhibited significantly higher kaolinite reductions than 90% and 95% DD chitosans (p <0.05), although all of them achieved reductions of > 91.4%. At dose 1 mg/L, kaolinite reductions were poor; only 70%, 80%
and 90% DD removed kaolinite from water, with reductions 47, 68 and 29%, respectively. However, the highest reduction at this dose by chitosan with 80% DD (68.5%) was not statistically significantly different than the reduction by 70% DD (47.3%).

For the same DD chitosans and at the same doses, removal of kaolinite was significantly lower than removal of bentonite at doses 1 and 3 mg/L (one-way ANOVA, Tukey’s Posttest, p <0.05). The exception was chitosan 80% DD at dose 1 mg/L, for which removals of bentonite (90.5% (±3.9)) and kaolinite (68.5% (±37.9)) were not statistically different (p>0.05).

**Dose 10 mg/L**

All chitosans tested gave turbidity reductions of 93% or more at a 10 mg/L dose. However, chitosans with DD >70% exhibited significantly higher bentonite reduction (~98%) compared to 70% DD chitosan (~93%) (p <0.05) at a dose of 10 mg/L, as shown in Figure 3.14. There were no statistically significant differences among mean reductions of chitosans DD >70% at this dose (p > 0.05).

When chitosan doses were increased from 3 mg/L to 10 mg/L, kaolinite removal decreased. At dose 10 mg/L, only high DD chitosan (85% DD - 95% DD) showed kaolinite
reductions ranging from about 52 to 75% and the 90% DD chitosan provided the highest kaolinite reduction of 75%. At dose 10 mg/L, low DD chitosans (70%, 75% and 80%) had very poor kaolinite reductions (<7%). For the same DD chitosan and at the same dose, removal of bentonite was significantly higher than removal of kaolinite at dose 10 mg/L (one-way ANOVA, Tukey’s Posttest, p <0.05).

![Graphs showing turbidity reduction of bentonite and kaolinite by different DD chitosans at 10 mg/L](image)

**Figure 3.14** Reduction of bentonite and kaolinite by chitosans with different degrees of deacetylation (similar MW of viscosity 5 mPa·s) at dose 10 mg/L

**Dose 30 mg/L**

When chitosan dose was increased to 30 mg/L, reduction of bentonite was significantly lower compared to the reductions at lower chitosan doses (Figure 3.15). The decrease was greatest for chitosan 70% DD, which showed only 22.8% reduction of bentonite compared to >90% reduction at doses between 1-10 mg/L. At dose 30 mg/L, none of the chitosans regardless of DD could remove kaolinite turbidity, with reductions <3%. For the same DD chitosan at dose 30 mg/L, removal of bentonite was significantly higher than removal of kaolinite (one-way ANOVA, Tukey’s Posttest, p <0.05).
Based on these results, dose 3 mg/L was the optimum dose that exhibited the highest removal of both bentonite and kaolinite turbidity. At this chitosan dose, removal of bentonite by different DD chitosans was similar, approximately 99%, and resulted in residual turbidity <5 NTU. The removals of kaolinite were also similar across DD chitosans, with approximately 90% reduction at dose 3 mg/L. Three DD chitosans, 70%, 85%, and 95% DD, were selected for further microbial coagulation studies as will be presented in later chapters of this dissertation. At dose 3 mg/L, these chitosans of differing DD could achieve >90% turbidity removal, and bring residual turbidity from 32-98 NTU to under 1 NTU for bentonite and under 5 NTU for kaolinite (Table 3.2).
Table 3.2 Residual turbidity (NTU) as a function of chitosan DD and dose in test water with kaolinite or bentonite clay

<table>
<thead>
<tr>
<th>DD (%)</th>
<th>Turbidity</th>
<th>Initial turbidity</th>
<th>Residual turbidity (NTU) at each chitosan dose (± 95% CI, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 mg/L</td>
</tr>
<tr>
<td>70</td>
<td>kaolinite</td>
<td>104.3 (±9.67)</td>
<td>97.7 (±76.65)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>35.7 (±3.09)</td>
<td>32.3 (±0.99)</td>
</tr>
<tr>
<td>75</td>
<td>kaolinite</td>
<td>50.3 (±4.38)</td>
<td>48.6 (±4.58)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>34.8 (±2.83)</td>
<td>33.6 (±1.67)</td>
</tr>
<tr>
<td>80</td>
<td>kaolinite</td>
<td>49.0 (±4.10)</td>
<td>47.7 (±18.41)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>38.1 (±1.44)</td>
<td>36.3 (±1.33)</td>
</tr>
<tr>
<td>85</td>
<td>kaolinite</td>
<td>43.6 (±1.99)</td>
<td>41.4 (±3.15)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>37.2 (±1.52)</td>
<td>35.9 (±2.08)</td>
</tr>
<tr>
<td>90</td>
<td>kaolinite</td>
<td>33.5 (±2.72)</td>
<td>33.7 (±38.76)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>37.0 (±3.26)</td>
<td>32.8 (±0.47)</td>
</tr>
<tr>
<td>95</td>
<td>kaolinite</td>
<td>38.4 (±4.14)</td>
<td>37.3 (±1.62)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>33.8 (±0.80)</td>
<td>34.0 (±1.03)</td>
</tr>
</tbody>
</table>

III. Effects of modified chitosans on turbidity removal

Six modified chitosans were tested: carboxymethyl chitosan, chitosan lactate, chitosan acetate, chitosan HCl and the commercial coagulants chitosan lactate-SK and chitosan acetate-SK. All modified chitosans except carboxymethyl chitosan showed high turbidity removal and both types and doses of modified chitosans significantly affected reductions of turbidity (p <0.0001). However, efficacy of each modified chitosan varied with test conditions. Highest reductions of bentonite were seen at dose 3 mg/L. Overall, modified chitosans demonstrated
bentonite reductions similar to those found with unmodified chitosans, though carboxymethyl chitosan showed lower reductions than other modified chitosan types (Figure 3.16).

Figure 3.16 Effects of modified chitosans (DD 80-95%, viscosity 2-300 mPa·S) on bentonite reductions

As seen with unmodified chitosan polymers, modified chitosans demonstrated poorer reductions of kaolinite compared to bentonite. Highest reductions of kaolinite were seen at dose 3 mg/L but only for some modified chitosans and not others (Figure 3.17). Carboxymethyl and acetate chitosans showed lower kaolinite reductions than the other modified types tested.
Modified chitosans in this study, except carboxymethyl chitosan, produced bentonite reductions of 97 – 98 % and residual turbidity 0.4 – 6.24 NTU at doses of 3 and 10 mg/L, as shown in Table 3.3 and Figure 3-18. The dose of 3 mg/L was the optimum dose; all modified chitosans showed high bentonite removals of 98 – 99% and there were no statistically significant differences among their mean reductions (p > 0.05) at this dose. Also at this dose, there were no statistically significant difference between the generic chitosan lactate and the commercial chitosan lactate-SK (a commercial coagulant) and between generic chitosan acetate and a commercial chitosan acetate-SK (p> 0.05). At the lowest chitosan dose of 1 mg/L, commercial chitosan acetate-SK had significantly higher reduction of bentonite (97%) than other modified chitosans (p-value < 0.05). Chitosan HCl and chitosan acetate exhibited bentonite removals of approximately 87 % and 90% at the 1 mg/L dose, respectively; there was no statistically
significant difference between them (p-value > 0.05). Chitosan lactate showed slightly but significantly better bentonite removal than a commercial coagulant chitosan lactate-SK with 81% and 77% reductions, respectively (p-value < 0.05). Residual bentonite turbidity (Table 3.3) was lower than 1 NTU at chitosan doses of 3 mg/L, with the exception of carboxymethyl chitosan, indicating this dose to be optimum among the doses tested.

Modified chitosans affected kaolinite removal differently from bentonite removal. Both the types and doses of modified chitosans influenced the removal of kaolinite from water (p < 0.0001). The optimum dose was 3 mg/L based on turbidity removal. Three modified chitosans, lactate (both laboratory grade and commercial coagulant) and chitosan HCl, produced relatively high kaolinite removals of 88 – 91% and residual turbidity from 3.80 – 5.45 NTU (Table 3.3 and Figure 3.18) that were not significantly different (p-value > 0.05). Kaolinite reductions were generally low for generic chitosan acetate at 16% or less. However, commercial chitosan acetate had a kaolinite removal of 82% and a residual turbidity of 11 NTU at its optimum dose of 1 mg/L, and was the only modified chitosan that was effective at this low dose. Chitosan HCl was also the only modified chitosan that reduced kaolinite turbidity to lower than 5 NTU (3.8 NTU), at a dose of 3 mg/L. However, the generic and commercial chitosan lactate reduced kaolinite turbidity to <6 NTU (Table 3.3). Carboxymethyl chitosan showed kaolinite removal of <2% at all doses tested.

For the same modified chitosans at the same doses, removal of bentonite was significantly higher than removal of kaolinite at dose 1 and 3 mg/L (one-way ANOVA, Tukey’s Posttest, p <0.05). The exception was chitosan HCl at dose 3 mg/L, for which removals of bentonite and kaolinite were not statistically significantly different (p>0.05).
Dose 10 mg/L

At a dose of 10 mg/L, modified chitosans in this study, except carboxymethyl chitosan, produced bentonite reductions of 97 – 98 % and residual turbidity 0.4 – 6.24 NTU, as shown in Figure 3.19. Bentonite reductions at the 10 mg/L dose were slightly lower (96 -99%) compared to reductions produced at the 3 mg/L dose for chitosan HCl, acetate, lactate and lactate-SK. However, at this dose, bentonite reduction by chitosan acetate-SK decreased substantially from approximately 99% at 1 and 3 mg/L doses to 81% at 10 mg/L dose. Lactate, lactate-SK and HCl chitosans at 10 mg/L dose also reduced turbidity to < 1 NTU. At dose 10 mg/L, chitosan HCl produced a kaolinite removal of 85% but the other modified chitosans produced reductions of only < 3%. For each of the same modified chitosans at dose 10 mg/L, removal of bentonite was significantly higher than removal of kaolinite (one-way ANOVA, Tukey’s Posttest, p <0.05).
**Dose 30 mg/L**

At dose 30 mg/L, bentonite removal by modified chitosans was lower compared to lower doses. Chitosan HCl was the best at this dose with reduction of 89%. Carboxymethyl chitosan was relatively ineffective for bentonite removal, with only 60% reduction and residual turbidity 12.79 NTU. At the 30 mg/L dose, all modified chitosans were ineffective in removing kaolinite from water (Figure 3.20), with reductions of <3%. For each of the same modified chitosans at dose 30 mg/L, removal of bentonite was significantly higher than removal of kaolinite (one-way ANOVA, Tukey’s Posttest, p <0.05).
Three modified chitosans were selected for further experiments on microbial coagulation-flocculation-sedimentation: chitosan HCl, chitosan lactate, and chitosan acetate. At dose 3 mg/L, these modified chitosans could decrease residual turbidity from 30 -60 NTU to under 1 NTU for bentonite and 6 NTU, for kaolinite, respectively as shown in Table 3.3, with the exception of chitosan acetate, which gave poor reductions of kaolinite turbidity.
Table 3.3: Residual turbidity (NTU) after coagulation-flocculation and sedimentation treatment with modified chitosans in test water containing bentonite or kaolinite turbidity

<table>
<thead>
<tr>
<th>Chitosan type</th>
<th>Turbidity</th>
<th>Initial turbidity</th>
<th>Residual turbidity (NTU) at each chitosan dose (mg/L) (± 95% CI, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mg/L)</td>
</tr>
<tr>
<td>Carboxymethyl chitosan</td>
<td>kaolinite</td>
<td>36.7</td>
<td>34.2 (±0.76)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>34.6</td>
<td>31.7 (±1.81)</td>
</tr>
<tr>
<td>Chitosan lactate</td>
<td>kaolinite</td>
<td>48.7</td>
<td>48.8 (±42.07)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>34.6</td>
<td>32.7 (±1.62)</td>
</tr>
<tr>
<td>Chitosan lactate (StormKlear)</td>
<td>kaolinite</td>
<td>41.2</td>
<td>41.8 (±9.51)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>36.3</td>
<td>35.0 (±2.20)</td>
</tr>
<tr>
<td>Chitosan acetate</td>
<td>kaolinite</td>
<td>34.0</td>
<td>33.4 (±2.10)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>33.6</td>
<td>32.3 (±5.79)</td>
</tr>
<tr>
<td>Chitosan acetate (StormKlear)</td>
<td>kaolinite</td>
<td>64.7</td>
<td>61.9 (±5.35)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>36.8</td>
<td>35.3 (±0.22)</td>
</tr>
<tr>
<td>Chitosan HCl</td>
<td>kaolinite</td>
<td>45.6</td>
<td>45.2 (±34.65)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>32.0</td>
<td>30.0 (±2.11)</td>
</tr>
</tbody>
</table>

3.3.2 Measurement of zeta potential

**I. Zeta potential of chitosan stock solutions and water samples**

Most chitosans studied showed positive zeta potentials but carboxymethyl chitosan showed negative zeta potentials as shown in Table 3.4. Comparing values across MW with similar DD (≈90%), the MW 5,000 Da had the smallest zeta potential of 6.61 mV which was not statistically different from that of MW 50,000 Da (19.8 mV) (p > 0.05), whereas chitosan
100,000 Da and 600,000 Da had the largest zeta potential of 88-89 mV (1 way-ANOVA, p > 0.05). However, chitosan MW 1,000,000 Da, the largest MW measured, showed only 38.41 mV.

Zeta potentials of chitosans with different DD but comparable MW (expressed as viscosity 5 mPa·S) were between 20 – 42 mV. Chitosan 70% DD had the highest zeta potential value of 42 mV but it was not statistically significantly different with chitosans of 80% and 90% DD with 38 and 37 mV (p > 0.05), respectively. Chitosan 95% DD had the lowest zeta potential among all DD chitosans with 20 mV and was significantly lower than zeta potential of chitosan DD 80 and 70% (p < 0.05).

Modified chitosans with different functional groups had different zeta potential values. Chitosan lactate-SK, a commercial coagulant, showed the highest zeta potential of 64.75 mV and it was significantly higher than the zeta potential of chitosan lactate (16.50 mV) (p<0.05). However, chitosan acetate-SK (a commercial coagulant) and chitosan acetate were not significantly different, with zeta potentials of 39.02 and 43.08 mV, respectively. Chitosan HCl also had a positive zeta potential of 30.37 mV. Carboxymethyl chitosan was the only water-soluble modified chitosan used in this study that had negative zeta potential of -29.64 mV.
Table 3.4 Zeta potentials of chitosan stock solutions (mV ± 95% CI, n = 9)

<table>
<thead>
<tr>
<th>Chitosans</th>
<th>Zeta potential, mV (± 95% CI, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MW 5,000 Da</td>
<td>6.61 (±5.28)</td>
</tr>
<tr>
<td>2. MW 50,000 Da</td>
<td>19.79 (±3.24)</td>
</tr>
<tr>
<td>3. MW 100,000 Da</td>
<td>88.93 (±10.88)</td>
</tr>
<tr>
<td>4. MW 600,000 Da</td>
<td>88.47 (±10.83)</td>
</tr>
<tr>
<td>5. MW 1,000,000 Da</td>
<td>38.41 (±6.28)</td>
</tr>
<tr>
<td>6. 70% DD</td>
<td>41.98 (±2.73)</td>
</tr>
<tr>
<td>7. 75% DD</td>
<td>31.86 (±8.08)</td>
</tr>
<tr>
<td>8. 80% DD</td>
<td>38.32 (±8.29)</td>
</tr>
<tr>
<td>9. 85% DD</td>
<td>24.43 (±5.98)</td>
</tr>
<tr>
<td>10. 90% DD</td>
<td>36.84 (±9.09)</td>
</tr>
<tr>
<td>11. 95% DD</td>
<td>19.84 (±12.00)</td>
</tr>
<tr>
<td>12. Chitosan acetate</td>
<td>43.08 (±20.00)</td>
</tr>
<tr>
<td>13. Chitosan acetate-SK</td>
<td>39.02 (±6.42)</td>
</tr>
<tr>
<td>14. Carboxymethyl chitosan</td>
<td>-29.64 (±2.20)</td>
</tr>
<tr>
<td>15. Chitosan HCl</td>
<td>30.37 (±10.07)</td>
</tr>
<tr>
<td>16. Chitosan lactate</td>
<td>16.50 (±9.67)</td>
</tr>
<tr>
<td>17. Chitosan lactate-SK</td>
<td>64.74 (±13.21)</td>
</tr>
</tbody>
</table>

Notes: *Chitosan stock solution—chitosan powders were dissolved in 0.5% (v/v) acetic acid or in deionized water for modified chitosans.

*Italic* and color fonts indicate zeta potential which were not significantly different in each group: MW, DD, and modified chitosans. Blue fonts were not significantly different from blue fonts and green fonts were not significantly different from green fonts.

**II. Zeta potentials of water samples**

All water samples exhibited negative zeta potential as shown in Table 3.5. Water samples with kaolinite turbidities of 30 and 300 NTU had the lowest zeta potentials (most negative...
values) with approximately -35 mV. Zeta potential values of test water with kaolinite were statistically different at varying turbidity levels (p<0.05). The zeta potential of water with kaolinite turbidity of 5 NTU differed significantly from zeta potential of waters with 30 and 300 NTU. Water samples containing bentonite turbidity, however, had only small differences in zeta potentials and all levels of turbidity in water (5, 30, and 300 NTU) had zeta potentials ranging from 24.99 – 26.24 mV that were not statistically significantly different (p>0.05). Water with kaolinite had more negative zeta potentials than water with bentonite at turbidity levels of 300 and 30 NTU. However, at 5 NTU turbidity, zeta potentials for water with bentonite and kaolinite were not significantly different (p>0.05).

Table 3.5 Zeta potential of water samples at different levels of turbidity

<table>
<thead>
<tr>
<th>Test water</th>
<th>Zeta potential, mV (± 95%CI, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water 5NTU kaolinite-pH4</td>
<td>-18.29 (±2.97)</td>
</tr>
<tr>
<td>Water 5NTU kaolinite-pH7</td>
<td>-27.83 (±1.27)</td>
</tr>
<tr>
<td>Water 30NTU kaolinite-pH7</td>
<td>-35.26 (±2.67)</td>
</tr>
<tr>
<td>Water 300NTU kaolinite-pH7</td>
<td>-35.59 (±0.78)</td>
</tr>
<tr>
<td>Water 5NTU bentonite-pH7</td>
<td>-24.99 (±3.63)</td>
</tr>
<tr>
<td>Water 30NTU bentonite-pH7</td>
<td>-26.24 (±2.19)</td>
</tr>
<tr>
<td>Water 300NTU bentonite-pH7</td>
<td>-25.28 (±1.10)</td>
</tr>
</tbody>
</table>

III. Isoelectric points during coagulation using chitosans

Zeta potential values and isoelectric points (IEP, point of zero charge) of chitosan coagulation were measured by the titration method. Several chitosans that provided high and low
turbidity removal were selected as representatives to study changes of zeta potential through the course of the coagulation process (Table 3.6).

The IEP of chitosans of 100,000 Da, 70% DD, 95% DD, and chitosan HCl were at doses of 4.61, 2.15, 1.88, and 2.19 mg/L, respectively, at neutral pH (pH 7.2 – 7.5), as shown in Table 3.6. These IEPs were close to the doses that gave the highest turbidity removals (3 mg/L dose) in jar test experiments. The IEPs of chitosan MW 100,000 Da for kaolinite (4.61 mg/l) was higher than that of bentonite (2.62), however both IEPs were still close to the jar test effective dose of 3 mg/L. The titration graphs of chitosan MW 100,000 Da and chitosan HCl are shown in Figure 3.21, and all titration graphs are in Appendix 9.

Table 3.6 Isoelectric points (IEPs) and pH values at IEPs of chitosans measured by a titration method

<table>
<thead>
<tr>
<th>Chitosan and test water clay and turbidity</th>
<th>Zeta potential of chitosan, mV (± 95%CI, n = 9)</th>
<th>IEP* at dose mg/L chitosan</th>
<th>pH at IEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW 5,000 Da and test water 5NTU kaolinite</td>
<td>6.61 (±5.28)</td>
<td>No IEP ζ-2.14 mV at 50 mg/L</td>
<td>pH 7.44 at dose 50 mg/L</td>
</tr>
<tr>
<td>MW 100,000 Da and test water 5NTU kaolinite</td>
<td>88.93 (±10.88)</td>
<td>4.61</td>
<td>≈ pH 7.2</td>
</tr>
<tr>
<td>MW 100,000 Da and test water 5NTU bentonite</td>
<td>88.93 (±10.88)</td>
<td>2.62</td>
<td>≈ pH 7.5</td>
</tr>
<tr>
<td>95DD and test water 5NTU kaolinite</td>
<td>19.84 (±12.00)</td>
<td>1.88</td>
<td>≈ pH 7.3</td>
</tr>
<tr>
<td>70DD and test water 5NTU kaolinite</td>
<td>41.98 (±2.73)</td>
<td>2.15</td>
<td>≈ pH 7.2</td>
</tr>
<tr>
<td>Carboxymethyl chitosan</td>
<td>-29.64 (±2.20)</td>
<td>No IEP</td>
<td>pH 8.08</td>
</tr>
<tr>
<td>test water 5NTU kaolinite</td>
<td>ζ -26 mV at 50 mg/L</td>
<td>at dose 50 mg/L</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>Chitosan HCl and test water 5NTU kaolinite</td>
<td>30.37 (±10.07)</td>
<td>2.19</td>
<td>≈ pH 7.2</td>
</tr>
</tbody>
</table>

3.4 Discussion

**Molecular weight of chitosans and turbidity removal**

Overall, the molecular weight (MW) of chitosan polymers influenced removal of turbidity from water and the higher MW chitosans were more effective than the lower MW chitosans. However, MW had minimal impacts on the optimum dose for coagulation and for most MW chitosans their optimum dose was 3 mg/L except MW 5,000 daltons.

Chitosans of higher MW, from 50,000 to 1,000,000 daltons, provided bentonite reductions from 80 – 99% between dose 1 - 10 mg/L. At the optimum dose, effectiveness of chitosan on bentonite removal was not improved with increasing MW above 100,000 daltons. Overall, higher MW chitosans were most effective; the smallest MW (5,000 daltons) performed poorly with less than 50% removal at every dose tested. Roussy et al (2005) also found similar results, reporting that bentonite removal increased as MW of chitosans increased but leveled off

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**Figure 3.21** Zeta potentials and isoelectric points (IEP) of turbidity coagulation by chitosan MW 100,000 daltons (A) and chitosan HCl (B)
above 100,000 daltons. In coagulation-flocculation using polymer coagulants, the size of the polymer plays a crucial role; larger size polymer chains can attach to more particles in water, forming bridges between polymers and particles. Chen et al (2003) observed better bentonite removal with higher MW chitosan, and the highest MW tested (300,000 Da) gave the best bentonite removal with $\approx 92\%$ at dose 2.5 mg/L whereas the smallest MW tested (27,900 Da) failed to remove bentonite. The authors also found that chitosan MW influenced bentonite removal more than chitosan DD. Since DD is related to charge density of chitosan (Renault et al, 2009) and MW corresponds to interparticle bridging (Faust & Aly, 1998), they concluded that the bridging mechanism was more important for bentonite coagulation than charge neutralization (a state at which a net electrical charge of colloidal particle is neutralized or lacks charge, resulting from association with an equal number of opposite charges). Our results also suggest that interparticle bridging may be a key mechanism of chitosan for removal of both bentonite and kaolinite turbidity at near neutral pH of water. MW of chitosans also influenced turbidity removal more than DD. Larger MW chitosans gave higher turbidity removal than lower MW chitosans, suggesting an important role for interparticle bridging between chitosans and clay particles in water.

**Degree of deacetylation of chitosan and turbidity removal**

According to the observed results, degree of deacetylation (DD) had minimal impact on turbidity removal at the same chitosan dose. At the optimum dose of 3 mg/L, removal of bentonite and kaolinite were not influenced by different DDs of chitosans. At this chitosan dose, all DD chitosans produced approximately 99% bentonite removal with residual turbidity $<5$ NTU, and approximately 90% kaolinite removals with residual turbidity $\leq 7$ NTU. Effects of DD on kaolinite removal at doses other than the optimum were variable. There was better
kaolinite removal by lower DD at dose of 1 mg/L, however at higher dose of 10 mg/L, higher DD were more effective than lower DD.

Degree of deacetylation (DD) indicates the amount of amino groups (-NH2) along the chitosan chain. These amino groups help create cationic sites along chitosan chains due to their protonation when dissolved in acidic solution. Positively charged chitosan sites attach to negatively charged turbidity constituents, resulting in removal. Because higher DD results in higher positive charge on the polymer, higher removal of bentonite and kaolinite would be expected from higher DD chitosans. However, our results showed minimal effects of different DD on turbidity removal. Chen et al (2003) used the pyrene-fluorescence probe method to study polarization of the microenvironment of chitosans due to chitosan DD and inferred the pyrene-fluorescence spectrum to their results on bentonite removal. They reported that varying chitosan DD had little effect on polarization of the microenvironment around chitosans and also had limited effects of chitosan DD on bentonite flocculation. Similar to their study, in our study it was found that the measured zeta potential of all chitosan DDs (70% - 95%) varied but did not differ significantly. This indicates that at similar MW, chitosan polymers possess equivalent electrical charge even though the DDs or number of –NH2 groups are different. As a result, different DD chitosans produced similar turbidity removals at the optimum dose. This observation suggests that charge neutralization is not the major mechanism for coagulation using chitosan coagulant. Possible reason for this outcome is that chitosans were not totally protonated at neutral water pH so that varying DD exhibited similar results. Another possible reason for the outcomes obtained is that coagulation using chitosan might need interaction between negative charges of colloids and positive charges of chitosan but it does not require complete charge neutralization. Roussy et al (2005) also suggested that complete charge neutralization was not the
major mechanism of colloidal coagulation. They tested chitosan on bentonite removal then calculated the amount of positive charges required for complete charge neutralization based on cationic exchange capacity (CEC) of bentonite at the concentration in the coagulation system. They found that effective coagulation occurred although amounts of positive charges from chitosan were much less than the amounts of negative charges of bentonite and therefore less than the amount required to induce complete charge neutralization. All these results suggested that charge neutralization was not the main mechanism of bentonite and kaolinite coagulation by chitosans at neutral water pH.

**Water-soluble modified chitosans and turbidity removal**

Overall, water-soluble (modified) chitosans performed similarly to acid-soluble (unmodified) chitosans. They efficiently removed bentonite turbidity between dose 1 – 10 mg/L and removed kaolinite at dose 3 mg/L. Chen and Chung (2011) reported that the optimum dose range of water-soluble chitosan for bentonite removal was broader than that of acid-soluble chitosans. However, in this present study similar optimum dose ranges were observed for bentonite removal between water-soluble and acid-soluble chitosans. As observed for acid-soluble chitosan, water-soluble modified chitosans demonstrated better removal of bentonite compared to kaolinite turbidity. However, the turbidity removal efficiency of each water-soluble chitosan varied. Among the modified chitosans tested, chitosan hydrochloride (HCl), chitosan acetate and chitosan lactate were more effective than carboxymethyl chitosan. Chitosan HCl produced constantly high bentonite removals (≈ 87% - 99%) between doses of 1 – 30 mg/L. Its overall performance was better than the other water-soluble chitosans tested and exhibited highest kaolinite removal at optimum dose 3 mg/L. Also, it could effectively remove kaolinite (85%) at a higher dose of 10 mg/L. The performance of chitosan HCl may relate to the –HCl
molecules that are added to the chitosan structure. HCl is a strong acid and has low pKa (<1) which means it is totally protonated in water. In water, HCl on chitosan chains will be ionized and react to water molecules to create $\text{H}_3\text{O}^+$ (shortly H$^+$). This reaction possibly creates a mildly acidic microenvironment around chitosan chains, which may benefit turbidity removal. This strong acid property together with high zeta potential ($\approx 30.37\ (\pm 10.07)\ \text{mV}$) may be factors helping the chitosan HCl molecules to react strongly with clay particles, providing high turbidity removal. Carboxymethyl chitosan which has a carboxymethyl group (-CH$_2$COOH) added to the polymer chain was the poorest for turbidity removal among water-soluble chitosans. The carboxymethyl group has a pKa of 4.64 but carboxymethyl chitosan possesses a high negative zeta potential of -29.64 ($\pm 2.20$) mV. The carboxymethyl group helps increase the solubility of the chitosan in water which also increases its availability to interact with other molecules. However, the high negative charges of this modified chitosan may repel negatively charged colloidal particles in water, resulting in poor removal of turbidity. Chitosan acetate has the acetate functional group (-CH$_3$COO$^-$) from acetic acid (pKa 4.75). Despite the fact that the pKa of acetic acid is close to the pKa of carboxymethyl group, the electrical charges and efficiency of turbidity removal of chitosan acetate and carboxymethyl chitosan were different. Chitosan acetate has a higher positive charge (43.08 ($\pm 20.00$) mV) than carboxymethyl chitosan (-29.64 ($\pm 2.20$)), which may facilitate its ability to coagulate and remove negatively charged clay turbidity. Chitosan lactate with the lactate group (-CH$_3$CH (OH) COO$^-$) from lactic acid (pKa 3.86) has a positive charge of 16.50 ($\pm 9.67$) mV. This positive charge is considered important for coagulation of turbidity by helping coagulant attachment to negatively charged turbidity colloids even though complete charge neutralization is not necessary, as was described before. Both chitosan acetate and chitosan lactate were effective in turbidity removal and performed similar to
chitosan HCl even though overall performance were less efficient than chitosan HCl. The observed results suggest that positive charges are crucial for water-soluble (modified) chitosans in coagulation of bentonite and kaolinite, but pKa of functional groups added onto the modified chitosan structure is unlikely to be significant indicator for efficiency of turbidity reduction by that modified chitosan. The pKa of functional groups added to chitosan molecules had variable impacts and gave no clear trend on turbidity removal, as seen from the turbidity reduction results of carboxymethyl chitosan and chitosan acetate. With similar pKa, chitosan acetate was effective for turbidity removal but carboxymethyl chitosan was not effective for turbidity removal at all.

Positive charge expressed as a zeta potential value, however, is important, as the results illustrated that chitosans with positive zeta potential were effective for removal of bentonite and kaolinite turbidity but chitosan with negative zeta potential was not effective. However, magnitude of positive zeta potential showed less impact on the efficiency of turbidity reduction by chitosans. For example, chitosan HCl with $\zeta = 30.37 \pm 10.07$ mV was as effective as MW 100,000 Da chitosan with $\zeta = 88.93 \pm 10.88$ mV in turbidity removal.

**Effects of chitosan doses on turbidity coagulation**

Overall, optimum dose for turbidity removal was 3 mg/L and the highest kaolinite and bentonite removals occurred at this dose. Increasing dose of chitosan especially from 1 -10 mg/L generally was effective for bentonite removal (approximately 80 – 99% removal and residual turbidity $\leq$ 5 NTU). Once chitosan dose exceeded 10 mg/L, bentonite removal dropped sharply. Kaolinite coagulation was more sensitive to chitosan doses than bentonite. Generally, removal of kaolinite occurred at only low chitosan doses of 1 and 3 mg/L ($\approx 82 – 93\%$ and residual turbidity down to 3-7 NTU although some chitosans exhibited some kaolinite removal at dose 10 mg/L. When chitosan dosage exceeds the optimum dose for turbidity coagulation, the surplus chitosan
may interfere with the attachment between chitosan and turbidity resulting in decreased removal and increased residual turbidity. Surplus chitosan molecules possibly surround turbidity particles, inducing the microenvironment around them to exhibit positive charge and causing repulsion between free chitosan molecules in water (that could attach to bentonite or kaolinite) and chitosan molecules surrounding clays. As a result, attachment between chitosan molecules and clays is inhibited, and further coagulation does not occur. In the coagulation process, doses of a coagulant influence colloid destabilization, a process in which the energy barrier of colloids is overcome so colloids become less stable and become easier to aggregate and then physically remove from water. At the optimum dose, destabilization of colloids occurs maximally at the lowest possible coagulant dose resulting in colloid aggregation and then removal from water. At a dose lower than the optimum, destabilization of colloids will not occur and coagulation will fail. Alternatively, if the coagulation dose exceeds the optimum dose, the excess coagulant will surround the colloids causing reversal of electrical charge around them, preventing destabilization and possibly inducing their re-stabilization (Faust & Aly, 1998). Similarly, overdosing of chitosan possibly causes the formation of re-stabilized particles by charge repulsion which prevents chitosan contact with bentonite or kaolinite particles.

**Differences between bentonite and kaolinite coagulation using chitosans**

Bentonite and kaolinite clays reacted differently to chitosan coagulants based on the condition of their removal from water. Chitosan showed a broader effective dose range for removal of bentonite than kaolinite. However, the optimum chitosan dose was similar at 3 mg/L for the highest removals of both bentonite and kaolinite. The coagulation-flocculation reaction of bentonite with chitosan was fast as flocs were large and they formed and settled quickly. Roussy et al (2004) also observed similar results. Kaolinite reacted more slowly with chitosan and
produced smaller flocs at the end of the slow mixing period. Figure 3.22 shows bentonite and kaolinite flocs formed from chitosan coagulation. At the same turbidity, chitosan and bentonite created more flocs than chitosan and kaolinite. Huang & Chen (1996) also reported different behavior of chitosan on bentonite and kaolinite removal. They concluded that chitosan was a potent coagulant for bentonite with optimum dose close to 2 mg/L but it was less effective on kaolinite removal. In this study, chitosan was effective for kaolinite removal but the reaction was more sensitive to chitosan doses compared to the reaction of chitosan and bentonite.

The observed differences in reaction of chitosans with bentonite and kaolinite turbidity may be related to the specific characteristics of these clays. Bentonite has a cationic exchange capacity (CEC) ranging between 0.8 – 1.2 milliequivalents per gram (meq/g), which is much higher than that of kaolinite which has a CEC between 0.03 – 0.15 meq/g (Grim & Kodama, 2014). This difference in CEC is likely to be a reason why bentonite reacts quickly with a cationic polymer like chitosan while kaolinite reacts slowly. Higher CEC possibly helps bentonite react with chitosan by attaching to the chitosan chain better than kaolinite can attach. Furthermore, bentonite has a specific surface area of 40 -800 square meters per gram while that of kaolinite is only 5 – 40 square meters per gram (Grim & Kodama, 2014). Greater clay surface area may increase the chances of interaction between 2 molecules.
A. Kaolinite flocs with chitosan of MW100K at 30 NTU turbidity and pH 7

B. Bentonite flocs with chitosan of MW100K at 30 NTU turbidity and pH 7

C. Kaolinite flocs with chitosan of MW100K at 300 NTU turbidity and pH 7

D. Bentonite flocs with chitosan of MW100K at 300 NTU and pH 7

Figure 3.22 Flocs generated by chitosan coagulation of bentonite and kaolinite turbidity in water

Zeta potentials of chitosans and clays and their roles in mechanisms of turbidity removal

Most of the chitosans tested showed positive zeta potential values, except carboxymethyl chitosan which was negative in potential. Theoretically, a positive zeta potential is preferred for coagulation because it will interact with negative charges of colloids in water to overcome the energy barrier at the colloid surface, destabilizing the colloids. Overall, the positive charge
magnitude of chitosan increased as molecular weight increased, except for MW 1,000,000 daltons, which had lower zeta potential than MW 100,000 and 600,000 daltons. Generally, turbidity removal by different molecular weights of chitosan polymers increased as MW and zeta potential of chitosans increased. Chitosans with very low positive charge may be ineffective for kaolinite and bentonite removal; the smallest chitosan of MW 5,000 Da and with the lowest zeta potential of 6.61 (±5.28) mV performed poorly in removing clay turbidity from test water.

Zeta potential values were not much different among chitosans of different DD although from the zeta potential results obtained, it seemed that chitosans of lower DD had higher positive charge than the higher DD chitosans. However, the zeta potential results were not statistically different across all DD chitosans tested. Zeta potential of water-soluble modified chitosans varied depending on the functional groups added. However, a modified chitosan with negative charges, carboxymethyl chitosan, was ineffective for turbidity removal. All modified chitosans with positively charged functional groups in this study effectively removed clay turbidity, but the effectiveness depended on types of clays and chitosan doses.

Generally, chitosan with higher positive charge produced higher turbidity removal than chitosan with much lower positive charge (close to zero charge) or chitosan with negative charge. However, chitosans with high and moderate positive charge ranging from 16.50 (±9.67) to 88.93 (±10.88) mV in this study, exhibited mixed results for both bentonite and kaolinite removal. Turbidity removal varied and did not correlate with the magnitude of the zeta potential of reaction constituents, thus giving no specific trend between value of positive zeta potential and percent turbidity removal. Using the magnitude of positive charge as a criterion for selection of chitosans for coagulation may not be a reliable parameter to predict turbidity reduction performance.
However, from the results of these coagulation-flocculation studies, a positive zeta potential of chitosan was observed to be crucial for effective turbidity coagulation even though the magnitude of the positive charge did not show a clear trend relating to chitosan efficacy in bentonite and kaolinite turbidity reduction. However, only positively charged chitosans produced removal of bentonite and kaolinite turbidity and removal efficacy varied among chitosan MWs and doses. Chitosan with very low positive charge such as chitosan MW 5,000 Da (6.61 (±5.28) mV), was ineffective for turbidity removal at low chitosan doses. However, the turbidity removal efficiency of this chitosan was improved at a high chitosan dose of 30 mg/L, possibly because this dose provided sufficient positive charges to overcome the energy barrier of the colloids to achieve their destabilization. Based on these results, charge neutralization appears to be an important mechanism for coagulation of turbidity by chitosans at neutral pH, but not solely. From the titration graphs it is seen that the negative zeta potentials of turbidity suspension decreased as positively charged chitosans were added and charges became zero at the IEP. Different chitosans produced an IEP at slightly different doses but all were close to the optimum dose of 3 mg/L as determined by jar test coagulation experiments. Above the optimum dose and the isoelectric point (IEP), turbidity removal decreased. At these conditions, it is possible that positive charges from the surplus chitosan saturated the clay surfaces causing reversal of charges and then restabilization (Huang & Chen, 1996). In this study, chitosans were tested in water at near neutral pH so it is possible that the interparticle bridging mechanism rather than charge neutralization plays a crucial role in coagulation and clay turbidity reduction. Roussy et al. (2004) also reported these dual mechanistic phenomena for the coagulating-flocculating effect of chitosan on clay turbidity. They suggested that charge neutralization was a coagulation mechanism at acidic pH of water, and interparticle bridging was a mechanism at neutral or mild
basic pH of water. In the present study the observed coagulation results for chitosan of MW 5,000 daltons indicated that complete charge neutralization was not the major mechanism for mineral clay coagulation. Bentonite and kaolinite turbidity removals at maximum were only 46.2% and 79.8%, respectively, even though the net zeta potential of the coagulation system showed negative values. Therefore, it can be inferred that chitosan produced turbidity removal by mechanisms other than charge neutralization. This smallest size chitosan of 5,000 daltons MW provided the highest removals of the mineral clays at the higher dosages of 10 mg/L for bentonite (46.2% removal) and 30 mg/L for kaolinite (79.8% removal). At these doses, the measured zeta potentials were still negative value between -9.63 mV and -2.63 mV. From these results, it is possible that interparticle bridging was a main coagulation mechanism.

3.5 Conclusions

- Chitosan molecular weight had significant effects on the efficacy of bentonite and kaolinite turbidity reduction from test water and the observed results suggested that higher molecular weight chitosans were more effective than lower molecular weight chitosan.
- The degree of deacetylation (DD) of chitosans had lesser impacts on the efficacy of bentonite and kaolinite turbidity removal from test water than did their molecular weight. At optimum chitosan doses, removals of bentonite and kaolinite turbidity by chitosans having different degrees of deacetylation were not significantly different.
- Chitosan dose had significant effects on bentonite and kaolinite turbidity reduction from test water. Only low doses of chitosan were needed for effective removal of kaolinite and bentonite turbidity from water, with chitosan doses for ≥ 80% turbidity removal ranging
between 1 – 10 mg/L for highest removals of kaolinite (93%) and bentonite (99%), respectively. Generally, a higher chitosan dose of > 10 mg/L was less effective and provided poorer bentonite and kaolinite turbidity removal than did lower doses.

- Acid-soluble chitosans were as effective as water-soluble chitosans for bentonite and kaolinite turbidity removals, with observed removals of 87 to 99%.
- The optimum dose range of acid-soluble chitosans for effective bentonite and kaolinite turbidity removal was as broad as that of water-soluble (modified) chitosans, with chitosan dose optima between 1-10 mg/L.
- Chitosans with positive zeta potential were effective for bentonite and kaolinite turbidity coagulation from water. Chitosans with negative zeta potential were not effective for bentonite and kaolinite turbidity removal from water.
- There was no clear trend of an effect from the magnitude of chitosan positive charge on bentonite and kaolinite turbidity removal from test water.
- The interparticle bridging mechanism was found to have a crucial role in bentonite and kaolinite turbidity coagulation using chitosans as turbidity removal increased with increasing chitosan molecular weight.
- Charge neutralization was also found to play a role in bentonite and kaolinite turbidity coagulation by chitosans because only positively charged chitosans but not negatively charged ones exhibited removals.
- The measured isoelectric points of chitosans on bentonite and kaolinite turbidity coagulation from water were close to the optimum chitosan doses obtained from jar test coagulation-flocculation experiments.
Chitosans as biopolymers with demonstrated coagulant properties have potential to serve as alternative coagulants of water at household and community levels, based on the results obtained in this study.
4.1 Introduction

Microbially contaminated drinking water can cause diarrheal and other enteric diseases especially in children age under 5 years old and immune compromised persons. However, supply of safe drinking water remains a challenging task in many developing countries. Nearly 800 million people, mostly in developing countries, lack access to improved sources of drinking water and are exposed to contaminated water. Advanced water treatment plants and distribution systems require substantial resources and time to develop. Meanwhile, appropriate technology to produce safe drinking water is needed immediately in many places. For microbial control measures of drinking water, the processes of coagulation, filtration and disinfection are important treatment barriers. Also, these three technologies can be used at home, either individually or together, as a stand-alone or a multi-barrier system to ensure microbial safety of water for people without access to conventional community water systems.

Coagulation is a simple physical-chemical technology that can be used to produce drinking water efficiently and safely. Like other suspended particles in water, bacteria and viruses in water also behave as colloids (Gerba, 1984), so coagulation can be employed to produce microbially safe drinking water at homes. However, the efficiency and safety of this technology mainly depends on the coagulant used in the coagulation-flocculation process and the achievement of an effective dose in the water. So, the first step is to select an appropriate
Coagulant and dose for household water treatment. Coagulation generally can remove microbes (bacteria, viruses and protozoa) by 1-2 logs, however performance may be highly variable depending on process conditions. Effects of coagulation on each type of microbes vary. Data about the performance of water treatment plants collected from various countries showed that for coagulation and sedimentation, average removal ranged between 27-74% for viruses, 32-87% for bacteria, and 0-94% for algal cells (WHO, 2004). However, stand-alone coagulation, flocculation and sedimentation at household level were not reported at that time. Nevertheless, coagulation of water at household level has been reported previously, using either inorganic coagulants such as salts of aluminum or iron and natural organic materials such as the contents of plant seeds such as those from *Moringa oleifera* (Sobsey, 2002). However, there is a lack of evidence documenting effective sustained performance in reducing microbial contamination of water using these coagulants for household water treatment. Although other naturally occurring organic polymers are also candidate coagulants, such as cactus plant extracts and chitosans, there is a lack of information on their use and effectiveness in microbial reduction from water.

In considering chitosan as a chemical coagulant of particles and microbes in water, there are two important chemical characteristics of chitosan to consider: molecular weight (MW) and degree of deacetylation (DD). These two parameters influence the physical-chemical characteristics of chitosan, such as its solubility, viscosity and electrical charge properties, and therefore, are important determinants of potential effectiveness in applications for water coagulation. For microbial removal by coagulation, MW is important as it influences the bridging mechanism of coagulation. Degree of deacetylation (DD) indicates the number of amino groups (-NH$_2$) along the chitosan chain. These amino groups help create cationic sites along chitosan chains, helping the charge neutralization mechanism of coagulation and
encouraging contact between negatively charged microbial particles and positively charged coagulant molecules. Therefore, variations in the molecular structure of chitosan, chiefly DD and MW, are expected to have effects on bacterial and viral removal through their influence on the dominant physical-chemical mechanisms of coagulation.

Studies on the effects of different MW of chitosan on microbial coagulation in water treatment processes are limited. Strand et al. (2002) evaluated efficiency of 3 chitosans—of MW 180,000 Da, 220,000 Da and 290,000 Da—for flocculation of different bacteria. The authors concluded that higher MW chitosans were more effective for flocculation of gram negative bacteria while lower MW chitosans were more effective for gram positive bacteria. The authors suggested that not only characteristics of chitosans but also types of microbes affected microbial removal. However, mechanisms of chitosan MW on removal of these microbes are unclear. From our experiments on turbidity removal, we observed that chitosans with high MWs were more effective for turbidity removal, and therefore we hypothesize that higher MW chitosans would exhibit similar results for microbial reductions. Three chitosans with MW ranging from approximately 50,000 – 1,000,000 daltons that showed extensive turbidity removal in our turbidity experiments were selected to study microbial reductions.

When dissolved, especially in acidic solution, protonation of the amine group on the chitosan chain will occur. As a result, chitosan exhibits positive surface charge, allowing it to readily react with negatively charged microbial cell surfaces and also viruses, thereby enhancing microbial reduction. However, Strand et al. (2001) studied different DD of chitosan hydrochloride (chitosan-HCl) on bacterial flocculation and indicated that at a lower DD (38%), the dose of chitosan-HCl necessary to flocculate *E.coli* in bacterial suspension was 10 times less than that of a higher DD (99%). They concluded that flocculation efficiency increases with
decreasing DD. However, this study investigated effects of chitosan DD after modification of the chitosan polymer to chitosan-HCl. Therefore, it was not clear whether coagulation efficacy was actually influenced by degree of deacetylation or by the use of a modified form of chitosan. The role of the DD of chitosan polymer in microbial removal from water needs to be further studied. Our previous study showed that DD had minimal impacts on turbidity removal and at optimum doses effects of DD on turbidity removal were not significant. Three different DDs (70, 85, and 95%) were selected for investigating the impact of low to high DD on bacteria and virus removals.

Modified chitosans, as previously mentioned, are water-soluble chitosans that easily dissolve in water over a broad pH range. This characteristic can benefit household water treatment because the liquids are easy to handle and store. Results from turbidity experiments, showed that modified (water-soluble) chitosans which possessed positive zeta potential exhibited high turbidity reductions (≈85-99%). Therefore, three modified chitosans, chitosan acetate, chitosan lactate, and chitosan hydrochloride, were chosen to test for microbial reduction efficiency by coagulation.

In this study the efficacy of chitosan coagulation was examined for the effects of different MW and DD of chitosan polymers on removal of bacteria (E. coli) and virus (coliphage MS2) using the jar test method, which was compared with removals by water-soluble modified chitosans.
4.2 Materials and Methods

Chitosan stock solutions

A total of 9 commercially available chitosans were tested: 6 acid-soluble chitosan polymers and 3 water-soluble modified chitosans. The information regarding MW and DD of all chitosans were obtained from the chitosan vendors. For effects of molecular weight on microbial coagulation, 3 chitosan polymers with different molecular weight and similar DD (≈90%) were compared: 50,000, 100,000, and 1,000,000 daltons (Da). These chitosans with different MW were bought from Heppe Medical Chitosan Gmbh (Halle, Germany) and Acros Organics (Bridgewater, NJ, USA). To test the effects of degree of deacetylation (DD), another set of chitosan polymers with different DD and approximately the same MW (≈ < 50,000 Da) with viscosity of 5 mPa·S was used: 70, 85, and 95% DD. These chitosans with different DD were bought from Heppe Medical Chitosan Gmbh (Halle, Germany). Three water-soluble modified chitosans which were bought from Heppe Medical Chitosan Gmbh (Halle, Germany): chitosan acetate, lactate, and HCl, were tested. These chitosans were chosen because they exhibited high turbidity removal (both bentonite and kaolinite) at low doses. Stock solutions of chitosans were made for all polymers at concentration 10,000 mg/L. Chitosan powder was dissolved in 0.5% acetic acid and stirred at room temperature until totally dissolved. Stock solutions were stored at room temperature (25°C). The stock solutions of modified (water-soluble) chitosans were prepared similarly, using deionized water instead of acetic acid. Characteristics of the tested chitosans (MW, DD, and viscosity) including zeta potential values obtained from measurement in this study were summarized in Appendix 11.
Test water

The test water was artificial surface water prepared based on the recommended parameters from USEPA, NSF and WHO for efficacy testing of POU technology as listed in Appendix 3 (EPA, 1987; NSF, 2008; WHO, 2011). For testing of microbial removal, the test water was created by spiking dechlorinated tap water with: 300 mg/L of total dissolved solids (TDS), 3 mg/L of total organic carbon (TOC) and 5 NTU of turbidity. Sodium chloride (NaCl), tannic acid and kaolinite were used as the added material for TDS, TOC and turbidity, respectively. Kaolinite is a type of clay commonly used as turbidity source. Results from our turbidity removal experiments showed that kaolinite had a narrow effective dose range and was more difficult to remove from water than bentonite clay, so it is not likely to interfere with microbial coagulation. Water pH was not adjusted after adding clay, NaCl, and tannic acid and water pH ranged pH 7 – 7.5. The test water was stirred overnight then simultaneously seeded with *E. coli* bacteria (target concentration $10^9$ CFU/ml) and bacteriophage MS2 (target concentration $10^{10}$ PFU/ml) and mixed for 30 minutes before starting the jar test. Bacterial culture was originally from frozen stock which was thawed and grown overnight in tryptic soy broth (TSB), then 100 µl of overnight culture was transferred to 50 ml fresh TSB media and incubated at 37°C to reach log phase. The log phase culture was centrifuged at 3000xG, 4°C for 10 minutes. The bacterial pellet of the 50 ml culture was re-suspended in 4 milliliters of test water and seeded into test water (5 liters) for the jar test. Viruses were from frozen stocks which were thawed at room temperature then 4 ml of virus stocks were seeded into 5 L of test water without dilution. Test water containing bacteria and virus was stirred continuously for 30 minutes before chitosan coagulation. The titer of virus and bacteria was evaluated as initial concentration before jar test experiment. Frozen stock of *E. coli* was prepared by growing *E. coli*
in 50 ml TSB overnight at 37°C with shaking, and the overnight culture was centrifuged at 3000xG, 4°C for 10 minutes. The bacterial pellet was recovered and re-suspended in 10 ml TSB with 20% glycerol; the stock was kept at -80°C. Frozen stock of bacteriophage MS2 was prepared on soft agar: 1 ml of MS2 with 1 ml of host bacteria (E. coli Famp) was grown on 30 ml soft agar overnight at 37°C. On the next day, top layer of the agar which had MS2 and host bacteria was recovered and placed into a centrifuge tube. Chloroform was added into the tube to equal half of the volume and the mixture was shaking vigorously for 5 minutes. Then the mixture was centrifuged at 3000xG, 4°C for 20 minutes. The top aqueous layer of supernatant was recovered to a new bottle then centrifugation was repeated by adding PBS to equal half of the volume of the remaining chloroform. Top layer was removed for second time and was added to the previously recovered aqueous layer. Then mixture which contained MS2 was poured into a large petri dish placing under the sterile hood for 30 minutes. After chloroform evaporated, the liquid contained MS2 was mixed with glycerol to equal 20% of the total volume of virus stock and kept at -80°C.

**Jar test experiment**

A jar test method of coagulation was used. The jar test is a common approach used for determining coagulation chemistry and for monitoring coagulation performance (WHO, 2004). All experiments were conducted at room temperature (25°C). Upon dosing with chitosan, the mixing conditions were: rapid mixing at 100 revolutions per minute (rpm) for 1 minute followed by slow mixing at 25 rpm for 15 minutes and then settling unmixed for 30 minutes. Supernatant was recovered at 2 centimeters from the top of the beakers for microbial and turbidity analysis using a pipette without disturbing the flocs. Infectivity assays were used to measure
concentrations and calculate reductions of \textit{E. coli} and MS2 by coagulation. Membrane filtration (MF) on MacConkey agar was used for \textit{E. coli} colony enumeration as described in the Standard Methods for the Examination of Water and Wastewater (APHA, AWWA & WAF, 1999). Phosphate buffered saline (PBS) was used as diluent for serial dilution. The MF on MacConkey agar was incubated at 37°C for 22 to 24 hours before counting colonies. The double agar layer (DAL) plaque assay with host bacteria \textit{E. coli F}_{amp} was used for enumeration of coliphage MS2 plaques, similar to EPA method 1601 (EPA, 2001). PBS was used as diluent for serial dilution of MS2 samples. Turbidity was measured by a turbidity meter (Hach 2100AN Turbidimeter, Hach, Loveland CO). Water pH was also measured before and after the jar test experiment with a pH meter. Appendix 4 and 5 show experiment protocols for bacteria and virus coagulation.

Doses of chitosans were: 1, 3, 10 and 30 mg/L. These doses were selected because they were in the ranges of optimum doses of conventional coagulants (2-5 mg/l for aluminum salts and 4-10 mg/L iron salt coagulants) (WHO, 2011), and encompassed the optimal dose range for turbidity removal by chitosans, as demonstrated in previous experiments. Three replicates were performed for each chitosan. Each replicate included four chitosan doses plus one control containing no chitosan for natural settling.

**Zeta potential measurement**

A Malvern Zetasizer nano ZS (Malvern, Worcestershire, UK) was used to study zeta potential of \textit{E. coli} bacteria and bacteriophage MS2 stock. Test water containing \textit{E. coli} and bacteriophage MS2 were also measured. Test water having 5 NTU kaolinite turbidity was seeded with \textit{E. coli} or MS2 and was prepared similar to the test water for jar test experiment. Zeta potential of \textit{E. coli} bacteria stock was measured in tryptic soy broth (TSB). Zeta potential of
MS2 was measured in a stock solution containing a mixture of phosphate buffer saline (PBS) and 20% glycerol. Each sample was evaluated for its zeta potential by Malvern Zetasizer nano ZS. Three replicates were performed for each sample.

**Data analysis**

Microbial removal was calculated as $\log_{10}$ reduction compared to the concentrations of experimental contaminants in the same water without added chitosans subjected to mixing and settling as shown below:

$$\text{Log}_{10} \text{ reduction} = \text{Log}_{10} \left( \frac{N_t}{N_0} \right)$$

When: $N_t$ = number of microbes in sample of supernatant from test beaker after jar test  
$N_0$ = number of microbes in sample of supernatant after jar test from control beaker of test water without added chitosans

Turbidity removal from coagulated water was calculated as percent turbidity removal relative to the control water after mixing and settling as shown below:

$$\text{Percent removal} = \left[1 - \frac{\text{sample turbidity}}{\text{control turbidity}} \right] \times 100$$

Statistical comparison of the effects of different chitosans on microbial removal was performed using GraphPad Prism (GraphPad, San Diego, CA). Two-way ANOVA and Tukey posttest analysis was employed for comparing effects of both chitosan types and chitosan doses and one-way ANOVA was used for comparing effects of chitosan doses of each chitosan tested.
4.3 Results

4.3.1 Bacteria coagulation from water by chitosans

I. Effects of chitosan molecular weight (MW) on bacteria removal

Chitosans of three MWs, 50,000, 100,000, and 1,000,000 daltons (Da), that were selected for their efficacy in removing bentonite and kaolinite turbidity, were tested for their efficacy in removing a representative enteric bacterium (*E. coli*) and a surrogate for enteric viruses (bacteriophage MS2) from test water representative of surface water used for drinking. The effects of chitosan MW on bacterial removal at varying doses are shown in Figure 4.1. Results suggested that both MW and dose influenced removal of bacteria from water (2-way ANOVA, p <0.0001). The medium size chitosan (MW 100,000 Da) exhibited higher bacterial removal than the smaller size (MW 50,000 Da) and the larger size (MW 1,000,000 Da). Bacterial removal was higher at dose of 3, 10 and 30 mg/L than the dose of 1 mg/L, and the highest bacterial removal occurred at dose 10 mg/L.

**Chitosan Doses 1 and 3 mg/L**

All 3 MW chitosans showed lower bacterial removal at doses of 1 and 3 mg/L than at 10 and 30 mg/L. At dose 1 mg/L, all three MW chitosans gave approximately 1 log₁₀ reduction of *E. coli*. When chitosan dose was increased to 3 mg/L, all 3 MW chitosans showed increasing bacterial removals (1.1-2.3 log₁₀). Chitosan MW 100,000 Da had the highest bacterial removal of 2.3 log₁₀ at this dose, with chitosan MW 50,000 Da and chitosan MW 1,000,000 Da exhibiting lower bacterial removals of 1.6 log₁₀ and 1.1 log₁₀, respectively, as shown in Figure 4.1. However, the differences in log₁₀ bacteria reductions among these different MW chitosans at these doses were not statistically significant (p > 0.05).
Chitosan Doses 10 and 30 mg/L

Overall, bacterial removal was greater at chitosan doses of 10 and 30 mg/L compared to 1 and 3 mg/L doses. Chitosan MW 100,000 Da provided the highest bacterial log$_{10}$ reduction (4.51 log$_{10}$ = 99.9969%) at dose 10 mg/L, significantly higher than other MW chitosans (p <0.001) at the same dose, (1.99 log$_{10}$ and 1.66 log$_{10}$ at MW 50,000 and 1,000,000, respectively). Chitosan 100,000 MW also provided high bacterial removal of 3.7 log$_{10}$ at dose 30 mg/L. However, *E. coli* removal by chitosan MW 100,000 was not significantly different between doses of 3, 10 and 30 mg/L (p > 0.05). The smallest size chitosan, MW 50,000 Da, and the largest size chitosan, MW 1,000,000 Da, also showed similar log$_{10}$ bacterial reductions at each dose (p > 0.05), with reductions about 2 log$_{10}$ for both 50,00 MW chitosan and 1,000,000 MW chitosan at dose 10, and 30 mg/L.

**Figure 4.1** Effects of molecular weight (≈ 90% DD) and doses of chitosans on bacteria log$_{10}$ reductions by coagulation
II. Effects of degree of deacetylation (DD) on bacteria removal

Three DD chitosans (70%, 85% and 95%) with comparable MW (approximately 5 mPa·s viscosity \( \approx < 50,000 \text{ Da} \)) were tested. Both degree of deacetylation (DD) and doses of chitosans showed significant effects on bacterial reduction (2-way ANOVA, \( p < 0.0001 \)), as shown in Figure 4.2. Overall, chitosan with higher DD exhibited higher bacteria removal. Higher bacterial reductions were also achieved at doses higher than 1 mg/L.

**Chitosan Doses 1 and 3 mg/L**

At dose 1mg/L, DD of chitosans had no difference in bacterial removals; all 3 DD chitosans (70%, 85% and 95%) had similar \( E. \ coli \) removals of approximately 1 log\(_{10}\) (90%). Bacterial removal increased to \( \geq 2 \log_{10} \) at dose 3 mg/L for higher DD chitosans (85% and 95%), and chitosan 95% DD had the highest \( E. \ coli \) removal of 2.86 log\(_{10}\). However, the low DD chitosan (70%) still showed 1 log\(_{10}\) \( E. \ coli \) reduction at both 1 and 3 mg/L dose.

**Chitosan Doses 10 and 30 mg/L**

Log\(_{10}\) reduction of bacteria was higher at chitosan doses of 10 and 30 mg/L than 1 and 3 mg/L. The highest DD chitosan (95%) gave the highest bacterial removals of approximately 3 – 4 log\(_{10}\) at doses 3, 10 and 30 mg/L. These removals were significantly greater than \( E. \ coli \) removals produced by chitosans with lower DD, particularly 70% DD chitosan at the same doses (\( p < 0.001 \)), for which removals were about 1 - 2 log\(_{10}\). Overall, chitosan 95% DD gave bacteria reductions of about 3 - 4 log\(_{10}\) with no statistically significant differences between doses 3, 10 and 30 mg/L (\( p > 0.05 \)). The low DD chitosan (70%) and the medium DD chitosan (85%) showed similar log\(_{10}\) reductions of about 2 log\(_{10}\) for bacteria, with no significant difference at dose 10 mg/L (\( p > 0.05 \)). However, chitosan 85% DD was greater than chitosan 70% DD for \( E. \)
coli removal at the high dose of 30 mg/L, with 2.87 log_{10} reduction compared to 1.99 log_{10} reduction, respectively.

![Figure 4.2](image)

**Figure 4.2** Effects of degree of deacetylation and doses of chitosans on bacteria log_{10} reductions by coagulation

**III. Effects of modified chitosans on bacteria removal**

Three modified chitosans, chitosan acetate, chitosan lactate and chitosan HCl, which exhibited high turbidity removal previously were selected for evaluation of bacterial reduction by jar test coagulation.

Results, as shown in Figure 4.3, indicated that doses of chitosans showed significant effects on bacterial removal from water (2-way ANOVA, p <0.0001). However, each modified chitosan exhibited similar removal of bacteria at the same doses (p = 0.1963).
Chitosan Doses 1 and 3 mg/L

Bacterial removals were low at chitosan doses of 1 and 3 mg/L. At 1 mg/L dose, chitosan acetate gave the highest bacterial removal (1.32 log\(_{10}\)) while chitosan lactate and chitosan HCl exhibited < 1 log\(_{10}\) reductions. However, at dose 3 mg/L, chitosan lactate and chitosan HCl gave higher bacteria reductions of about 1.2- 1.3 log\(_{10}\) than the 0.8 log\(_{10}\) reduction of chitosan acetate. Overall, the effects of all 3 modified chitosans on *E. coli* removals were not statistically significantly different at the same dose, both at 1 and 3 mg/L doses (p > 0.05), with reductions of about 0.6 to 1.3 log\(_{10}\).

Chitosan Doses 10 and 30 mg/L

Bacterial removals were higher at the higher chitosan dose of 10 mg/L but they were lower at the 30 mg/L dose as shown in Figure 4.3. At dose 10 mg/L, all 3 modified chitosans showed the highest reduction of *E. coli* ranging from 3 – 4.28 log\(_{10}\) reduction. Chitosan HCl gave the highest *E. coli* reduction of 4.28 log\(_{10}\) at this dose, which was significantly higher than that of chitosan lactate at 3 log\(_{10}\) (p < 0.01) but was statistically similar to that of chitosan acetate at 3.45 log\(_{10}\) reduction (p > 0.05). When applied at high dose of 30 mg/L, all 3 modified chitosans had similar effects on *E. coli* reductions (p > 0.05), with average reductions lower at approximately 2 log\(_{10}\) .
Effects of modified chitosans on \textit{E. coli} removal

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Effects of types and doses of modified chitosans (DD 80-95\%, viscosity 2-300 mPa\cdot S) on bacterial coagulation}
\end{figure}

**Comparisons of all 9 chitosans for bacteria removal by coagulation**

\(\log_{10}\) reductions of \textit{E. coli} bacteria by coagulation with all candidate chitosans as well as turbidities of coagulated and settled test water are shown in Figure 4.4. The 10 mg/L chitosan dose appears to be the optimum for bacterial removal, with 100,000 Da chitosan giving 4.51 \(\log_{10}\) reduction, followed by 4.28 \(\log_{10}\) reduction by chitosan HCl and 3.45 \(\log_{10}\) reduction by chitosan acetate. These reductions were not different statistically (p>0.05). Turbidity removals from test water originally with 5 NTU kaolinite turbidity plus \textit{E. coli} occurred at low chitosan doses 1 of and 3 mg/L with approximately 60 -70\% removal. However, at the 10 mg/L chitosan dose, where bacterial reductions were highest, turbidity removals by most chitosans were poor. Only chitosan 85\% DD and chitosan HCl exhibited appreciable turbidity removals of 50 -60\% at this dose (see Appendix 8).
Figure 4.4 *E. coli* removal by different doses of chitosans having different molecular weight (MW 50,000 – 1,000,000 Da), degree of deacetylation (DD 70-95%) and with chemical modifications
4.3.2 Virus coagulation from water by chitosans

I. Effects of chitosan molecular weight (MW) on virus removal

The same MW chitosans used for bacterial coagulation were also used for virus coagulation. Results for the effects of 50,000, 100,000, and 1,000,000 MW dalton (Da) chitosans on reduction of MS2 virus at varying doses are shown in Figure 4.5.

Different molecular weights (MW) and doses of chitosans had significant effects on virus removal from water (2-way ANOVA, p < 0.0001). Overall, both higher MW chitosans, MW 100,000 and 1,000,000 Da, gave higher virus removals from water (2.7 to 3.6 log_{10}) than MW 50,000 Da chitosan (1.9 – 2.6 log_{10}). Doses of 3 mg/L and higher provided greater virus removals (1.9 to 3.6 log_{10}) compared to the 1 mg/L dose (< 0.7 log_{10}).

Chitosan Doses 1 and 3 mg/L

At dose 1mg/L, all three different MW chitosans gave minimal virus removals of < 1 log_{10} viral reduction (Figure 4.5). At dose 3 mg/L, virus removal was substantially greater for all MW chitosans. Chitosans of MW 100,000 and 1,000,000 DA exhibited similar virus removals of 3.56 and 3.53 log_{10} (p > 0.05), respectively. The smaller chitosan MW 50,000 Da gave a lower virus removal of 2.55 log_{10} at dose 3 mg/L as shown in Figure 4.5.

Chitosan Doses 10 and 30 mg/L

At doses of 10 and 30 mg/L, chitosan MW 100,000 Da had significantly higher virus removals of 3.47 and 3.41 log_{10}, respectively, than both the lower MW 50,000 Da (2 and 1.85 log_{10}) and the larger MW 1,000,000 Da (2.97 and 2.69 log_{10}) chitosans. Virus removals by the three higher doses of chitosan MW 100,000 Da (3, 10 and 30 mg/L) were not significantly different (1-way ANOVA, p > 0.05). Chitosan MW 50,000 Da exhibited significantly lower
virus removals of \(<0.5 \log_{10}\) than MW 100,000 and 1,000,000 Da chitosans at dose 10 and 30 mg/L.

![Figure 4.5](image)

**Figure 4.5** Effects of chitosan molecular weight (≈ 90% DD) and dose on virus reductions

### II. Effects of degree of deacetylation (DD) on virus removal

Three chitosans having different degrees of deacetylation (DDs) of 70%, 85% and 95% were tested for virus coagulation. Results, as shown in Figure 4.6, indicate that both DD and dose of chitosans had significant effects on viral reduction by coagulation (2-way ANOVA, \(p < 0.0001\)). Chitosan with higher DD gave higher virus removals of 2.4 to 4.1 \(\log_{10}\) and higher doses of chitosans, starting at dose 3 mg/L, produced higher virus removals of 1.2 to 4.1 \(\log_{10}\) than the 1 mg/L dose.

**Chitosan Doses 1 and 3 mg/L**

At dose 1 mg/L, the higher DD chitosans of 85 and 95% were ineffective for virus removals (<0.5 \(\log_{10}\)) but the low (70%) DD chitosan gave a somewhat greater virus removal of
1.2 log$_{10}$ (Figure 4.6). At dose 3 mg/L, virus removals by the higher DD chitosans of 85% and 95% were markedly higher at 2.4 to 2.8 log$_{10}$ than the removals by the lowest DD chitosan of 70%, which remained at about 1 log$_{10}$. Virus removals by chitosans 85% and 95% DD were not significantly different at dose 3 mg/L (p > 0.05).

**Chitosan Doses 10 and 30 mg/L**

At dose 10 and 30 mg/L, higher DD chitosans of 85% and 95% gave significantly greater virus removals of 2.7 to 4.1 log$_{10}$ compared to the low DD chitosan of 70% that gave removals of only 1.2 log$_{10}$ (p < 0.001) (Figure 4.6). The highest chitosan of 95% DD gave the best virus reductions by coagulation among those tested, with approximately 3.4 – 4.1 log$_{10}$ reduction. At the highest dose of 30 mg/L, virus removals were significantly different, with chitosan 95% DD giving 3.41 log$_{10}$ reduction compared to 2.65 log$_{10}$ and 1.17 log$_{10}$ reductions for chitosans 85% and 70% DD, respectively (p < 0.05). However, at dose 10 mg/L, chitosans 85% and 95% DD gave similar virus reductions of 4.02 and 4.09 log$_{10}$.

![Effects of degree of deacetylation of chitosans on MS2 removal](image)

**Figure 4.6** Effects of chitosan degree of deacetylation (viscosity 5 mPa•S, MW < 50,000 Da) and dose on virus reductions by coagulation
III. Effects of modified chitosans on virus removal

Consistent with chitosan choices for bacteria coagulation, the three modified chitosans of chitosan acetate, chitosan lactate and chitosan HCl were selected for virus coagulation experiments. Results (in Figure 4.7) indicated that types and doses of modified chitosans gave significantly different virus removals from water (2-way ANOVA, p <0.0001). Chitosan acetate achieved highest virus removals of 0.7 to 3.8 log\(_{10}\) at every dose tested compared to chitosan HCl (0.2 to 2.9 log\(_{10}\) reductions) and chitosan lactate (0.1 to 3.4 log\(_{10}\) reductions). Virus reductions were greater at higher doses of modified chitosans from 3 to 30 mg/L compared to 1 mg/L doses (Figure 4.7).

**Chitosan Doses 1 and 3 mg/L**

All modified chitosans were ineffective for virus removal at dose 1 mg/L, with <1 log\(_{10}\) reductions ranging from 0.69 log\(_{10}\) for chitosan acetate, to 0.16 log\(_{10}\) for chitosan HCl, and 0.10 log\(_{10}\) for chitosan lactate. At the higher chitosan dose of 3 mg/L, virus removals increased considerably to about 2 log\(_{10}\) or higher, especially by chitosan acetate with >3 log\(_{10}\) reductions, while chitosan HCl and chitosan lactate exhibited similar virus reductions of about 2 log\(_{10}\) or higher (p > 0.05).

**Chitosan Doses 10 and 30 mg/L**

Virus removals were greater at the high chitosan doses of 10 and 30 mg/L, especially particularly compared to the 1 mg/L dose. Virus removals by chitosan acetate did not differ significantly among doses of 3, 10, and 30 mg/L (p > 0.05), ranging from 3.3 to 3.8 log\(_{10}\). Chitosan HCl and chitosan lactate gave higher virus reductions of 2.5 to 3.4 log\(_{10}\) at the higher doses of 10 and 30 mg/L compared to reductions of 0.1 to 1.9 log\(_{10}\) at lower doses of 1 and 3
mg/L. Modified chitosans HCl and lactate gave similar virus removals of 2.5 to 3.4 log$_{10}$ at doses 10 and 30 mg/L (p > 0.05) and were less effective than chitosan acetate at every dose tested.

![Figure 4.7](image)

**Figure 4.7** Effects of modified chitosans (DD 80-95%, viscosity 2-300 mPa·S) and their doses on virus reduction by coagulation

**Comparisons of all 9 chitosans for virus removal by coagulation**

Log$_{10}$ reductions of bacteriophage MS2 for all candidate chitosans tested are shown in Figure 4.8 and turbidity levels of coagulated test water supernatant are shown in Appendix 8. The chitosan dose range for effective virus removal was from 3-30 mg/L. Chitosan acetate and chitosan MW 100,000 Da exhibited highest viral removals, with 3 – 4 log$_{10}$ reductions at doses 3, 10 and 30 mg/L and both gave similar reductions across these doses. Chitosan 95% DD and chitosan 1,000,000 daltons also gave 3-4 log$_{10}$ virus reductions beginning at dose 3 mg/L and higher, with similar removals to chitosan acetate and chitosan MW 100,000 Da. Generally, higher MW and higher DD chitosans gave greater virus removals. At dose 10 mg/L chitosan 100,000 Da, chitosan 85DD, chitosan 95DD and chitosan acetate gave similar MS2 removals of
> 3 log_{10} (p > 0.05). Removal of test water turbidity, which was initially 5 NTU kaolinite, was achieved by most chitosans at the 3 mg/L dose, with 40 -70% removal, but chitosan 85DD, chitosan 95DD and chitosan HCl also removed turbidity at the 10 mg/L dose.
Figure 4.8 Removal of bacteriophage MS2 by coagulation treatment with different doses of chitosans having different molecular weights (MW 50,000 – 1,000,000 Da) and degrees of deacetylation (DD 70–95%) and by chemically modified chitosans
4.3.3 Zeta potential of *E. coli* and bacteriophage MS2

Zeta potentials of *E. coli* bacteria and bacteriophage MS2 were measured in stock solutions (a mixture of phosphate buffer saline (PBS) and 20% glycerol) and *E. coli* zeta potential was also measured in tryptic soy broth (TSB). *E. coli* displayed more negative zeta potential of -38.06 mV in test water and -16.6 mV in TSB than MS2 with zeta potentials of -6.87 mV in stock to -19.84 mV in test water as shown in Table 4.1. Zeta potential of test water containing both *E. coli* and MS2 was not measured.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Zeta potential (mV±95% CI, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18. <em>E. coli</em> B in TSB</td>
<td>-16.60 (±0.78)</td>
</tr>
<tr>
<td>19. <em>E. coli</em> B in test water</td>
<td>-38.06 (±2.72)</td>
</tr>
<tr>
<td>20. MS2 stock</td>
<td>-6.87 (±0.33)</td>
</tr>
<tr>
<td>21. MS2 in test water</td>
<td>-19.84 (±4.14)</td>
</tr>
</tbody>
</table>

4.3.4 Microbial removals by chitosan coagulants in natural surface waters

Chitosan MW 100,000 daltons (Da) was tested for microbial coagulation efficacy in natural surface water from the Chattahoochee River (Atlanta, GA) and University Lake (Chapel Hill, NC) compared to artificial surface water made as described above. As indicated by the results in Figure 4.9, *E. coli* removals by chitosan MW100,000 Da were not different statistically between natural surface water and artificial test water (p = 0.26). Between natural water samples, bacterial removal from the Chattahoochee River and University Lake were similar (p>0.05) with > 3 log<sub>10</sub> reduction. Water quality parameters of the test water, both natural surface water and laboratory water, are in Table 4.2.
Data for reductions of bacteriophage MS2 in test water and Chattahoochee River water by coagulation with 100,000 MW chitosan are shown in Figure 4.10. At doses of 3 and 10 mg/L MS2 was reduced by 2.4 to 3 $\log_{10}$ in Chattahoochee River water and by 3.5 to 4.9 $\log_{10}$ in test waters with kaolinite turbidities of 5 or 10 NTU. Overall, removals of bacteriophage MS2 were lower in Chattahoochee River water than in artificial test water, but at the 10 mg/L dose the differences in virus reduction were small and not statistically significant ($p>0.05$).

**Figure 4.9** *E. coli* coagulation using chitosan MW 100,000 daltons in artificial and natural surface water samples
Table 4.2 Quality characteristics of water samples used for microbial coagulation studies comparing natural surface water and laboratory water

<table>
<thead>
<tr>
<th>Water parameters</th>
<th>Chattahoochee River</th>
<th>University Lake</th>
<th>Test water 10 NTU</th>
<th>Test water 5 NTU</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.52</td>
<td>7.41</td>
<td>7.34</td>
<td>7.23</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>18.9</td>
<td>10.9</td>
<td>11.22</td>
<td>4.69</td>
</tr>
<tr>
<td>TOC (mg/L)</td>
<td>6.28</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>TDS (mg/L)</td>
<td>-</td>
<td>-</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>E. coli bacteria, CFU/ml (seeded)</td>
<td>$10^9$</td>
<td>$10^9$ (natural background 158 CFU/100 ml)</td>
<td>$10^9$</td>
<td>$10^9$</td>
</tr>
<tr>
<td>MS2, PFU/ml (seeded)</td>
<td>$10^9$</td>
<td>$10^9$</td>
<td>$10^9$</td>
<td>$10^9$</td>
</tr>
</tbody>
</table>

4.4 Discussion

The results of these jar test coagulation experiments suggest that chitosans are efficacious for microbial removal from water, and that efficacy is influenced by chitosan MW, DD, and functional group modifications of the chitosan polymer. Coagulation with chitosans could remove *E. coli* bacteria at high efficacy ($2.0 – 4.5 \log_{10}$), which are values comparable to

**Molecular weight of chitosan polymers and microbial removal**

*Effects of chitosan molecular weight on bacterial removal*

Molecular weight (MW) of chitosan polymers influenced removal of bacteria from water, with chitosan in the middle of the MW range at 100,000 Da being more effective than the lower MW (50,000 Da) or higher (1,000,000 Da) MW chitosans. At the optimum dose 10 mg/L, effectiveness of chitosan for E. coli removal did not appear to improve with increasing MW beyond 100,000 daltons. The medium MW chitosan of 100,000 daltons gave E. coli reductions of 2.27 – 4.51 log$_{10}$ between dose 3 -30 mg/L.

*Effects of chitosan molecular weight on viral removal*

Overall, higher MW chitosans (100,000 and 1,000,000 Da) were more effective for virus removal than the lower MW chitosan of 50,000 Da. Both medium MW (100,000 Da) and high MW (1,000,000 Da) exhibited similar virus reductions of 2.7 to 3.6 log$_{10}$. Similar to bacteria removal, higher chitosan MW of 1,000,000 Da did not further increase virus removal. These results suggest that a middle MW chitosan of 100,000 Da is efficacious for both bacterial and viral removal by coagulation.

The size of polymer normally plays a crucial role in coagulation-flocculation using polymer coagulants. MW influences the interparticle bridging mechanism of coagulation, with larger size polymer chains able to attach to more particles in water, forming bridges, resulting in
the removal of more particles. Our study showed increasing coagulation efficacy with increasing MW up to a certain point. Strand et al (2001) also reported a similar trend in a study of bacteria flocculation by chitosan. It is possible that beyond a certain point, increasing polymer size has diminishing returns for improving coagulation. Similar outcomes were seen in our previous turbidity coagulation studies, where higher MW chitosans also showed better removal of bentonite clay than smaller MW chitosans. Other studies (Roussy et al, 2005, Chen et al, 2003) have suggested that bentonite removal using chitosan coagulant was not increased when MW of chitosan increased beyond 100,000 daltons. These results suggest that bridging may be a key mechanism of chitosan for microbial removal at near neutral pH of water. This is because overall bacteria and virus removal were improved as MW of chitosans was increased, but impacts of MW on microbial removal plateaued after 100,000 daltons. The largest MW chitosans (1,000,000 daltons) exhibited lower E. coli and virus removals of 1.1 - 2.2 and 2.7 – 3.5 log_{10}, respectively, than the medium MW chitosan (100,000 daltons) removals of 2.3 - 4.5 and 3.4 - 3.6 log_{10}, respectively. However, at the optimum dose of 3 mg/L for virus coagulation, the removal did not differ significantly between MW 100,000 and 1,000,000 Da chitosan. The virus removal by the high MW chitosan decreased and was lower than the medium MW chitosan at doses beyond this optimum dose. This removal trend based on MW suggests the bridging mechanism between chitosans and microbes in water. Microbial removal increases from low MW chitosan to higher MW chitosan and then plateaus when chitosan MW exceeds a certain value. In this study removal decreased when chitosan MW increased from 100,000 to 1,000,000 Da. Very high MW chitosan may be not a good candidate for microbial coagulation because the high MW chitosan as a long chain may fold possibly during coagulation process. Such folding of the chitosan chain can decrease availability of chitosan functional sites to attach to microbes. It is also speculated
that folding of chitosan chain can bring monomer units into close contact with each other and this event can possibly interfere with the interaction between the chitosan and microbes because of intra-chain hydrogen bonding. Intra-chain hydrogen bonds of chitosan can occur between –OH group at C3 and –O at C4 and between –OH at C6 and –N (of –NH₂) at C2 on chitosan monomers (Rinaudo, 2006).

Effects of degree of deacetylation of chitosan on microbial removal

Effects of chitosan degree of deacetylation (DD) on bacteria removal

Overall, higher chitosan DD exhibited higher bacterial removal in the coagulation experiments of this study. The highest DD chitosan (95%) gave the highest bacteria (*E. coli*) removal by coagulation, with 3 – 4 log₁₀ reduction at doses 3, 10 and 30 mg/L. These *E. coli* removals were significantly better than those produced by chitosans with lower DD, particularly 70% DD chitosan at the same doses. With the highest chitosan DD of 95%, bacteria removal was not dose dependent because chitosan doses from 3 – 30 mg/L did not differ in bacteria removal.

The results suggested that with higher DD chitosan, smaller doses for coagulation achieved as high as 3 log₁₀ reduction of bacteria compared to medium DD (85%) chitosan. The lowest DD chitosan tested, 70%, showed significantly improved bacteria removal with the increasing doses, with 1.16 (±0.33) log₁₀ removal at 1 mg/L and 1.99 (±0.32) log₁₀ removal at 30 mg/L.

Negatively charged bacteria may react more with the protonated amine group (-NH₂) on the chitosan molecule at higher dose, giving increased bacteria removal with more –NH₂ groups in the coagulation system, as occurs with high DD chitosan.

Effects of degree of deacetylation (DD) on virus removal

At the same chitosan dose, the degree of deacetylation (DD) influences virus removal by coagulation. Chitosans with higher DD exhibited greater virus removals than the lowest DD
chitosan. The chitosans with the higher DDs of 95% and 85% produced similar virus removals at all doses with exception of dose 30 mg/L. Both of the higher DD chitosans exhibited high virus removals of 4 log_{10} at 10 mg/L. The virus removal by the lowest DD chitosan tested, 70%, was not dose dependent and gave about 1 log_{10} virus removal across doses of 1 – 30 mg/L. Overall, results indicated minimal impact of low DD or low –NH_{2} groups on the chitosan polymer chain for virus removal and increasing the low DD chitosan concentration did not improve virus reduction efficiency. The high concentration of the low DD chitosan had less impact on virus removal than low concentrations of the chitosan of higher DD (85% and 95%).

Degree of deacetylation (DD) indicates the amount of amino groups (-NH_{2}) along the chitosan polymer chain. These amino groups provide cationic sites along chitosan polymer chains due to their protonation when dissolved in acidic solution. Positively charged chitosan sites attach to negatively charged surfaces of microbes, facilitating their coagulation and removal. Theoretically, higher chitosan DD provides a more highly positively charged polymer and stronger affinity between positive charged chitosan polymer and the negative charged microbes is expected. Therefore, higher removals of bacteria and virus would be expected from higher DD chitosans because of their greater number of positively charged sites to react with negatively charged microbe surfaces. Our results support this charge neutralization mechanism because high DD (95%) chitosan gave higher bacteria and virus removal than low DD chitosan. Previous studies of synthetic cationic polymers also showed that charge neutralization was a likely mechanism for adsorption and flocculation removal of particles by highly charged polymers (Durand-Piana et al, 1987; Eriksson et al, 1993 cited in Strand et al, 2001). The measured positive charges of the DD chitosans tested ranged from 19.84 (±12.00) to 41.98 (±2.73) mV. The results for microbial removals provide evidence that these positive charge
values of the chitosans were high enough to initiate charge neutralization by the reactions of the positive charged chitosans with the negative charged test microbes as a plausible basis for microbial removal by coagulation.

Chitosan DD is related to the charge density on the molecule, with low DD having lower positive charge density (lower number of positive charged sites on chitosan chains), which may limit the capacity to remove viruses from water. As seen from the results of coagulation experiments with 70% DD chitosan, virus removal did not improve when coagulation dose was increased. In contrast, the effects of low DD chitosan on bacteria removal were different, with 70% DD chitosan showing increased bacteria removal with increased doses.

Based on the difference in virus and bacteria removals by chitosans with different DDs, it is possible that charge density on the chitosan polymer chain may be more critical for virus removal than bacteria removal and that low DD chitosan is not effective for virus coagulation. Further study on effects of low DD chitosan on virus removal is recommended.

From measurements of zeta potentials of chitosans with DDs of 70% - 95%, 70% DD chitosan had significantly higher zeta potential (41.98 (±2.73) mV) than chitosans of 85 and 95% DD (19.84 to 24.43 mV) (p < 0.05) and the zeta potential of chitosans 85 and 95% DD did not differ significantly (p > 0.05). However, for different chitosan DDs with different zeta potentials, chitosan polymers still exhibited equivalent electrical charges in their microenvironments even though the DD or number of –NH₂ groups were different (Chen et al, 2003). Polarization of the microenvironment of chitosans as an indication of charge density due to chitosan’s DD was studied by Chen et al (2003), who reported that varying chitosan DD had little effect on polarization of the microenvironment around chitosans with similar electrical potentials for chitosans of different DD. These findings, together with our results, suggest that the magnitude
of charge density may not be crucial to alter electrical potential of the microenvironment around chitosans, thereby not strongly affecting attachment between chitosan and microbes. Positive electrical charge, however, is important for the interaction of chitosan and negatively charged particles like bacteria cells and viruses.

Different effects of low DD chitosan on removals of bacteria and virus possibly involved the surface electrical charge of these microbes as measured by zeta potential. The test *E. coli* bacteria possessed a surface potential of approximately -16.6 (±0.78) mV whereas the test surrogate virus, bacteriophage MS2, had a surface potential of about -6.87 (±0.33) mV. Because the zeta potential measurements showed that *E. coli* bacteria possessed higher zeta potential than bacteriophage MS2, *E. coli* should require higher positive charges to destabilize than MS2. Results from coagulation experiments with low DD chitosan (70%) indicated that removals of bacteria and virus were consistent with this explanation. Bacteria removals by chitosan 70% DD were greater with increasing dose from 1 to 30 mg/L but virus removals by chitosan 70% DD were not improved as chitosan doses increased, with log_{10} virus removals similar for doses of 1 to 30 mg/L. It is hypothesized that the low surface potential of bacteriophage MS2 and high zeta potential of chitosan 70% DD have a role in this interaction. Perhaps the positive charges of chitosan DD 70% greatly exceed the positive surface potential required for destabilizing MS2, thus causing repulsion of the like charges between positive charges of chitosans attached to MS2 and free positive charges on chitosans. Therefore, virus removals were not improved with increasing doses. These results suggest that charge neutralization plays a role in microbial coagulation using chitosan. Type of microbe and its surface charges appear to affect the coagulation mechanism and removal efficacy by coagulation.
**Water-soluble modified chitosans and their microbial removal by coagulation**

*Effects of modified chitosans on bacteria removal by coagulation*

At the same doses, different types of modified chitosans: chitosan acetate, chitosan lactate and chitosan HCl, exhibited similar removal of bacteria from test water by coagulation. Bacteria removals were higher as doses of modified chitosans were increased, although removals decreased when the dose exceeded 10 mg/L for all three of them. Therefore, excess chitosan dose beyond the optimum had a negative impact on bacteria removal by coagulation. For all 3 modified chitosans, the highest *E. coli* reductions of 3 – 4.28 log_{10} were at optimum dose of 10 mg/L, and chitosan HCl produced the highest *E. coli* removal of 4.28 log_{10}. Based on results of this study, the different chemical groups making the chitosan salts showed minimal effects on bacteria removal by coagulation except at the optimum dose of 10 mg/L. These modified chitosans are salts produced from different acids, namely hydrochloric, acetic and lactic acids and they have different functional groups. The pKas of these acids differ, with \text{pH} <1, 3.86, and 4.75 for HCl, lactic and acetic acid, respectively, and all are much lower than the pKa of chitosan at 6-6.5. The pKa properties of the modified chitosan salts may limit the effects of their different functional groups on bacteria removals at the neutral pH of test water. Results from this study indicate that the functional counter ion groups associated with modified chitosans are not crucial for their removal of bacteria by coagulation.

*Effects of modified chitosans on viral removal*

Virus removals from water by coagulation with modified chitosans differed from bacteria removals because they differed among the types of modified chitosans. The dose of three modified chitosans for effective virus removal was as low as 3 mg/L to achieve $\approx 2$-$3$ log_{10} reductions. Chitosan acetate gave highest virus removals of $> 3$ log_{10} at every dose tested from 3
mg/L or higher, compared to removals of 1.9 to 3.4 log_{10} for chitosan HCl and chitosan lactate. Chitosan lactate and chitosan HCl performed similarly, with both chitosans exhibiting lower virus removal than chitosan acetate.

Overall, modified (water-soluble) chitosans performed similarly to unmodified (acid-soluble) chitosans for microbial reductions by coagulation. They efficiently removed bacteria at the 10 mg/L dose, with chitosan HCl giving the highest bacteria removals of > 4 log_{10}. The effects of modified chitosans and their doses on virus removal differed from bacteria removals. The type of salt associated with the modified chitosan structure influenced virus removal efficiency by coagulation, with chitosan acetate giving the best virus removal of 3.8 log_{10}. The effective dose range of modified chitosans for effective virus removals was broader than that for effective bacteria removal. Virus removal by coagulation with modified chitosans was about 2 log_{10} at the 3 mg/L dose and increased up to 3.8 log_{10} as doses increased. Chitosan acetate produced consistently high virus removals of 3.3 – 3.8 log_{10} at doses between 3 – 30 mg/L and they were greater than other modified chitosans, for which virus reductions were 1.9 to 3.4 log_{10}. Overall, chitosan acetate gave the highest viral removals of 3.8 log_{10} and chitosan HCl gave the highest bacteria removals of 4.3 log_{10}. The pH of modified chitosan stocks differed, with pH 3.7 for chitosan HCl and chitosan lactate, and pH 4.7 for chitosan acetate. The pH values of waters treated with modified chitosans were not much different and remained near pH 7. Chitosan HCl is derived from the reaction between chitosan polymer and hydrochloric acid while chitosan acetate is produced the reaction of chitosan polymer with acetic acid. From the results of this study, chitosan modified with the strong acid HCl gave highest log_{10} bacteria removals and chitosans modified with the weak acetate and lactate gave the highest viral removals. HCl is a strong acid and has a low pKa <1 (Benjamin, 2002), which means it is totally protonated in water.
HCl ionizes in water creating $\text{H}_3\text{O}^+$ (shortly $\text{H}^+$) and this reaction possibly creates a mild acidic microenvironment around chitosan polymer chains that promotes bacteria removal. It is possible that the coagulating effect of the $\text{H}^+$ cannot go far from its origin so its acidic effects occur only in a small area around the chitosan polymer. This strong acid property together with a high zeta potential of $\approx 30.37 \ (\pm 10.07) \ \text{mV}$ may help the chitosan HCl molecule attach strongly to the bacteria cell. Chitosan acetate has the acetate functional group (-CH$_3$COO$^-$) from acetic acid and a pKa of 4.76 (Benjamin, 2002). The measured zeta potential of chitosan acetate was a high positive charge of 43.08 ($\pm 20.00$) mV, which could explain why chitosan acetate exhibited the highest log$_{10}$ virus removals by coagulation. Although chitosan acetate it is a chitosan salt from the weak acid acetate, its high positive electrical potential may explain why it has a strong affinity for microbe particles.

The results of this study provide evidence that having a positive charge and a high DD ($> 80\%$) are chitosan properties critical for effective coagulation of viruses and bacteria. The surface charge of a virus (bacteriophage MS2 for this study) is hypothesized to have a role in virus coagulation. The low negative surface charge of MS2 (-6.87 ($\pm 0.33$) mV) may make this virus rather sensitive to the magnitude of positive charge of a chitosan and influence coagulation and removal efficiency. Chitosan lactate with the lactate group (-CH$_3$CH (OH) COO$^-$) from lactic acid has a pKa of 3.86 and a positive charge of 16.50 ($\pm 9.67$) mV. Among the three modified chitosans tested, HCl with the lowest pKa performed best for bacteria reduction by coagulation and acetate with the highest pKa worked best for virus reduction by coagulation. The pKa of lactate was in between that of HCl and acetate and its performance for both bacteria and virus removal by coagulation was not better than those of HCl and acetate. The pKa of functional groups on chitosan molecules appears to impact microbial reduction by coagulation and it may
serve as an initial guide for selecting the type of modified chitosan for effective microbial coagulation.

**Effects of chitosan doses on microbial reduction by coagulation**

In the coagulation process the dose of a coagulant influences the extent of destabilization of colloids, including bacteria and viruses. When a sufficient dose of coagulant is added, the energy barrier of colloids is overcome so they become less stable and easier to aggregate and physically remove from water (Binnie & Kimber, 2009). In this study the dose of chitosan influenced the magnitude of microbial removal although its impacts on bacteria and virus removal were somewhat different. Chitosan showed a wider range of effective doses for virus removal but a narrower range for bacteria removal. A chitosan dose of 1 mg/L appeared to be too low and did not produce extensive removals of both bacteria and viruses ($\leq 1 \log_{10}$). These results indicate that at a coagulant dose lower than the optimum, destabilization of colloids such as microbes may not occur efficiently and therefore, coagulation is not very effective. Overall, bacteria removal required higher doses of chitosans than virus removal. Low chitosan doses (1 and 3 mg/L) produced about $1 \log_{10}$ bacteria reductions, except chitosan MW100,000 daltons and chitosan DD85 and 95% at dose 3 mg/L that gave about $2 \log_{10}$ reductions. Increasing chitosan dose from 1 to 10 mg/L, generally increased bacteria removals from $\leq 1 \log_{10}$ to $4 \log_{10}$. Most chitosans exhibited the highest bacteria removal at dose 10 mg/L. However, at a chitosan dose of 30 mg/L, bacteria removal by coagulation was lower. In contrast, effective removal of viruses occurred at a low chitosan dose of 3 mg/L, with many chitosans achieving as high as $3 \log_{10}$ virus reductions. Most chitosans tested gave the greatest virus removals at doses of 3 and 10 mg/L, and their virus removal efficiency was lower at dose 30 mg/L. At an optimum coagulation dose, destabilization of colloids occurs maximally at the lowest possible coagulant dose resulting
in effective colloid aggregation and removal from water (Shammas, 2005). On this basis, the chitosan optimum dose for bacteria removal from water derived by jar test coagulation was 10 mg/L and the optimum doses for virus removal were 3 and 10 mg/L. However, overdosing of chitosan (at 30 mg/L) showed lesser negative effects than under dosing (at 1 mg/L) for microbial removals by coagulation. It is likely that the excess chitosan minimally interfered with attachment between chitosan and microbes. Another hypothesis is that attachment between chitosan and microbes (bacteria or virus) was sufficiently strong that there was minimal impact by surplus chitosan on microbe removal. This phenomenon was different from reaction of chitosan and mineral clays for which strong negative effects on removal were observed from overdosing of chitosan.

**Zeta potential and chitosan mechanisms of microbial removal by coagulation**

All 9 chitosans tested had positive zeta potential values, ranging between 16 – 90 mV. Positive zeta potential of chitosan is preferred for the coagulation processes because it will facilitate interactions with negative charges on the microbial surface, overcoming the energy barrier at the surface of the microbe and inducing interactions and attachment between chitosan and microbes. Overall, the positive charge of the chitosan polymer increases from 19.79 (±3.24) to 88.93 (±10.88) mV) as molecular weight increases from 50,000 Da to 100,000 Da. However, the zeta potential of MW1,000,000 Da chitosan is 38.41 (±6.28) mV, which is lower than the zeta potential of MW100,000 Da chitosan. The results of coagulation experiments showed that higher MW chitosans (often with higher zeta potential) generally performed better than lower MW chitosans (typically lower zeta potential) for both bacteria and virus removal. Microbial removal by chitosans of different molecular weight increased as chitosan zeta potential increased. Theoretically, the positive charge of chitosan increases as chitosan DD increases.
However, results of measured zeta potentials from this study decreased from 41.98 (±2.73) to 19.84 (±12.00) mV as chitosan DD increased from 70 to 95%, with 70% DD chitosan having significantly higher zeta potential than 85 and 95% DD chitosan (p < 0.05). Nevertheless, microbial reduction results of this study indicated that higher DD chitosans were more effective for both bacteria and virus removal than lower DD chitosan. Therefore, while zeta potential and chitosan DD gave conflicting trends for microbial removals by coagulation, the actual mechanisms of microbial reductions by chitosan coagulation remain uncertain and require further study. While the results of these studies support a role for chitosan positive charges in microbial removals by coagulation, it is possible that there is a threshold or minimum positive potential required to initiate effective microbial coagulation and removal using chitosan. The results of these studies also showed that charge neutralization is an important mechanism for chitosan coagulation and microbial removals, but not solely for microbial coagulation at neutral pH of water. This is because the MW of chitosan also played a role in the coagulation and removal of bacteria and virus, which indicates that the bridging mechanism of coagulation also plays a role in these processes.

**Microbial coagulation by chitosans in natural surface water**

Chitosan of MW100,000 daltons was selected for testing the efficiency of chitosan coagulation of microbes in natural surface water. In natural surface water seeded with *E. coli* bacteria and bacteriophage MS2, the 100,000 Da MW chitosan removed *E. coli* as effectively as when applied to lab test water, with about 2-4 log₁₀ reductions. Its performance in microbial reductions from natural surface waters (both river and lake waters) did not differ statistically from the lab made test water. Effective coagulant doses of 3 and 10 g/L obtained from experiments using lab water also produced ≥ 3 log₁₀ *E. coli* removals in surface waters.
However, virus removals in natural water were significantly lower than in lab water with $2.4 - 3 \log_{10}$ compared to $3.5 - 4.9 \log_{10}$, respectively. There might have been some water quality constituents in natural surface waters that adversely influenced virus but not bacteria reductions by chitosan coagulation. More bench scale and field studies are needed for further information to elucidate and explain these differences in virus and bacteria removals that appear to be related to natural water quality conditions. Nevertheless, virus reductions by chitosan coagulation were still as high as $2 - 3 \log_{10}$ in natural water.

### 4.5 Conclusions

Overall, the results of this study provide evidence that chitosan coagulation has potential as an effective household water treatment technology, producing extensive microbial removals at relatively low doses. Microbially contaminated drinking water particularly in developing countries can likely be improved by using chitosan coagulant. In rural Thailand, two important sources of drinking water are rainwater and shallow wells. Rainwater could contain up to 5 fecal coliforms (FC) per 100 ml and shallow wells could have as many as 30 FC per 100 ml (Pinfold et al, 1993). A study in Pakistan showed dug wells as sources of drinking water contained at least 54,300 total coliforms per 100 ml (Jehan et al, 2006). Because chitosan coagulation can produce $\geq 3 \log_{10}$ bacteria reductions, it should be possible to improve poor water quality to the safe or low risks level of the WHO drinking water guidelines by this process.

- Coagulation of water with 3 to 10 mg/L doses of chitosans in the jar test procedure efficiently removed bacteria by $2.0 - 4.5 \log_{10}$ and viruses by $2.0 - 4.1 \log_{10}$.
- Chitosan molecular weight (MW) had significant effects on bacteria and virus reductions by chitosan coagulation and results suggested that medium molecular weight chitosans
(100,000 Da) gave better microbial removals than either lower (50,000 Da) or higher (1,000,000 Da) molecular weight chitosan polymers.

- Chitosan degree of deacetylation (DD) also influenced microbial removals from water by coagulation. Higher DD chitosans gave higher removals of both bacteria and virus by coagulation.

- Modified water-soluble chitosans removed bacteria and virus from water by coagulation as efficiently as acid soluble chitosan polymers.

- Chitosan hydrochloride was the most effective chitosan polymer for coagulation-flocculation of bacteria and chitosan acetate was the most effective for virus removal.

- Dose of chitosans had significant effects on microbial removal efficiency by coagulation, but the effects on bacteria removals were somewhat different from the effects on virus removals. Relatively low doses of chitosans of 3 – 10 mg/L were able to achieve 2 – 4 log_{10} reductions of both bacteria and viruses from water.

- Bridging mechanisms appeared to play a crucial role in microbial coagulation from water using chitosans, which was inferred from the effects of chitosan molecular weight on bacteria and virus removals.

- Charge neutralization also appeared to play a role in microbial coagulation because degree of deacetylation, which is indicative of the magnitude of positive charge of the chitosan, significantly affected microbial removal from water by coagulation.

- The chitosan biopolymers studied had coagulant properties that achieved appreciable removals of bacteria, viruses and turbidity from both model and natural waters, thus indicating considerable potential to serve as alternative coagulants at household level to
improve the microbial quality of water and thereby reduce health risks related to contaminated drinking water.
CHAPTER 5
EFFECTS OF WATER QUALITY PARAMETERS ON CHITOSAN PERFORMANCES FOR REMOVAL OF TURBIDITY, BACTERIA AND VIRUS

5.1 Introduction

Treating water of varying quality in the home is a challenge because water quality parameters may vary according to source, and even the same household’s water quality parameters may vary if people change water sources. Differences in water chemistry that are source and site-specific have critical impacts on contaminant removal efficiency by coagulation-flocculation. Coagulation conditions such as dose, pH, turbidity, temperature, alkalinity, and level and type of natural organic matter affect efficiency of microbial removal (WHO, 2004). In many areas, surface water is a main source of drinking water, and its quality varies over space and time. For example, turbidity of natural water may change between wet and dry periods and seasons. Water turbidity also varies from place to place according to soil type, land cover and the conditions and activities of the surrounding area. Generally, turbidity in surface water increases after rainfall because particles are washed into water bodies from surrounding areas. In Thailand, turbidity of source water for drinking in rural area can be 30 - 40 NTU but the level can substantially increase in the rainy season to much greater than 100 NTU (Vater, 2008). Salinity of surface water in Thailand also varies, ranging from 0.1 to 1 g/L (PCD, 2014).

Efficacy of chitosan as a household water treatment may be influenced by the quality of raw water to be treated. Previous research has reported impacts of water quality parameters on
chitosan efficacy. In studies on bentonite turbidity removal the required chitosan doses were higher at higher initial bentonite turbidity (Huang & Chen, 1996; Roussy et al, 2004). In contrast, bacterial flocculation using chitosan exhibited decreased performance at lower bacterial concentration (Strand et al, 2001). Water pH is also considered a critical parameter for the coagulation process. It is expected that chitosan coagulation will perform better at water pH levels lower than the pKa of amino groups (–NH₂) on the chitosan chain. The –NH₂ group is the functional group that provides electrical positive charge for chitosan molecules, and its pKa is approximately 6.3 – 6.5 (Guibal & Roussy, 2007; Yadav et al, 2011). Chitosan efficacy for coagulation-flocculation is predicted to be greater at water pH below the pKa of chitosan because protonation of amino groups provides cationic sites for the coagulation process. However, data from previous coagulation studies were not entirely consistent with this prediction. Roussy et al (2004) reported that relatively high chitosan doses were required at pH 9 while lower chitosan doses were needed at pH 5 (acidic region) for bentonite coagulation. In contrast, in studies of the effects of water pH on bacteria flocculation, Strand et al (2001) reported insignificant differences in the efficiency of _E. coli_ bacteria flocculation over the pH range of 4 – 7.4. Chitosan can coagulate particles by both charge neutralization and bridging mechanisms, depending on conditions present in the coagulation system and both of these mechanisms can explain the varying effects of water quality on coagulation using chitosan.

From previous chapters, it was found that chitosan effectively removed turbidity and microbes over a broad range of optimum doses. This characteristic is beneficial for household water treatment where precise measurement of dose is difficult for non-skilled users. In rural areas of developing countries where the quality of water for consumption varies and there are limited water treatment options, ability of chitosan to maintain efficacy in waters of diverse
quality makes it a strong candidate for household water treatment. While ease of use and efficacy in various waters support chitosan coagulation in field applications, the impacts of water quality parameters on chitosan performance requires further investigation, especially for virus removal efficiency. In this chapter, the impacts of the water quality parameters pH, turbidity, and salinity on chitosan coagulation will be examined in the context of removals of viruses, bacteria and turbidity.

5.2 Materials and Methods

Chitosan stock solution

Chitosan (MW 100,000 daltons, ≈ 90% DD) was chosen for these studies based on its demonstrated effectiveness for reduction of turbidity (kaolinite and bentonite) and microbes (E. coli bacteria and MS2 virus) in previous experiments of this study. It was a commercially available chitosan and was bought from Acros Organics (Bridgewater, NJ, USA). Stock chitosan solution was prepared at 10,000 mg/L by dissolving chitosan powder in 0.5% (v/v) acetic acid and stirring at room temperature until totally dissolved. Stock solution was stored at room temperature (25°C).

Test water

The test water was a chemically defined model for surface water prepared based on the recommended parameters from USEPA, NSF and WHO for efficacy testing of POU technology as listed in Appendix 1 (EPA, 1987; NSF, 2008; WHO, 2011). The test water was prepared by spiking dechlorinated tap water with: 300 mg/L of total dissolved solids (TDS) for NaCl, 3 mg/L of total organic carbon (TOC) from tannic acid and 5 NTU of clay turbidity from bentonite and kaolinite; and was use as the control condition for test water in coagulation-flocculation.
experiments. Prepared test water was stirred at 1000 rpm overnight at 25°C prior to chitosan coagulation experiments. Three chemical water quality parameters were tested: pH, turbidity, and salinity and microbial water quality was evaluated by simultaneously seeding test water with *E. coli* bacteria (target concentration $10^9$ CFU/ml) and bacteriophage MS2 (target concentration $10^{10}$ PFU/ml) and mixing for 30 minutes before starting coagulation jar tests. Seeded bacteria were from frozen stock (containing 20% glycerol-TSB media) that was thawed, grown overnight in tryptic soy broth (TSB), followed by inoculating 100 µl of overnight culture into 50 ml fresh TSB media, incubating at 37°C to reach log phase, then centrifuging at 3000 revolutions per minute (rpm), 4°C for 10 minutes and resuspending the bacterial pellet in 4 ml of test water for seeding into the 5 L of test water of jar test experiments. Viruses were from frozen stocks (containing 20% glycerol-PBS) which were thawed at room temperature then seeded directly into test water. Water quality parameters were adjusted separately, one condition at a time, in the control water to study their impacts on coagulation as follows:

**pH of water**

Water at pH 6 and pH 9 was compared with the standard test water control at neutral pH of 7.0 -7.5. The pH was adjusted using 1M hydrochloric acid (HCl) and 1M sodium hydroxide (NaOH).

**Turbidity**

The mineral clays kaolinite and bentonite were used to create water turbidity. Effects of turbidity level on removal of water turbidity by chitosan coagulation were tested at 5, 30 and 300 NTU for both types of mineral clays. Turbidity 5 and 30 NTU are recommended for testing water treatment technologies of possible untreated sources (WHO, 2011). A high turbidity of 300 NTU was studied because this turbidity level often occurs in surface waters impacted by
stormwater or during rainy seasons (WPA, 2014; Annadurai et al, 2004 cited in Baghvand et al, 2010). For effects of turbidity level on microbial coagulation, turbidity was set at 10 and 30 NTU compared to control water turbidity of 5 NTU and kaolinite was used as the turbidity source. Turbidity 10 NTU was the turbidity level which modeled natural surface water used in previous experiments, i.e. University Lake in North Carolina and the Chattahoochee River upstream of Atlanta, Georgia.

**Salinity**

Salinity was tested at 0.1 part per thousand (ppt) (100 mg/L) and 1 ppt (1,000 mg/L) compared to the standard test water control of 0.3 ppt (300 mg/L). NaCl (Fisher Scientific, Fair Lawn, NJ) was used to adjust water salinity.

**Jar test coagulation experiment procedure**

The jar test method of coagulation-flocculation and sedimentation used mixing conditions of: rapid mixing at 100 revolutions per minute (rpm) for 1 minute followed by slow mixing at 25 rpm for 15 minutes and then settling with no mixing for 30 minutes. All experiments were conducted at 25°C. Supernatant for turbidity and microbial analysis was recovered at 2 centimeters below the surface of the water using a pipette without disturbing the floc. Turbidity was measured by a turbidity meter (HACH 2100AN Turbidimeter, HACH, Loveland CO). Water pH was also measured before and after the jar test experiments using a pH meter. Culture methods were used to enumerate the concentrations of *E. coli* and MS2 and then calculate log_{10} reductions. Enumeration of *E. coli* B was by the membrane filtration (MF) method on MacConkey agar and after overnight incubation at 37°C, *E. coli* colonies were counted and concentration was expressed as colony forming units per unit volume (APHA, AWWA & WAF, 1999). MS2 was enumerated by the double agar layer (DAL) plaque assay with host bacteria *E.*
coli F<sub>amp</sub> with overnight incubation at 37°C for plaque development. MS2 concentrations were expressed as plaque-forming units per unit volume (EPA, 2001).

Doses of chitosans were: 1, 3, 10 and 30 mg/L, for comparison with doses previously evaluated in turbidity and microbial removal experiments using chitosan MW 100,000 Da. Three replicates, plus one control for natural coagulation-flocculation and settling, were performed for each set of experimental conditions.

**Data analysis**

Turbidity removal was calculated as percent turbidity removal relative to the natural coagulation-flocculation and settling control as shown below:

\[
\text{Percent removal} = [1 - \text{sample turbidity/control turbidity}] \times 100
\]

Microbial removal was calculated as log<sub>10</sub> reduction compared to the natural water coagulation-flocculation and settling control as shown below:

\[
\text{Log}_{10} \text{ reduction} = \log_{10}(N_t/N_0)
\]

When: \(N_t\) = number of microbes in chitosan coagulated-flocculated and settled supernatant sample after jar test

\(N_0\) = number of microbes in control after jar test

Statistical comparison of different conditions of test water on both turbidity and microbial removal was performed using GraphPad Prism (GraphPad, San Diego, CA) and SPSS (IBM, Armonk, NY). Two-way ANOVA and Tukey posttest analysis was employed for comparing effects of water quality parameters and chitosan doses and one-way ANOVA was used for comparing effects of chitosan doses.
5.3 Results

5.3.1 Effects of water quality parameters on turbidity coagulation

I. Effects of water pH and coagulant doses

Low water turbidity (5 NTU)

Effects of water pH and coagulant dose on kaolinite removal by chitosan

Effects of water pH on removal of kaolinite (5 NTU) turbidity at varying chitosan doses are shown in Figure 5.1 and Table 5.1. Overall, chitosan removal by kaolinite from water was poor, but kaolinite removal differed significantly at each water pH (2-way ANOVA, p < 0.0001). For neutral pH 7, maximum reduction in kaolinite turbidity was only 14% at 1 mg/L dose. At pH 6, about pKa of chitosan, kaolinite removal did not occur at any dose. At pH 9, kaolinite removal was highest among the 3 pH levels but removal was only 33% at dose 10 mg/L. Over-dosing and under-dosing at each pH increased residual water turbidity due to chitosan added into coagulation system as shown in Figure 5.1.

![Figure 5.1 Kaolinite turbidity removal by coagulation using chitosan MW 100,000 Da at water pH 6, 7 and 9, from initial 5 NTU water](image-url)
Effects of water pH and coagulant dose on bentonite removal by chitosan

The effects of water pH on reduction of bentonite turbidity at varying chitosan doses are shown in Figure 5.2 and Table 5.1. Bentonite removal differed significantly by pH of water and chitosan doses (p < 0.0001). At neutral pH 7, the highest reductions in bentonite turbidity were at doses of 1 mg/L with approximately 85% removal. At pH 6, the highest bentonite removal was achieved at dose 1 mg/L and removal decreased with increasing chitosan doses. At pH 9, chitosan produced highest bentonite removal at dose 10 mg/L with 76% reduction. For all pH levels tested, 6, 7, and 9, bentonite removal did not occur at the highest chitosan dose of 30 mg/L.

![Figure 5.2: Effects of water pH on 5 NTU bentonite removal from initial 5 NTU test water at pH 6, 7 and 9 by coagulation using chitosan MW 100,000 Da](image)

**Dose 1-3 mg/L (Figure 5.3)**

At chitosan doses of 1 and 3 mg/L, kaolinite removal was poor for all pH levels with pH 7 giving the highest kaolinite removal of only 14%. Bentonite removals at 3 mg/L dose were different statistically between each pH. Bentonite removal at pH 7 was also the best among 3 pH
tested, with 85% removal at dose 1 mg/L. At pH 6, bentonite removal did not occur when chitosan dose increased to 3 mg/L. At pH 9, chitosans showed poor bentonite reduction between 1 and 3 mg/L doses, with ≤ 23% removal.

**Figure 5.3** Kaolinite and bentonite removal by coagulation using chitosan MW 100,000 Da at dose 1 and 3 mg/L from initial turbidity 5 NTU, tested at water pH 6, 7 and 9 (error bars = 95% CI, n = 9)

### Dose 10-30 mg/L (Figure 5.4)

At dose 10 mg/L, kaolinite removal at pH 6 and pH 7 did not occur and at pH 9 turbidity the highest removal was only 33% at dose 10 mg/L. At dose 30 mg/L, chitosan failed to remove kaolinite turbidity at all tested water pH levels.

Bentonite removal at dose 10 mg/L did not occur at pH 6 and 7 but at pH 9, it was significantly higher (one-way ANOVA, Tukey’s Posttest, p < 0.05) with 76% removal. At dose 30 mg/L and all pH levels, bentonite removal was poor, even conditions that gave some removal at doses of 1-10 mg/L.
Figure 5.4 Kaolinite and bentonite removal by coagulation using chitosan MW 100,000 Da at doses of 10 and 30 mg/L from turbidity 5 NTU test at water at pH 6, 7 and 9 (error bars = 95% CI, n = 9)

Chitosan exhibited poor kaolinite turbidity removal from 5 NTU water at every pH tested (6, 7, and 9) and residual turbidity was removed only minimally. However, chitosan effectively removed bentonite turbidity from 5 NTU water by ≥ 75% at all tested pH levels and brought turbidity to <1 NTU at chitosan dose 1 mg/L, at pH 7 (Table 5.1).
Table 5.1 Residual turbidity in chitosan coagulated 5 NTU water at different pH levels (used chitosan MW100,000 Da)

<table>
<thead>
<tr>
<th>Water pH</th>
<th>Turbidity</th>
<th>Initial turbidity</th>
<th>Residual turbidity (NTU) at each chitosan dose (mg/L) (± 95% CI, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>pH 6</td>
<td>kaolinite</td>
<td>4.52 (±0.41)</td>
<td>4.46 (±0.27)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>4.91 (±0.09)</td>
<td>4.63 (±0.19)</td>
</tr>
<tr>
<td>pH 7</td>
<td>kaolinite</td>
<td>4.77 (±0.10)</td>
<td>4.48 (±0.28)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>5.10 (±0.07)</td>
<td>4.84 (±0.10)</td>
</tr>
<tr>
<td>pH 9</td>
<td>kaolinite</td>
<td>4.65 (±0.53)</td>
<td>4.31 (±0.19)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>4.85 (±0.17)</td>
<td>4.73 (±0.15)</td>
</tr>
</tbody>
</table>

Intermediate water turbidity (30 NTU)

Effects of water pH and coagulant dose on kaolinite removal by chitosan

Effects of water pH on removal of kaolinite turbidity at varying chitosan doses are shown in Figure 5.5 and Table 5.2. Overall, kaolinite removal differed significantly at each water pH (2-way ANOVA, p < 0.0001). Generally, kaolinite removal occurred most effectively at pH 6 with 1 mg/L dose and at pH 7 with 1 and 3 mg/L chitosan doses, with 87 to 90% reductions. At pH 6 and chitosan doses of 3 mg/L or higher, kaolinite removals were poor. At pH 9, kaolinite removals were about 45-50% at doses of 1-10 mg/L, but were only 3% at dose 30 mg/L.
Effects of water pH on kaolinite removal

Figure 5.5 Kaolinite turbidity removal by coagulation using chitosan MW 100,000 Da from initial 30 NTU water at pH 6, 7 and 9

Effects of water pH and coagulant dose on bentonite removal by chitosan

The effects of pH on reduction of the intermediate 30 NTU bentonite turbidity level at different chitosan doses are shown in Figure 5.6 and Table 5.2. Bentonite removal differed significantly by water pH and chitosan doses (2-way ANOVA, p < 0.0001). At pH 7, reductions in bentonite turbidity were achieved at doses of 1 and 3 mg/L with approximately 98% removal and at 10 mg/L dose with about 80% removal, but they were much lower when at chitosan dose 30 mg/L. At pH 6, bentonite removal was greatest at dose 1 mg/L with 99% removal and decreased progressively to about 85, 50 and 20% with the increasing chitosan doses of 3, 10 and 30 mg/L, respectively. At pH 9, bentonite removal was highest at 96% for the 10 mg/L chitosan dose, reasonably high at about 85% for lower chitosan doses of 1 and 3 mg/L, and decreased to only about 10% at the 30 mg/L dose. For all pH levels tested (pH 6, 7, and 9), bentonite removal was poor at highest chitosan dose of 30 mg/L with ≤ 20% reduction.
Dose 1-3 mg/L (Figure 5.7)

At chitosan dose 1 mg/L, kaolinite removals were high at 87-90% and not significantly different at pH 6 and 7 (p>0.05). Kaolinite removal at pH 9 was significantly lower than at pH 6 and pH 7 with only 46% removal. At chitosan dose 3 mg/L, kaolinite removal remained high at 90 at pH 7, did not occur at pH 6 and was only modest at about 43% at pH 9.

Bentonite removals at 1 mg/L chitosan dose were high at 98-99%, not significantly different at pH 6 and pH 7 and significantly (p, 0.05) lower but still quite high at 86% at pH 9. At 3 mg/L chitosan dose, bentonite removal remained high at 98 at pH 7 and somewhat lower at 82% at pH 6 and 86% at pH 9. Overall, bentonite removals by chitosan were generally similar at doses 1 and 3 mg/L.
Figure 5.7 Kaolinite and bentonite removal by coagulation using chitosan MW 100,000 Da from intermediate turbidity 30 NTU water at dose 1 and 3 mg/L at pH 6, 7 and 9 (error bars = 95% CI, n = 9)

Dose 10-30 mg/L chitosan (Figure 5.8)

At chitosan dose 10 mg/L, kaolinite removal at pH 6 and pH 7 did not occur and at pH 9 it was only 45% (Figure 5.8). At dose 30 mg/L, there was no removal of kaolinite turbidity by chitosan. Overall, kaolinite reductions were significantly lower as chitosan dose became higher, increasing from 3 to 10 and 30 mg/L (p< 0.05). All pH levels, even those for which there were kaolinite turbidity removals at 1-3 mg/L doses, kaolinite reduction at 10 and 30 mg/L doses either did not occur or were low at no more than about 40%.

Similar to results for kaolinite, bentonite removals were lower at the higher chitosan dose of 30 mg/L compared to the 10 mg/L dose at all pH levels. For dose 10 mg/L, removal of bentonite at pH 9 was significantly higher at 96% than the removals of 74% at pH 7 and 47% at
pH 6 (one-way ANOVA, Tukey’s Posttest, p <0.05). Bentonite reduction by chitosan coagulation was significantly lower at the highest chitosan dose of 30 mg/L, with < 20% reduction.

Figure 5.8 Kaolinite and bentonite removal by coagulation using chitosan MW 100,000 Da for intermediate 30 NTU turbidity water at doses of 10 and 30 mg/L at water pH 6, 7 and 9 (error bars = 95% CI, n = 9)

Overall, chitosan effectively removed both kaolinite and bentonite turbidity from 30 NTU water at pH 6 and pH 7 using low coagulant doses of 1 and 3 mg/L. At these pH levels, chitosans could achieve ≥ 98% bentonite removal and reduce initial turbidity of 30-40 NTU to <5 NTU and could achieve 90% kaolinite removal and reduce initial turbidity to < 5 NTU (Table 5.2).
Table 5.2 Residual turbidity of coagulated, flocculated and settled water at different pH levels and initially 30 NTU turbidity after treating with chitosan MW100,000 Da

<table>
<thead>
<tr>
<th>Water pH</th>
<th>Turbidity</th>
<th>Initial turbidity</th>
<th>Residual turbidity (NTU) at each chitosan dose (mg/L) (± 95% CI, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>pH 6</td>
<td>kaolinite</td>
<td>34.75 (±7.43)</td>
<td>21.12 (±12.70)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>37.33 (±0)</td>
<td>30.97 (±2.60)</td>
</tr>
<tr>
<td>pH 7</td>
<td>kaolinite</td>
<td>34.20 (±14.21)</td>
<td>32.50 (±14.52)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>35.57 (±0)</td>
<td>19.96 (±2.57)</td>
</tr>
<tr>
<td>pH 9</td>
<td>kaolinite</td>
<td>34.30 (±0)</td>
<td>23.56 (±5.48)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>36.37 (±0)</td>
<td>15.31 (±14.19)</td>
</tr>
</tbody>
</table>

High water turbidity (300 NTU)

Effects of water pH and coagulant dose on removal of kaolinite 300 NTU turbidity by chitosan

Effects of water pH on removal of high kaolinite turbidity of 300 NTU at varying chitosan doses are shown in Figure 5.9 and Table 5.3. Overall, kaolinite removal differed significantly among water pH levels (2-way ANOVA, p < 0.0001). For highly (300 NTU) turbid water at neutral pH 7, the high kaolinite turbidity removal of 94% was achieved at dose 3 mg/L. However, relatively high turbidity removals also occurred at the 1 mg/L dose at pH 6 (~85%) and pH 7 (about 73%). At all pH levels, there were no kaolinite reductions at the highest chitosan dose of 30 mg/L.
Effects of water pH on kaolinite removal

The effects of water pH and coagulant dose on bentonite removal by chitosan MW 100,000 Da are shown in Figure 5.10 and Table 5.3. Bentonite removal differed significantly by water pH and chitosan doses (2-way ANOVA, $p < 0.0001$), but all removals were 90% or more. At pH 6 and 7, bentonite removals were high and 99% removal was achieved at chitosan doses of 1, 3 and 10 mg/L. At pH 6, the highest turbidity removal of 99.93% was achieved at pH 6 at dose 3 mg/L. At pH 9, the highest water pH tested, bentonite removals were highest at chitosan doses of 3, 10 and 30 mg/L but they were also quite high at 91.9% at the 1 mg/L dose. Bentonite removals were still high ($\geq 94\%$) at all pH levels at highest chitosan dose of 30 mg/L. For the same water pH, removal of bentonite was significantly better than removal of kaolinite (one-way ANOVA, Tukey’s Posttest, $p < 0.05$).

**Figure 5.9** Kaolinite removals from high initial turbidity (300 NTU) water at pH 6, 7 and 9 by coagulation using chitosan MW 100,000 Da.
Effects of water pH on bentonite removal

Figure 5.10 Effects of water pH on bentonite removal from initially high (300 NTU) turbidity water at pH 6, 7 and 9 by coagulation using chitosan MW 100,000 Da

Doses 1 and 3 mg/L (Figure 5.11)

At chitosan dose 1 mg/L, highest kaolinite removal of 85% was at pH 6 and was better than removal at pH 7 (about 73%) but these removals were not different statistically (p>0.05). Removals were lowest at pH 9 at about 35%. At chitosan dose 3 mg/L, kaolinite removal at pH 6 was much lower at only 14% and at pH 7 it was much higher at 94%. Kaolinite removal by 3 mg/L chitosan dose at pH 9 remained at about 35% as it was at the 1 mg/L dose.

Bentonite removals were greater than 90% at all pH levels at chitosan doses of 1 and 3 mg/L and removals at pH 6 and pH 7 were not different statistically (p>0.05). However, bentonite removals of 92% or more at pH 9 were significantly lower than those at pH 6 and pH 7 for both chitosan doses (p<0.05).
Effects of water pH on 300 NTU kaolinite removal at dose 1 mg/L

Effects of water pH on 300 NTU bentonite removal at dose 1 mg/L

Effects of water pH on 300 NTU kaolinite removal at dose 3 mg/L

Effects of water pH on 300 NTU bentonite removal at dose 3 mg/L

Figure 5.11 Kaolinite and bentonite turbidity removal by coagulation using chitosan MW 100,000 Da at doses of 1 and 3 mg/L from initially high (300 NTU) turbidity water at pH 6, 7 and 9 (error bars = 95% CI, n = 9)

**Doses 10 and 30 mg/L**

At high chitosan doses, kaolinite removals from high turbidity water were poor to none, although there were some removals at pH 7 (43%) and pH 9 (about 60%) at the 10 mg/L dose (Figure 5.12). Bentonite removals from high turbidity water at 10 and 30 mg/L chitosan doses were consistently >90%.

At the 10 mg/L dose, the 99.84% bentonite removal at pH 7 was significantly higher than removals at other pH levels (one-way ANOVA, Tukey’s Posttest, p <0.05), but all bentonite removals at all pH levels were > 99% (Figure 5.12). At the 30 mg/L chitosan dose, bentonite removals were somewhat lower than at the 10 mg/L dose, but all removals were ≥94 at all pH levels.
At pH 6 and 7, chitosan effectively removed bentonite from test water of 300 NTU turbidity at low doses of 1 and 3 mg/L, achieving ≥ 98% removal and bringing residual turbidity to <1 NTU or <5 NTU. Low doses of chitosan were less effective for kaolinite turbidity of 300 NTU, with > 85% removal and bringing residual turbidity to < 20 NTU at optimum dose (Table 5.3). At pH 9, chitosan removed bentonite by >99% at the optimum dose of 10 mg/L to give a residual turbidity of <1 NTU, but removal of kaolinite was generally low, with maximum removal of 89% at the 10 mg/L dose giving about 35 NTU residual turbidity.
Table 5.3 Residual turbidity at different water pH after treating with chitosan coagulant (MW100,000 Da), initial turbidity 300 NTU

<table>
<thead>
<tr>
<th>Water pH</th>
<th>Turbidity</th>
<th>Initial turbidity</th>
<th>Residual turbidity (NTU) at each chitosan dose (mg/L) (±95% CI, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>pH 6</td>
<td>Kaolinite</td>
<td>324.67 (±78.57)</td>
<td>95.42 (±136.78)</td>
</tr>
<tr>
<td></td>
<td>Bentonite</td>
<td>466.00 (±0)</td>
<td>322.89 (±48.89)</td>
</tr>
<tr>
<td>pH 7</td>
<td>Kaolinite</td>
<td>299.00 (±157.09)</td>
<td>201.00 (±116.17)</td>
</tr>
<tr>
<td></td>
<td>Bentonite</td>
<td>322.67 (±0)</td>
<td>213.44 (±62.58)</td>
</tr>
<tr>
<td>pH 9</td>
<td>Kaolinite</td>
<td>332.22 (±28.95)</td>
<td>96.98 (±81.79)</td>
</tr>
<tr>
<td></td>
<td>Bentonite</td>
<td>334.00 (±0)</td>
<td>204.11 (±47.80)</td>
</tr>
</tbody>
</table>

**II. Effects of water turbidity and chitosan coagulant doses**

*Effects of water turbidity and chitosan coagulant dose on kaolinite removal*

For the 3 kaolinite turbidity levels of 5, 30, and 300 NTU, effects on removal at varying chitosan doses were tested at water pH 7 and are shown in Figure 5.13 and Table 5.4. Both turbidity level and doses of chitosan significantly affected reductions of kaolinite (p < 0.0001). Chitosan at the lowest doses of 1 and 3 mg/L gave highest kaolinite removals of 70% or more from waters with the higher turbidity levels of 30 and 300 NTU. At the same chitosan dose, kaolinite removals were not significantly different from waters with 30 and 300 NTU turbidities. Highest reductions of kaolinite were at dose 3 mg/L with 90.2 and 94.7% at water turbidities of 30 and 300 NTU, respectively (Figure 5.13). Kaolinite removal was poor (< 15 %) at the low turbidity level of 5 NTU. However, removal of kaolinite increased significantly when water turbidity increased to 30 and 300 NTU and removals for these turbidity levels were not
statistically different at the same chitosan dose. Chitosan demonstrated lower reductions of kaolinite compared to bentonite.

Figure 5.13 Kaolinite removal after coagulation using chitosan MW 100,000 Da from initial turbidity: 5, 30, and 300 NTU

Effects of water turbidity level and chitosan coagulant dose on bentonite removal

The effects of turbidity level on removal of bentonite at varying chitosan doses are shown in Figure 5.14 and Table 5.4. Bentonite removal differed significantly by turbidity level and chitosan doses (p < 0.0001). Chitosan gave greater bentonite removals from water with higher turbidity levels. Bentonite removal at the low turbidity level of 5 NTU was significantly lower than bentonite removal at higher turbidity levels of 30 and 300 NTU. The highest bentonite removal at the low turbidity level of 5 NTU was 85% at dose 1 mg/L. Bentonite removal increased significantly from water with higher turbidities of 30 and 300 NTU and these removals were not statistically different at the low chitosan doses of 1 and 3 mg/L. Highest bentonite removals were 99% for turbidity level 300 NTU at chitosan doses of 1 - 10 mg/L. For turbidity
At chitosan doses of 1 and 3 mg/L, kaolinite removal was less than 15% for water with a low turbidity level of 5 NTU. However, chitosan coagulation effectively removed kaolinite when water turbidity levels were higher at 30 and 300 NTU, with 77 – 95% removals at doses of 1-3 mg/L, as shown in Figure 5.15.

Bentonite removals by coagulation at low 1 and 3 mg/L chitosan doses were generally high at >75 to >90% but differed significantly among turbidity levels, as shown in Figure 5.15. Bentonite removal from low 5 NTU turbidity water was much higher than kaolinite removal at the same turbidity level with 79 – 85% removal. In waters with turbidities of 30 and 300 NTU, turbidity level did not affect bentonite removals, with high removals of 97% to nearly 100%. At
the same chitosan dose, bentonite removals were statistically similar for waters with 30 and 300 NTU turbidity (p > 0.05). For the same turbidity level at the same chitosan dose, removal of bentonite was significantly higher than removal of kaolinite at chitosan doses of 1 and 3 mg/L, (one-way ANOVA, Tukey’s Posttest, p < 0.05), except for water of turbidity 300 NTU at the chitosan dose of 3 mg/L.

Figure 5.15 Kaolinite and bentonite removal by coagulation using chitosan MW 100,000 Da at doses of 1 and 3 mg/L from waters of initial turbidity 5, 30 and 300 NTU (error bars = 95% CI, n = 9)

**Chitosan Dose 10-30 mg/L (Figure 5.16)**

At the 10 mg/L chitosan dose, kaolinite removals were poor, with no removals from waters with 5 and 30 NTU turbidity levels. Only at the high turbidity level of 300 NTU did chitosan give some kaolinite turbidity removal of 45% at the 10 mg/L chitosan dose (Figure 5.16).
At the 10 mg/L dose, chitosan did not remove bentonite from water of low (5 NTU) turbidity, but bentonite removals from waters at 30 and 300 NTU turbidity were 79 and 99.85%, respectively. At the highest chitosan dose of 30 mg/L, bentonite removal was poor for 5 and 30 NTU waters (≤2%) but, for the highest turbidity water of 300 NTU removal of bentonite was significantly higher at 94% compared to removals from lower turbidity waters (one-way ANOVA, Tukey’s Posttest, p <0.05).

Chitosan exhibited poor kaolinite removal at every dose for low turbidity (5 NTU) water and residual turbidity levels were only minimally lower after coagulation. Highest kaolinite removals of 95% occurred from water at the highest turbidity level of 300 NTU at chitosan dose 3 mg/L (95%) which brought residual kaolinite turbidity to 11 NTU. For water of intermediate 30 NTU turbidity, kaolinite removals by coagulation were relatively effective at chitosan doses of 1 and 3 mg/L which reduced turbidities by about 88-90% to about 3 and 4 NTU, respectively.

**Figure 5.16** Kaolinite and bentonite removal by coagulation using chitosan MW 100,000 Da at doses 10 and 30 mg/L from waters of initial turbidity 5, 30 and 300 NTU (error bars = 95% CI, n = 9)
Chitosan coagulation was more effective for bentonite removal than kaolinite removal for all 3 water turbidity levels tested. In low (5 NTU) turbidity water, bentonite turbidity was reduced to about 0.7 and 1 NTU at chitosan doses of 1 and 3 mg/L. In intermediate turbidity water of 30 NTU, bentonite turbidity was reduced to as low as 0.4 NTU or nearly 90% by chitosan doses of 1 and 3 mg/L. At the highest water turbidity of 300 NTU, bentonite reductions were about 99.9% and residual bentonite turbidities were < 1 NTU at chitosan doses of 3 and 10 mg/L (Table 5.4).

### Table 5.4 Residual turbidity after coagulation using chitosan MW 100,000 Da at different levels of water turbidity

<table>
<thead>
<tr>
<th>Water turbidity, NTU</th>
<th>Turbidity</th>
<th>Initial turbidity</th>
<th>Residual turbidity (NTU) at each chitosan dose (mg/L) (±95% CI, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>kaolinite</td>
<td>4.77 (±0.10)</td>
<td>4.48 (±0.28)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>5.10 (±0.07)</td>
<td>4.84 (±0.10)</td>
</tr>
<tr>
<td>30</td>
<td>kaolinite</td>
<td>34.20 (±14.21)</td>
<td>32.50 (±14.52)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>35.57 (±0.00)</td>
<td>19.96 (±2.57)</td>
</tr>
<tr>
<td>300</td>
<td>kaolinite</td>
<td>299.00 (±157.09)</td>
<td>201.00 (±116.17)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>322.67 (±0.00)</td>
<td>213.44 (±62.58)</td>
</tr>
</tbody>
</table>

**III. Effects of water salinity and coagulant doses**

*Effects of water salinity and coagulant dose on kaolinite removal by chitosan*

Effects of water salinity on removal of kaolinite (5 NTU) turbidity at varying chitosan doses are shown in Figure 5.17 and Table 5.5. Overall, chitosan poorly removed kaolinite from water at all 3 salinity levels. Chitosan could remove kaolinite minimally at dose 1 mg/L but
coagulation did not occur when chitosan doses exceeded 1 mg/L. Maximum reductions in kaolinite turbidity was only 23% at salinity level of 1 ppt (part per thousand).

Figure 5.17 Effects of salinity (0.1 – 1.0 ppt) on kaolinite removal from water of 5 NTU turbidity by coagulation using chitosan MW 100,000 Da at doses of 1-30 mg/L

Effects of water salinity on reduction of bentonite turbidity at varying chitosan doses are shown in Figure 5.18 and Table 5.5. Water salinity and chitosan doses affected bentonite removal significantly (p < 0.0001). However, chitosan only removed bentonite turbidity by 77 – 86% at low chitosan doses of 1 and 3 mg/L and higher salinity levels showed higher bentonite removals (p < 0.05). At salinity 1 ppt, chitosan exhibited the highest bentonite removals at both 1 and 3 mg/L doses of 86 and 84%, respectively. Bentonite removals at lower salinities of 0.1 and 0.3 ppt were >75% at 1 and 3 mg/L chitosan doses. Chitosan failed to remove bentonite at higher chitosan doses of 10 and 30 mg/L.
Figure 5.18 Effects of water salinity on bentonite removal from water of 5 NTU turbidity by coagulation using chitosan MW 100,000 Da at doses of 1-30 mg/L

Chitosan Doses of 1 and 3 mg/L (Figure 5.19)

At chitosan dose 1 mg/L, kaolinite removal was poor (14 – 23 %) for all water salinity levels and the removals were not significantly different (One-way ANOVA, p > 0.05). Kaolinite removal did not occur at chitosan dose 3 mg/L for all water salinities as shown in Figure 5.19.

Bentonite removals at the low 1 and 3 mg/L doses were much higher than those of kaolinite at the same salinity levels. Chitosan removed bentonite by ≥ 77 % at all salinity level. Bentonite removal at salinity 1 ppt was the highest among 3 salinity levels, with 86% removal at dose 1 mg/L and 84% at dose 3 mg/L.
Effects of water salinity on 5 NTU kaolinite removal at chitosan dose 1 mg/L

Effects of water salinity on 5 NTU bentonite removal at chitosan dose 1 mg/L

Effects of water salinity on 5 NTU kaolinite removal at chitosan dose 3 mg/L

Effects of water salinity on 5 NTU bentonite removal at chitosan dose 3 mg/L

**Figure 5.19** Kaolinite and bentonite removals from water of 5 NTU turbidity after coagulation using chitosan MW 100,000 Da at doses of 1 and 3 mg/L at varying salinities of 0.1 to 1 ppt and pH 7 (error bars = 95% CI, n = 9)

**Chitosan Doses of 10-30 mg/L (Figure 5.20)**

At doses of 10 and 30 mg/L, chitosan failed to remove kaolinite from water of 5 NTU turbidity at all salinities tested. As for kaolinite, bentonite was not removed at chitosan doses of 10 and 30 mg/L from 5 NTU waters of all three salinity levels.
In water of low (5 NTU) turbidity, chitosan coagulation gave poor kaolinite removals at all water salinities tested (0.1, 0.3, and 1 ppt) with remaining turbidities essentially unchanged from the initial turbidities, as shown in Table 5.5. However, chitosan effectively removed bentonite turbidity by 77-86% at low chitosan doses and could bring residual bentonite turbidity to less than 1 NTU at chitosan doses of 1 and 3 mg/L.
Table 5.5 Residual turbidities in coagulated-flocculated and settled waters of initial 5 NTU turbidity and different salinities after coagulation using chitosan MW 100,000 Da at different doses

<table>
<thead>
<tr>
<th>Water salinity, ppt</th>
<th>Turbidity</th>
<th>Initial turbidity</th>
<th>Residual turbidity (NTU) at each chitosan dose (mg/L) (±95% CI, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>Kaolinite</td>
<td>4.55 (±0.00)</td>
<td>4.28 (±0.17)</td>
</tr>
<tr>
<td></td>
<td>Bentonite</td>
<td>6.39 (±0.48)</td>
<td>6.26 (±0.43)</td>
</tr>
<tr>
<td>0.3</td>
<td>Kaolinite</td>
<td>4.77 (±0.10)</td>
<td>4.48 (±0.28)</td>
</tr>
<tr>
<td></td>
<td>Bentonite</td>
<td>5.10 (±0.07)</td>
<td>4.84 (±0.10)</td>
</tr>
<tr>
<td>1.0</td>
<td>Kaolinite</td>
<td>5.87 (±0.37)</td>
<td>5.78 (±0.29)</td>
</tr>
<tr>
<td></td>
<td>Bentonite</td>
<td>4.76 (±0.13)</td>
<td>4.36 (±0.24)</td>
</tr>
</tbody>
</table>

5.3.2 Effects of water quality on microbial coagulation by chitosan

I. Effects of water pH and coagulant dose on microbial coagulation

Effects of water pH and chitosan dose on bacteria removal

Similar to experiments for turbidity removal, the same 3 pH levels of test water, pH 6, 7 and 9, were evaluated for bacterial removal by chitosan coagulation of test water with an initial turbidity of 5 NTU. As shown in Figure 5.21, bacterial removal was not significantly affected by water pH levels of 6, 7 or 9 at the different chitosan coagulant doses of 1-30 mg/L (2-way ANOVA, p>0.05). Chitosan exhibited highest E. coli removals at the higher coagulants doses of 10 and 30 mg/L for all 3 water pH levels tested, with about 3 – 5 log₁₀ reductions as shown in Figure 5.21.
Effects of water pH on E. coli removal

Figure 5.2 Log$_{10}$ reductions of E. coli bacteria by coagulation with different doses of chitosan MW100,000 Da at different pH levels of test water with initial turbidity of 5 NTU.

Effects of water pH and chitosan dose on virus removal

Overall virus removal was significantly affected by the different pH levels of test water with turbidity 5 NTU (2-way ANOVA, p<0.0001). At all pH levels of test water, virus removals were high ($\approx$3-5 log$_{10}$ reduction) at chitosan doses 10 and 30 mg/L (Figure 5.22). For the same chitosan dose, viral removal was higher at pH 6 than pH 7 and 9 test water. Chitosan gave significantly better virus removals at pH 6 than virus removal at pH 9 at every chitosan dose tested (p<0.01). Chitosan dose somewhat affected virus removals at pH 6 compared to pH 7 test water. Virus removal at pH 6 was greater than removal at pH 7 at 1 and 10 mg/L doses but the removals were not statistically different at 3 and 30 mg/L dose.
Effects of water pH on MS2 removal

Figure 5.22 Log$_{10}$ reductions of bacteriophage MS2 after coagulation with chitosan MW100,000 Da at different doses in test waters with different pH levels and turbidity 5 NTU.

Chitosan Doses of 1 and 3 mg/L (Figure 5.23)

At the lowest dose of 1 mg/L, there were no differences in bacteria removals from 5 NTU waters at different pH levels, with approximately 1 log$_{10}$ reduction of *E. coli*. However, at chitosan dose 3 mg/L, bacterial removals were higher at 3.65 and 3.91 log$_{10}$ for waters at pH 6 and 7, respectively, and were significantly lower at pH 9 (0.64 log$_{10}$).

Virus removals were significantly higher at low pH than higher pH in 5 NTU test water. At the lowest chitosan dose of 1 mg/L, chitosan gave 1.64, 0.26 and 0.03 log$_{10}$ virus reductions at pH 6, 7 and 9, respectively. Similar to bacteria removal, virus removal was higher at higher chitosan doses, with 3.85 to 3.65 log10 reductions at the 3 mg/L chitosan dose at pH 6 and 7 respectively. However, viruses were not removed from test water at pH 9 with the low 1 and 3 mg/L chitosan doses tested.
**Figure 5.23** *E. coli* and bacteriophage MS2 reduction from 5 NTU test waters at pH 6, 7 and 9 by coagulation using chitosan MW 100,000 Da at doses of 1 and 3 mg/L. (error bars = 95% CI, n = 3)

### Chitosan Doses 10 and 30 mg/L (Figure 5.24)

Overall, bacterial removals from 5 NTU test waters at pH 6, 7 and 9 were greater at chitosan doses of 10 and 30 mg/L (range 3.20 – 4.82 log\textsubscript{10}) than removals at 1 and 3 mg/L doses (0.6 to 3.9 log\textsubscript{10}). At pH 6, chitosan gave the highest bacterial log\textsubscript{10} reductions of 4.51 and 4.82 log\textsubscript{10} at doses 10 and 30 mg/L, respectively. However, bacteria reductions at the different water pH levels were not significantly different (p > 0.05) and at the same chitosan doses, bacterial removals were not significantly different at each pH level.

Virus removals from 5 NTU test water were also greater at chitosan doses of 10 and 30 mg/L (range 2.91 – 4.86 log\textsubscript{10}) compared to removals at 1 and 3 mg/L doses (range 0.03 – 3.85 log\textsubscript{10}). Virus removals were significantly higher at pH 6 and were lower at higher pH levels. At
both 10 and 30 mg/L chitosan doses, virus removals were highest at pH 6 with 4.86 and 4.53 \( \log_{10} \), respectively, compared to reductions of 3 to 3.5 \( \log_{10} \) at higher pH levels.

![Graphs showing E. coli and MS2 reductions at different pH levels.](image)

**Figure 5.24** *E. coli* and bacteriophage MS2 reductions from 5 NTU test waters at pH 6, 7 and 9 by coagulation using chitosan MW 100,000 Da at doses of 10 and 30 mg/L (error bars = 95% CI, n = 3)

**II. Effects of water turbidity and chitosan dose on microbial coagulation**

Effects of water turbidity and chitosan dose on bacteria removal by coagulation

Bacteria removals were not significantly different in pH 7 test waters with different turbidities of 5, 10 and 30 NTU (p = 0.5432), as can be seen in Figure 5.25. However, *E. coli* reductions from test waters differed among chitosan doses (see Figure 5.25). At the lowest chitosan dose of 1 mg/L, bacterial removals were consistently low at <1.5 \( \log_{10} \) at all water turbidities tested. However, at higher chitosan doses of 3, 10, and 30 mg/L, bacterial removals were higher, at about 2.5 to 3.5 \( \log_{10} \) for the 3 mg/L dose and about 4 to 5 \( \log_{10} \) for the 10 and 30 mg/L doses.
Effects of water turbidity on E. coli removal

![Graph showing Log₁₀ reductions of E. coli bacteria from pH 7 test waters of 5, 10 and 30 NTU turbidities by coagulation using chitosan MW 100,000 Da at different doses.](chart)

**Figure 5.25** Log₁₀ reductions of E. coli bacteria from pH 7 test waters of 5, 10 and 30 NTU turbidities by coagulation using chitosan MW 100,000 Da at different doses

Effects of water turbidity and chitosan dose on virus removal by coagulation (Figure 5.26)

Water turbidity generally did not affect the extent of virus removal by coagulation at each chitosan dose tested, as can be seen in Figure 5.26. Although virus removals were significantly different (p < 0.001) among water turbidities at chitosan dose 3mg/L, all virus reductions were extensive (>3.5 log₁₀), with 3.56, 4.89 and 4.71 log₁₀ removals from waters of turbidity 5, 10 and 30 NTU, respectively (see Figure 5.26). At all chitosan doses tested, there were no significant differences in virus removal from waters of 10 and 30 NTU turbidity, with virus removals of >3.5 log₁₀.
Figure 5.26 Log$_{10}$ reductions of bacteriophage MS2 from pH 7 test waters of 5, 10 and 30 NTU turbidity by coagulation using chitosan MW 100,000 Da at doses of 1-30 mg/L.

**Chitosan Doses 1 and 3 mg/L (Figure 5.27)**

Bacterial removals were lower at chitosan dose 1 mg/L than 3 mg/L in all 3 test waters of different turbidities, with *E. coli* removals of about 1 log$_{10}$ at the 1 mg/L dose to between 2.90 – 3.91 log$_{10}$ removals at the 3 mg/L dose. Bacterial removals were somewhat lower from water of 30 NTU turbidity than other turbidity levels, but the differences were not statistically significant.

At the chitosan dose of 1 mg/L, virus removals were consistently low for all three turbidities, with 0.26, 0.38 and 0.66 log$_{10}$ removals at turbidities of 5, 10 and 30 NTU, respectively. Viral removals were significantly higher at the 3 mg/L chitosan dose, with > 3.56 log$_{10}$ removal and removals were significantly lower at turbidity 5 NTU (about 3.6 log$_{10}$) than at 10 and 30 NTU (4.7-4.9 log$_{10}$).
Chitosan Doses of 10 and 30 mg/L (Figure 5.28)

Bacterial removals were greater at chitosan doses of 10 and 30 mg/L at 4 log_{10} or more compared to the low removals of <1.5 log_{10} at the 1 mg/L dose. Chitosan gave the highest bacterial reduction of 4.95 log_{10} at dose 10 mg/L from water of 10 NTU turbidity, although the removals were not different significantly among three turbidity levels tested (p > 0.05). Chitosan also effectively removed bacteria at the 30 mg/L dose by > 3.20 log_{10} for all water turbidities tested and the removals were not significantly different among 3 water turbidities of 5, 10 and 30 NTU.
Virus reductions by the 10 and 30 mg/L chitosan doses were >3 log$_{10}$ and at the same
dose, removals were similar for waters of different turbidities. Viral removals were not
significantly different for chitosan doses of 10 to 30 mg/L (p > 0.05), ranging from 3.47 -3.89
log$_{10}$ for dose 10 mg/L and 3.41 – 3.73 for dose 30 mg/L.

**Figure 5.28** *E. coli* and bacteriophage MS2 reduction from pH 7 water of 5, 10 and 30 NTU
turbidity by coagulation using chitosan MW 100,000 Da at doses of 10 and 30 mg/L (error bars =
95% CI, n = 3)

**III. Effects of water salinity and coagulant dose on microbial coagulation**

*Effects of water salinity and chitosan dose on bacteria removal (Figure 5.29)*

At the 3 salinities of 0.1, 0.3 and 1.0 part per thousand (ppt) in water at pH 7 and 5 NTU
turbidity, log$_{10}$ *E. coli* removals at each chitosan dose were not significantly different (2-way
ANOVA p = 0.8872), although removals differed among the chitosan doses tested. All three
salinity levels showed similar trends of much lower bacteria removals of $<1.2 \log_{10}$ at 1 mg/L dose and much higher ($>3.5 \log_{10}$) removals at higher chitosan doses of 3, 10 and 30 mg/L.

![Figure 5.29](image)

**Effects of water salinity on E. coli removal**

<table>
<thead>
<tr>
<th>Chitosan dose (mg/L)</th>
<th>Log$_{10}$ Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1ppt-E.coli</td>
<td>0.3ppt-E.coli</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>1ppt-E.coli</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>0.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**Figure 5.29** Log$_{10}$ reductions of *E. coli* bacteria from pH 7 and 5 NTU turbidity waters of different salinities by coagulation using chitosan MW 100,000 Da at doses of 1, 3, 10 and 30 mg/L.

Water salinity influenced virus removal somewhat, with greater reductions of about 4 log$_{10}$ at the highest 1 ppt salinity than the slightly lower 3-3.5 log$_{10}$ reductions at the low water salinity of 0.1 ppt (see Figure 5.30). At all three salinity levels, virus removals were much lower ($<0.8 \log_{10}$) at 1 mg/L dose and much higher ($>3 \log_{10}$) at the higher chitosan doses of 3, 10 and 30 mg/L.
Effects of water salinity on MS2 removal

Figure 5.30 Log$_{10}$ reductions of bacteriophage MS2 virus in pH 7 and 5 NTU test waters of different salinities of 0.1, 0.3 and 1 ppt by coagulation using chitosan MW 100,000 Da

**Chitosan Doses 1 and 3 mg/L (Figure 5.31)**

At all 3 salinity levels of 0.1, 0.3 and 1 ppt, chitosan coagulation gave much lower bacterial removals of 1.2 log$_{10}$ at the low dose of 1 mg/L then the much higher removals of >3.9 log$_{10}$ at the higher chitosan dose of 3 mg/L. Chitosan doses had greater impacts on bacteria removals than did water salinity. At the 1 mg/L chitosan dose bacteria removals were higher at 1 log$_{10}$ in water with 0.3 ppt than in waters with 0.1 and 1.0 ppt salinities with removals of <0.3 log$_{10}$. However, bacteria removals from water of different salinities were not statistically different at the 3 mg/L chitosan dose, with removals of about 4-5 log$_{10}$ at all water salinities tested.

Similar to bacterial removals, virus removals by chitosan were much lower at dose 1 mg/L (< 0.8 log$_{10}$) for all three water salinities. Viral reductions were significantly higher at >3.56 log$_{10}$ at the higher chitosan dose of 3 mg/L (> 3.56 log$_{10}$). At these low chitosan doses of 1 and 3 mg/L, water salinity minimally influenced the extent of virus removal but doses of
chitosan greatly influenced virus removals, with much greater removals at the 3 mg/L dose than the 1 mg/L dose as shown in Figure 5.31.

![Graphs showing E. coli and MS2 removal at dose 1 mg/L and 3 mg/L](image)

**Figure 5.31** E. coli and bacteriophage MS2 reductions from pH 7 and 5 NTU turbidity waters at salinities of 0.1, 0.3 and 1 ppt after coagulation using chitosan MW 100,000 Da at doses of 1 and 3 mg/L (error bars = 95% CI, n = 3)

**Dose 10 and 30 mg/L (Figure 5.32)**

At all 3 salinity levels tested, chitosan provided higher bacterial removals at doses of 10 and 30 mg/L (3.20 – 5.15 log\(_{10}\)) than at dose 1 mg/L. Chitosan provided the highest bacterial reduction (5.15 log\(_{10}\)) at dose 10 mg/L and at salinity 1.0 ppt. However, bacterial removals were not different significantly among three salinities (p > 0.05). Moreover, increasing of chitosan dose from 10 to 30 mg/L did not significantly increase bacterial removals as shown in Figure 5.32.
At the same 3 water salinities, viral removals were not different at chitosan doses of 3, 10 or 30 mg/L, with log_{10} reductions of about 3 to 4 log_{10}. However, virus reductions differed somewhat with water salinity, between high salinity 1.0 ppt and low salinity 0.1 ppt. At chitosan dose 10 mg/L, virus removal was significantly better at salinity 1 ppt (3.96 log_{10}) than at salinity 0.1 ppt (3.19 log_{10}) (p<0.01). Also at dose 30 mg/L, virus removal was significantly better at salinity 1 ppt (3.99 log_{10}) than salinity at 0.1 ppt (2.79 log_{10}) (p<0.001). Virus removals were not statistically different in waters with 0.3 and 1 ppt salinities (p>0.05 value).

![Effects of water salinity on E. coli removal at dose 10 mg/L](image1)

![Effects of water salinity on E. coli removal at dose 30 mg/L](image2)

![Effects of water salinity on MS2 removal at dose 10 mg/L](image3)

![Effects of water salinity on MS2 removal at dose 30 mg/L](image4)

**Figure 5.32** *E. coli* and bacteriophage MS2 reduction after coagulation using chitosan MW 100,000 Da at dose 10 and 30 mg/L at different water salinity (error bars = 95% CI, n = 3)
5.4 Discussion

**Effects of water pH on chitosan performance**

*Water pH effects on turbidity removal*

Amino groups (–NH₂) on the glucosamine monomer of chitosan are a key factor for the coagulation process. Chitosan is expected to be more effective in water with acidic pH which approximates the chitosan pKa of 6-6.5 (Roussy et al, 2004; Kong et al, 2010), as most amine groups can be protonated to produce more positive charges for the coagulation process. At low (5 NTU) turbidity, kaolinite removals were poor regardless of water pH and chitosan dose. For this level of bentonite turbidity, to achieve similar turbidity removal (76-78%), bentonite coagulation at pH 6 (≈ pKa of amino group) required a lower chitosan dose (1 mg/L) than coagulation at pH 9 (dose 10 mg/L). Coagulation in water at pH 7 had a wider range of optimum chitosan dose than water at pH 6 and 9. Generally, effects of water pH on medium (30 NTU) and high (300 NTU) turbidity had similar trends for low (5 NTU) turbidity water, with the optimum doses of chitosan for turbidity removal usually higher at the higher pH of the test water. Lower doses of chitosans were effective at lower water pH levels; optimum doses for turbidity removal were 1, 3, and 10 mg/L for waters at pH 6, 7 and 9, respectively. Roussy et al (2005) also found the same trend of lower chitosan doses being more effective for bentonite removal at pH 5 than at pH 7 and that chitosan coagulation of bentonite turbidity was effective at both the low pH of 5 and at neutral pH 7. Another study also reported that removal of bentonite by chitosan coagulation was similar between pH 4 and pH 7 (Huang & Chen, 1996).

In this study chitosan effectively removed turbidity from test waters at all pH levels tested (pH 6- pH 9) but optimum chitosan doses differed. At higher water pH higher chitosan dose was required for effective turbidity reduction. These results suggest that more positive
charges were needed in the coagulation system because protonation of chitosan is less efficient at higher water pH. The effectiveness of chitosan coagulant was likely affected by protonation of amine groups at each water pH. Only limited previous research has reported testing the effects of water pH on chitosan coagulation efficiency. Roussy et al (2004) suggested that at high water pH levels ≥ 9, the majority of amine groups on the chitosan structure were de-protonated and precipitated. The authors suggested that turbidity removal at high pH possibly occurred because particles were entrapped by the precipitating chitosan instead of charge neutralization. At an acidic low pH of 6, effective turbidity removal was achieved with only a low chitosan dose of 1 mg/L dose and the turbidity removal was significantly lower at higher chitosan dose. This observation suggests strong effects of excess chitosan at acidic pH. At pH 6 (≤ pKa of amine), most amine groups are likely protonated so this condition favors charge neutralization between chitosan and clays because more positive charges from protonation of amine groups will attach to clays and neutralize their negative surface charge, thereby producing coagulation. Increasing dose of chitosan means an excess of positive charges in the colloidal system causing repulsion of the same charged particles and reducing the effectiveness of coagulation because particles and coagulant have fewer contacts, resulting in less efficient coagulation at this low pH. For chitosan coagulant, pH influences protonation but not speciation transformation (change of ions or molecules by forming a series of products due to hydrolysis reaction) of chitosan, unlike metal salt coagulants. Normally, the effectiveness of metal salt coagulants, such as alum or ferric salts, depends on water pH because it influences speciation transformation of metal salts. Metal salt coagulants will undergo hydrolysis reactions after being added to water, resulting in different products or metal species (e.g. at low pH hydrolyzed Al (III) can form Al₂(OH)₂⁴⁺ or Al₃(OH)₄⁵⁺ and at high pH Al(OH)₃↓). The amount or distribution of each metal species varies and is
dependent on the pH of the water (Yan et al, 2008). The effectiveness of coagulation using metal salts is therefore significantly affected by the pH of the water. For coagulation using chitosan, protonation and de-protonation were likely critical for coagulation reactivity, but the speciation of chitosan was unchanged and not an influencing factor for the coagulation process.

*Effect of water pH on microbial removal*

From the results of this study pH influenced the extent of bacterial removal by chitosan coagulation under some dose conditions. At an acidic pH of 6 and neutral pH 7, bacterial removals were similar at all chitosan doses. At the basic pH of 9, however, higher chitosan doses were needed to achieve comparable bacterial removals as at pH 6 and pH 7. Chitosan could effectively remove *E. coli* bacteria (3-5 log$_{10}$) at pH 6-9 although a chitosan dose as high as 10 mg/L was needed for basic water at pH 9. Chitosan was less protonated at pH 9 so more chitosan was needed to achieve coagulation efficacy.

Based on the results of this study, virus removal was different from bacteria removal by chitosan coagulation and virus removal was significantly affected by waters at different pH levels. Significantly greater virus removals were achieved at acidic pH 6 than at basic pH 9 at every chitosan dose tested. Like bacterial removal, removal of viruses at pH 9 required higher chitosan doses of 10-30 mg/L to achieve ≈ 3 log$_{10}$ microbial reductions.

At the lowest chitosan dose of 1 mg/L, bacteria removals were generally low and water at acidic pH 6 did not give better bacteria removals than water at to higher pH levels of 7 and 9. However, virus removals at the acidic pH of 6 were much greater than at neutral pH 7 and basic pH 9. These results illustrate that at the low 1 mg/L dose, the protonation of chitosan at acidic pH was apparently effective for virus removal but was not effective for bacteria removal. The measured zeta potential of bacteriophage MS2 in test water was -19.86 mV whereas the zeta
potential of *E. coli* bacteria in test water was -38.06 mV. It is likely that positive charges initiate microbial coagulation using chitosan. At acidic pH, chitosan is likely to destabilize colloids through charge neutralization and the positive charges produced by a 1 mg/L dose of chitosan may be sufficient to destabilize MS2 in water but are not enough to destabilize the bacteria in the water with their higher negative surface charges.

**Effects of water turbidity on chitosan performance**

*Water turbidity level effects on turbidity removal*

Water turbidity causes aesthetic problems in drinking water and also can affect performance of a coagulant during coagulation-flocculation. In general, higher initial water turbidity helps improve the effectiveness of coagulation-flocculation because there are more particles in water that increase the chances of contacts between coagulant and particles. In low turbidity water there are fewer particles and this characteristic can reduce the effectiveness of a coagulant. Fewer particles in water provide fewer chances for coagulant and colloidal particles to come in contact, so fewer flocs are formed and coagulation-flocculation is less effective. It is expected that the increasing water turbidity increases the optimum dose of a coagulant because there will be more colloidal particles which will need more coagulant for effective destabilization. From the results of this study, chitosan was less effective for coagulation for water with low initial turbidity ≤ 5 NTU and the effect was more pronounced for kaolinite than bentonite. The effect of low turbidity water on coagulation efficiency is an expected phenomenon as described before because there are fewer chances for coagulant and particles to contact in a low turbidity suspension. Turbidity removals increased as initial turbidity increased to 30 and 300 NTU. However, optimum chitosan doses did not change related to initial turbidity. This observation differed from a study by Huang & Chen (1996) who reported
increases of optimum chitosan doses for water of increased initial turbidity. In the present study turbidity removals were not significantly different between water of turbidity 30 and 300 NTU at the optimum coagulant dose. It is possible that there is a threshold for turbidity particles and coagulant to produce effective coagulation. Too few particles result in less contact between turbidity particles and coagulant but once particles in water are at a sufficient level for chitosan to adsorb and produce coagulation, the amount of particles beyond that level is less crucial. It is also possible that increasing in the amount of turbidity particles accelerates natural settling in high turbidity water during the treatment process so that medium and high turbidity water show similar overall removal (Chen & Chung, 2011).

Water turbidity effects on microbial removal

From the results of this study water turbidity generally did not influence microbial removal by coagulation. Bacteria and virus removal efficiencies did not change in waters with 5 and 30 NTU initial turbidity. This phenomenon makes coagulation different from water disinfection. For water disinfection, water turbidity usually impairs effectiveness of microbial disinfection. Based on the results of this study, higher water turbidity did not decrease efficacy of chitosan for bacteria and virus removals. Also, effects of overdosing on microbial removals were minimal, and high removals (≥ 3 log₁₀) were achieved for chitosan doses from 3 to 30 mg/L. However, we observed that turbidity level had some effects on virus removal at the chitosan dose of 3 mg/L. At this chitosan dose, virus removals from water at higher turbidity levels (10 and 30 NTU) were significantly higher than the removals at low turbidity (5 NTU). However, virus removals at all 3 turbidity levels were still extensive at ≥ 3.5 log₁₀.

It is possible that combined effects of the 3 mg/L chitosan dose which is the optimum for turbidity removal at neutral pH and higher numbers of clay particles at 10 and 30 NTU, together
create better conditions for virus removal. In this case, it seems that virus responds more like kaolinite turbidity than do bacteria. So at this dose, higher turbidity levels improve virus removals.

**Effects of water salinity on chitosan performance of coagulation**

*Water salinity effects on turbidity removal by coagulation*

Salinity relates to inorganic salts dissolved in water. It affects palatability of drinking water and possibly influences efficacy of a coagulant. Based on the results of this study, removal of turbidity from low turbidity water of 5 NTU seemed to increase as water salinity increased in range of 0.1-1 ppt (100 -1,000 mg/L) salinity, but the removals were not statistically significantly different. However, a small variation was observed at chitosan dose 3 mg/L where bentonite removals were different between low salinity (0.1 ppt) and high salinity (1.0 ppt) water. In the coagulation process, water salinity or salts affect compression of the electrical double layer of colloids. The double layer of colloids represents the energy around colloidal particle which keeps colloids away from each other. The double layer helps in stabilizing colloids and makes it difficult to form particle clumps or flocs and remove them from water. To destabilize colloids and minimize the double layer, salts can be added into the coagulation system to help compress the double layer so colloidal particles can overcome repulsive forces and induce particle clumping and formation of flocs. So, higher a salt concentration should be better for coagulation. However, a clear improvement of coagulation as a result of water salinity in a range that is acceptable for drinking water (< 1.0 ppt) was not observed. Possibly a higher salinity beyond 1 ppt is required to improve coagulation significantly but such elevated salinity might create taste issues. No previous work was found that evaluates the effects of salt concentration on water treatment by chitosan coagulation, so the findings of this current study are new and unique.
Water salinity effects on microbial removal by coagulation

Overall, water salinity statistically significantly influenced chitosan performance for virus removal but not bacteria removal. Although chitosan efficacy for virus removal was affected by water salinity, virus removal was effective from low (0.1 ppt) to high salinity (1.0 ppt). Inorganic solutes or salts, theoretically, will help improve coagulation but we did not clearly observe such distinct impacts for microbial coagulation. Varying levels of water salinity did not compromise microbial removal and low doses of 3–10 mg/L chitosan can achieve 3 to 5 log\textsubscript{10} removal of *E. coli* bacteria and indicator virus MS2.

Analyzing all experimental conditions (i.e. varying water pH, turbidity and salinity) by linear regression model, gave $r^2$ values of 0.323 and 0.3 for turbidity and microbial removal respectively. These results suggest that water quality parameters (combined together) in the range tested, which are within the acceptable water quality parameter ranges for drinking water, had limited effects on the efficacy of chitosan coagulation for turbidity and microbial removals. At varying water quality conditions, chitosan still gave high removals of turbidity ($\geq$85%) and microbes (3-5 log\textsubscript{10}) at optimum chitosan doses of 3 and 10 mg/L.

5.5 Conclusions

Water quality parameters have minimal impact on overall chitosan performance. At varying water quality conditions, chitosan can effectively remove turbidity ($\geq$ 85%) and bacteria and virus (3-5 log\textsubscript{10}) at optimum doses of 3 and 10 mg/L. Therefore, chitosan can be a useful and effective candidate for point of use water treatment.
• Water quality influences chitosan performance and the effects are different for the removals of bentonite, kaolinite, bacteria, and viruses.

• Generally, optimum doses of chitosan for turbidity removal increased with increasing pH of water (pH 6 to 9) and the same trend was observed for increasing water turbidity (5 - 300 NTU). However, percent turbidity removals were extensive (≥ 85%) at these optimum coagulant doses at every pH tested (pH 6-9).

• Water pH had minimal effects on bacterial removal. Chitosan produced high bacterial removal (> 3 -5 log$_{10}$) between doses of 3-30 mg/L although more chitosan was required to initiate effective microbial coagulation at high pH 9.

• Virus removal by chitosan was significantly influenced by water pH and virus removal at acidic pH was greater than the removal at basic pH. Like bacteria removal, however, virus removal was considerable (≥ 3-5 log$_{10}$) at all pH levels tested.

• It is likely that water pH influences the effectiveness of chitosan coagulant because it relates to protonation of chitosan (H$^+$) but not the chemical speciation of the chitosan coagulant.

• Initial water turbidity had minimal effects on chitosan efficacy for turbidity removal. Chitosan was less effective for water with low initial turbidity ≤ 5 NTU and the impact was more prominent for kaolinite than bentonite turbidity. However, chitosan performance was not significantly different between turbidity 30 and 300 NTU at the optimum dose, with > 90% removal.

• Water turbidity generally did not influence microbial removals. Increasing initial water turbidity from 5 NTU to 30 NTU did not change bacteria and virus removals statistically.
Reductions of $3-5 \log_{10}$ were achieved at chitosan doses of 3-30 mg/L for all water turbidity levels.

- Higher initial water turbidity did not increase the optimum chitosan doses for effective coagulation.

- Water salinity in range of 0.1-1 ppt (100 -1,000 mg/L) did not significantly change removals of kaolinite and bentonite from low turbidity water of 5 NTU by chitosan coagulation.

- Salinity levels influenced the efficacy of chitosan performance for virus removal but not bacteria removal. However, varying levels of water salinity did not compromise microbial removal and low doses of 3–10 mg/L chitosan achieved 3 to 5 $\log_{10}$ removal of *E. coli* bacteria and indicator virus MS2.
CHAPTER 6
ANTIMICROBIAL EFFECTS OF CHITOSANS

6.1 Introduction

Chitosan possesses broad spectrum antimicrobial properties, and has been shown to both inhibit growth and kill bacteria, fungi (Rabea et al, 2003; Goy et al, 2009; Kong et al, 2010) and viruses (Chirkov, 2002; Ly-Chatain et al, 2013). The antimicrobial activity of chitosan against bacteria and fungi has been of interest to the food and textile industries for years. For example, chitosan treated fabrics have been shown to have lower bacterial growth and better deodorant activity (Lim & Hudson, 2003).

To study the antimicrobial properties of molecules, the minimum inhibitory concentration (MIC) is a common measurement. A review of the literature by Liu et al (2001) reported MICs of chitosans were between 10 to 1,000 mg/L against bacteria and 10 to 5,000 mg/L against fungi depending on types of bacteria and fungi. The MICs of chitosans against Escherichia coli or Staphylococcus aureus were ~20 mg/L. The polycationic nature of chitosan is believed to play a critical role in its antimicrobial activity. The mechanisms of microbial inhibition and inactivation are not well understood but it is hypothesized that the positively charged chitosan interacts with the negatively charged cell surface of microbes disturbing the stability of the lipopolysaccharide layer and thus causing alterations of the cell surface and cell permeability. The final results are cell damage causing leakage of intracellular substances such as electrolytes, proteins, glucose, and lactate dehydrogenase (Lim and Hudson, 2003). Fahnestock et al (2011) also suggested that
antimicrobial properties of chitosan relate to its amino groups in the polymer chains. The authors proposed two key mechanisms of chitosan against microbes. The first mechanism was electrostatic membrane binding which is attachment between chitosan and microbes due to opposite surface charges. The second mechanism was cell nucleus permeation which is penetration of chitosan into microbial cells causing damage associated with nucleus permeation. This suggests that molecular weight (MW) and degree of deacetylation (DD) of chitosan influence its antimicrobial properties although this relationships is not yet well described.

Chitosan properties such as degree of deacetylation (DD) and molecular weight (MW) also influence their antimicrobial activity. A study on effects of DD of chitosan against *E. coli* by Liu et al (2001) showed that its antibacterial activity increased as its DD increased. These results were possibly from the increase of amino groups (-NH$_2$) as DD increased. Interaction time needed between chitosan and *E. coli* also decreased as DD increased. The authors described that protonation of -NH$_2$ to -NH$_3^+$ made chitosan attach to the negatively charged bacterial surface, then disturbed the cell membrane and caused cell death. Effects of molecular weight (MW) of chitosan on antimicrobial property were varied and provided no clear conclusions. Some studies showed stronger antimicrobial effects by low MW chitosans but some studies reported the opposite results, in which high MW chitosan exhibited stronger antimicrobial properties (Lim and Hudson, 2003). Liu et al (2006) tested chitosan with MW from 55,000 to 155,000 daltons against *E. coli* and reported that antibacterial activity was higher at low MW. Qin et al (2006) studied chitosans MW 1,400 to 400,000 daltons on growth of *Staphylococcus aureus, E. coli,* and *Candida albicans* and found that mid-range MW of approximately 50,000 daltons had higher inhibitory effects on the test microbes compared to smaller or larger MW than $\approx$ 50,000 Da.
There are few studies on antiviral effects of chitosans. Chirkov (2002) reported effects of chitosans against bacteriophages of plants and animals and concluded that higher MW and cationic derivatives of chitosan were more effective in inhibiting phage infection than low MW and anionic derivatives. Ly-Chatain et al (2013) studied antiviral effects of chitosan against bacteriophage MS2 and reported that 2 log_{10} reductions of MS2 was observed at dose 1,000 mg/L and 1 minute contact time.

Research has been focused on the antimicrobial activity of chitosans against bacteria and fungi for applications in food, agricultural, paper and textile industries (Romanazzi et al, 2012; Lim & Hudson, 2003). Studies on chitosan application as a natural antimicrobial agent for water treatment are limited. It is unclear whether chitosan will exhibit similar antimicrobial effects when it is applied in water. Chung et al (2003) studied the effects of natural water parameters on the antibacterial activity of chitosan against waterborne pathogens *E. coli* and *Staphylococcus aureus*. The results showed that chitosan at dose 2,500 mg/L had bactericidal activity against waterborne pathogens. Describing more fully and carefully the antimicrobial properties of chitosan will help to determine if chitosan in water is acting simultaneously by antimicrobial and physical removal mechanisms.

6.2 Materials and Methods

**Chitosan stock solutions**

Nine chitosans which had already been evaluated for their efficacy in turbidity and microbial coagulation were studied to evaluate antimicrobial effects. Studies were done using 3 chitosan polymers with different MW and similar DD (≈ 90%DD): 50,000, 100,000, and 1,000,000 daltons; 3 chitosan polymers with different DD and approximately the same MW (<
50,000 Da) with viscosity of 5 mPa·S: DD70%, 85%, and 95%; and 3 water-soluble modified chitosans (DD 80-95%, viscosity 2-200 mPa•S): chitosan acetate, chitosan lactate, and chitosan HCl. The information regarding MW and DD of all chitosans were obtained from the chitosan vendors. These chitosans with different MW were bought from Heppe Medical Chitosan GmbH (Halle, Germany) and Acros Organics (Bridgewater, NJ, USA). Characteristics of the tested chitosans (MW, DD, and viscosity) including zeta potential values obtained from measurement in this study were summarized in Appendix 11.

Stock solutions of chitosans were made for all polymers at concentration 10,000 mg/L. Chitosan powder was dissolved in 0.5% acetic acid and stirred at room temperature until totally dissolved. Stock solutions were stored at room temperature (25°C). The stock solutions of modified (water-soluble) chitosans were prepared similarly, using deionized water instead of acetic acid.

**Minimum Inhibitory Concentration (MIC) of chitosans**

The minimum inhibitory concentration (MIC) against *E. coli* bacteria and antiviral effects against bacteriophage MS2 were studied.

For MIC of chitosans against *E. coli*, the broth macro-dilution method was performed using Mueller Hinton Broth (MHB) as described in methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard-7th edition (CLSI, 2006). A series of two-fold chitosan concentrations (range 1.95 – 10,000 µg/ml) were prepared from chitosan stock solution using MHB as a test medium. Then 1 ml of prepared chitosan concentration was added into tubes containing 1 ml of *E. coli* (≈10⁶ CFU/ml). A negative control tube did not have chitosan. A positive control tube had only chitosan without microbes. All tubes
were incubated at 37°C for 24 hours. Turbidity of media was used as indicator for growth of *E. coli* bacteria. The first concentration at which tubes had no visible growth (clear tube) was considered the MIC.

For antiviral effects of chitosan candidates against bacteriophage MS2, a series of two-fold chitosan concentrations (range 1.95 – 10,000 µg/ml) was prepared from chitosan stock solution using 10-fold diluted PBS as a test medium. Then 1 ml of chitosan was added into tubes containing 1 ml of bacteriophages MS2 (≈ 10⁶ PFU/ml). The tubes were incubated at room temperature for 1 hour to mimic the exposure time of a jar test experiment. Then samples were diluted serially 10-fold in TSB. Bacterial host *E. coli F*amp was added into the sample-TSB mixtures and the tubes were incubated overnight for coliphage replication and enrichment. The enriched dilutions were centrifuged at 5000xG for 10 minutes to remove bacterial host and supernatants were recovered. Growth of MS2 in the centrifuged supernatant was determined by spot plate lysis method with host *E. coli F*amp on soft Tryptic Soy agar (EPA, 2001). The concentration of chitosan with the absence of lysis in the spot was counted as the MIC against MS2. See Appendix 6 for protocol.

**Evaluation of culturability of *E. coli* and infectivity of bacteriophage MS2 after coagulation using chitosan**

Chitosan 100,000 daltons at doses 3 and 10 mg/L, chosen for optimal performance in microbial removal, was used to study infectivity of *E. coli* and bacteriophage MS2 in the treated water and the settled flocs after coagulation using chitosan. After performance of the jar test under the same conditions used for microbial coagulation, the level of microbes in the treated
water was measured. Then the treated water and the settled flocs were recovered and were
processed as shown in Figure 6.1 and 6.2.

For culturability of *E. coli* bacteria, samples of both treated water and flocs were
centrifuged at 3000xG at 4° C for 10 minutes to sediment the bacteria and get bacterial pellets.
Then the pellets were resuspended in beef extract eluent (3% (w/v) beef extract at pH 9.5 with
0.1% Tween 80) followed by sonication (Sonic Dismembrator Model500, Fisher Scientific,
Pittsburgh, PA) to disperse *E. coli* bacteria. Samples were sonicated at 30% amplitude for a total
of 2 minutes, with intervals of 30 seconds sonication and 30 seconds pause. After sonication, *E.
coli* levels were quantitated by the MPN method using Colilert Quantitray (IDEXX, Westbrook,
ME).

For infectivity of bacteriophage MS2 after jar test coagulation, the treated water and
settled flocs were recovered then centrifuged at 3000xG at 4° C for 10 minutes. The supernatant
of treated water and the flocs pellets were recovered and resuspended in beef extract eluent (3%
(w/v) beef extract at pH 9.5 with 0.1% Tween 80, followed by sonication (Sonic Dismembrator
Model500, Fisher Scientific, Pittsburgh, PA) to disperse bacteriophage MS2. The eluted viruses
were quantitated by enrichment-spot plate MPN as modified from EPA Method 1601 (EPA,
2001). Four replicate experiments were performed.
Figure 6.1 Diagram for *E. coli* elution and culturability test of treated water and coagulated flocs

- **treated water (mixture of water & chitosan) with bacteria**
  - Sample 30 ml of treated water and centrifuge to get bacterial pellet
  - Resuspend pellet with 10 ml eluent (3% (w/v) beef extract at pH 9.5 + 0.1% Tween 80)
  - Mixing thoroughly then sonicate
  - Test culturability of *E. coli* quantitatively by MPN method with Colilert Quantitray

- **flocs with *E. coli* from jar test**
  - Recover flocs then centrifuge to get bacterial-floc pellet
  - Resuspend pellet with 10 ml eluent (3% (w/v) beef extract at pH 9.5 + 0.1% Tween 80)
  - Mix thoroughly then sonicate
  - Test culturability of *E. coli* quantitatively by MPN method with Colilert Quantitray
Figure 6.2 Diagram for bacteriophage MS2 elution and infectivity testing of treated water and flocs

**Test water**

The test water was artificial surface water prepared based on the recommended parameters from USEPA, NSF and WHO for efficacy testing of POU technology as listed in Appendix 3. For testing of microbial removal, the test water was created by spiking dechlorinated of tap water with: 300 mg/L of total dissolved solids (TDS), 3 mg/L of total
organic carbon (TOC) and 5 NTU of turbidity. Sodium chloride (NaCl), tannic acid and kaolinite were used as an adjusting material for TDS, TOC and turbidity, respectively. The test water was stirred overnight then seeded with \textit{E. coli} bacteria (≈10^9 CFU/ml) and bacteriophage MS2 (≈10^{10} PFU/ml) and mixed for 30 minutes before starting the jar test.

\textbf{Jar test experiment}

A jar test method of coagulation testing was used. The mixing conditions were: rapid mixing at 100 revolutions per minute (rpm) for 1 minute, followed by slow mixing at 25 rpm for 15 minutes and settling for 30 minutes. All experiments were conducted at 25°C. Supernatant for microbial and turbidity analysis was recovered using a pipette without disturbing the flocs. The viable cell count method was used to measure reduction of \textit{E. coli} B and the plaque assay was used to measure reduction of MS2. Membrane filtration (MF) then culture on MacConkey agar was used for \textit{E. coli} B colony enumeration; double agar layer (DAL) plaque assay with host bacteria \textit{E. coli} \textit{F}_{amp} was used for MS2 enumeration. Turbidity was measured by a turbidity meter (Hach 2100AN Turbidimeter, Hach, Loveland CO). Water pH was also measured by a pH meter before and after the jar test experiment.

Doses of chitosans were 3 and 10 mg/L. These doses were selected because they were in the optimum dose range for microbial coagulation from results of a previous study.

\textbf{Data analysis}

Microbial removal was calculated as log_{10} reduction compared to the natural settling control as shown below:

$$\text{Log}_{10} \text{ reduction} = \text{Log}_{10} \left( \frac{N_t}{N_0} \right)$$
When: \( N_t \) = number of microbes in sample supernatant after jar test  
\( N_0 \) = number of microbes in control supernatant after jar test  

Turbidity removal was calculated as percent turbidity removal relative to the natural settling control as shown below:

\[
\text{Percent removal} = [1 - \text{sample supernatant turbidity/control supernatant turbidity}] \times 100
\]

6.3 Results

6.3.1 Antibacterial effects of chitosan

I. Minimum inhibitory concentration (MIC) of chitosans against bacteria

Nine chitosans which were used previously for microbial coagulation were evaluated for their antimicrobial properties against \textit{E. coli} bacteria. Minimum inhibitory concentrations (MIC) of chitosans are shown in Table 6.1. Chitosan MW 50,000 daltons exhibited the lowest MIC of 250 mg/L while chitosan MW 1,000,000 daltons and chitosan acetate had the highest MIC of 1,000 mg/L.

Among 3 different MW chitosans, smallest MW chitosan showed the lowest MIC of 250 mg/L and the MIC increased as MW of chitosan increased, with MICs of 500 and 1000 mg/L for chitosans of 100,000 and 1,000,000 Da MWs, respectively. These results suggest that small MW chitosan had stronger antimicrobial activity against \textit{E. coli} bacteria. Chitosans with different DDs, however, showed similar MICs of 500 mg/L. For modified (water-soluble) chitosans, the MICs differed somewhat, with chitosan acetate exhibiting weaker antibacterial activity with an MIC of 1,000 mg/L than chitosan HCl and chitosan lactate, with MICs of 500 mg/L.
Table 6.1 The MIC of acid-soluble and water-soluble chitosans against *E. coli* bacteria

<table>
<thead>
<tr>
<th>Chitosans</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MW 50,000 Da</td>
<td>250</td>
</tr>
<tr>
<td>2. MW 100,000 Da</td>
<td>500</td>
</tr>
<tr>
<td>3. MW 1,000,000 Da</td>
<td>1,000</td>
</tr>
<tr>
<td>4. 70% DD</td>
<td>500</td>
</tr>
<tr>
<td>5. 85% DD</td>
<td>500</td>
</tr>
<tr>
<td>6. 95% DD</td>
<td>500</td>
</tr>
<tr>
<td>7. Chitosan acetate</td>
<td>1,000</td>
</tr>
<tr>
<td>8. Chitosan HCl</td>
<td>500</td>
</tr>
<tr>
<td>9. Chitosan lactate</td>
<td>500</td>
</tr>
</tbody>
</table>

**II. Culturability of E. coli bacteria in treated water and settled flocs**

Results showed that after microbial coagulation by a chitosan coagulant at doses of 3 and 10 mg/L in test water, culturable *E. coli* bacteria were found in the treated water as shown in Table 6.2. Concentrations of *E. coli* in the water treated with chitosan were much lower than the concentrations of *E. coli* in the control without chitosan. Culturable *E. coli* concentrations also decreased with increasing chitosan doses from 3 to 10 mg/L. These results coincided with log\(_{10}\) reductions of *E. coli* by chitosan coagulation, which also exhibited greater bacterial removal at greater dose (10 mg/L). The settled flocs were harvested and tested for culturability of *E. coli* bacteria using by MPN method with Colilert Quantitray. Culturable *E. coli* bacteria were also found in coagulating flocs as showed in Table 6.2. At the same chitosan doses, more bacteria were found in flocs than in the treated water.
Table 6.2 The culturability of *E. coli* bacteria in **treated water and flocs** after coagulation with chitosan MW 100,000 daltons by MPN method with Colilert Quantitray

<table>
<thead>
<tr>
<th>Trials</th>
<th>Chitosan doses (mg/L)</th>
<th>Initial <em>E. coli</em> concentration in test water (CFU/100 ml)</th>
<th>Log\textsubscript{10} reductions of <em>E. coli</em> in test water by coagulation-flocculation</th>
<th>MPN (<em>E. coli</em> in treated water per 100 ml)</th>
<th>MPN (<em>E. coli</em> in flocs per 100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>0</td>
<td>3.05 x 10\textsuperscript{6}</td>
<td>-0.02</td>
<td>&gt; 2419.6</td>
<td>547.5</td>
</tr>
<tr>
<td>Control 2</td>
<td>0</td>
<td>3.05 x 10\textsuperscript{6}</td>
<td>-0.02</td>
<td>&gt; 2419.6</td>
<td>547.5</td>
</tr>
<tr>
<td>Control 3</td>
<td>0</td>
<td>2.58 x 10\textsuperscript{6}</td>
<td>-0.15</td>
<td>&gt; 2419.6</td>
<td>&gt; 2419.6</td>
</tr>
<tr>
<td>Control 4</td>
<td>0</td>
<td>2.58 x 10\textsuperscript{6}</td>
<td>-0.15</td>
<td>&gt; 2419.6</td>
<td>&gt; 2419.6</td>
</tr>
<tr>
<td>Control 5</td>
<td>0</td>
<td>7.67 x 10\textsuperscript{6}</td>
<td>0.15</td>
<td>&gt; 2419.6</td>
<td>&gt; 2419.6</td>
</tr>
<tr>
<td>Control 6</td>
<td>0</td>
<td>7.67 x 10\textsuperscript{6}</td>
<td>0.15</td>
<td>&gt; 2419.6</td>
<td>&gt; 2419.6</td>
</tr>
<tr>
<td>Water 3-1</td>
<td>3</td>
<td>3.05 x 10\textsuperscript{6}</td>
<td>3.02</td>
<td>41.4</td>
<td>&gt; 2419.6</td>
</tr>
<tr>
<td>Water 3-2</td>
<td>3</td>
<td>3.05 x 10\textsuperscript{6}</td>
<td>3.36</td>
<td>95.9</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Water 3-3</td>
<td>3</td>
<td>2.58 x 10\textsuperscript{6}</td>
<td>4.46</td>
<td>1203.3</td>
<td>&gt; 2419.6</td>
</tr>
<tr>
<td>Water 3-4</td>
<td>3</td>
<td>2.58 x 10\textsuperscript{6}</td>
<td>3.78</td>
<td>816.4</td>
<td>&gt; 2419.6</td>
</tr>
<tr>
<td>Water 3-5</td>
<td>3</td>
<td>7.67 x 10\textsuperscript{6}</td>
<td>3.45</td>
<td>&gt; 2419.6</td>
<td>&gt; 2419.6</td>
</tr>
<tr>
<td>Water 3-6</td>
<td>3</td>
<td>7.67 x 10\textsuperscript{6}</td>
<td>3.40</td>
<td>1986.3</td>
<td>&gt; 2419.6</td>
</tr>
<tr>
<td>Water 10-1</td>
<td>10</td>
<td>3.05 x 10\textsuperscript{6}</td>
<td>4.98</td>
<td>&lt; 1</td>
<td>7.4</td>
</tr>
<tr>
<td>Water 10-2</td>
<td>10</td>
<td>3.05 x 10\textsuperscript{6}</td>
<td>5.28</td>
<td>1.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Water 10-3</td>
<td>10</td>
<td>2.58 x 10\textsuperscript{6}</td>
<td>5.34</td>
<td>38.4</td>
<td>980.4</td>
</tr>
<tr>
<td>Water 10-4</td>
<td>10</td>
<td>2.58 x 10\textsuperscript{6}</td>
<td>5.34</td>
<td>25.3</td>
<td>686.7</td>
</tr>
<tr>
<td>Water 10-5</td>
<td>10</td>
<td>7.67 x 10\textsuperscript{6}</td>
<td>5.21</td>
<td>3.1</td>
<td>920.8</td>
</tr>
<tr>
<td>Water 10-6</td>
<td>10</td>
<td>7.67 x 10\textsuperscript{6}</td>
<td>5.12</td>
<td>6.3</td>
<td>111.9</td>
</tr>
</tbody>
</table>

Note: average Log\textsubscript{10} reduction at chitosan dose 3 mg/L = 3.58 ± 0.52 (95%CI)
average Log\textsubscript{10} reduction at chitosan dose 10 mg/L = 5.21 ± 0.15 (95%CI)
average initial *E. coli* concentration in test water = 4.43 x 10\textsuperscript{8} CFU/ml

6.3.2 Antiviral effects of chitosan

*I. Minimum inhibitory concentration (MIC) of chitosans against bacteriophage MS2*

Seven chitosans identified from previous microbial coagulation experiments were evaluated for their antimicrobial properties against bacteriophage MS2. Minimum inhibitory concentrations (MIC) of chitosans against virus are shown in Table 6.3. Chitosan 95% DD exhibited the lowest MIC with 5,000 mg/L while chitosan 70% DD showed the highest MIC with 20,000 mg/L. Among 3 different DD chitosans, lower DD showed higher MIC and the MIC...
was lower with chitosans of higher DD. For modified (water-soluble) chitosans, the MICs against virus varied but chitosan acetate and chitosan HCl exhibited more potent antiviral property with 10,000 mg/L MIC than chitosan lactate with 15,000 mg/L MIC. Chitosan MW 100,000 daltons showed more antiviral activity of 7,500 MIC than other chitosans except chitosan 95% DD with 5,000 mg/L MIC.

Table 6.3 MICs of Acid-soluble and water-soluble chitosans against bacteriophage MS2

<table>
<thead>
<tr>
<th>Chitosans</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MW 100,000 Da</td>
<td>7,500</td>
</tr>
<tr>
<td>2. 70% DD</td>
<td>20,000*</td>
</tr>
<tr>
<td>3. 85% DD</td>
<td>10,000*</td>
</tr>
<tr>
<td>4. 95% DD</td>
<td>5,000*</td>
</tr>
<tr>
<td>5. Chitosan acetate</td>
<td>10,000*</td>
</tr>
<tr>
<td>6. Chitosan HCl</td>
<td>10,000*</td>
</tr>
<tr>
<td>7. Chitosan lactate</td>
<td>15,000*</td>
</tr>
</tbody>
</table>

Note: * experiments run by Wilson (2015)

II. Infectivity of bacteriophage MS2 in treated water and settled flocs

After microbial coagulation by a chitosan coagulant, infectious bacteriophages MS2 were found in the treated water as shown in Table 6.4. Concentrations of MS2 in the water treated with chitosan (3 and 10 mg/L doses) were much less than the concentration of MS2 in the control jars without chitosan. However, infectious MS2 concentrations were somewhat higher at lower chitosan dose of 3 mg/L than at the higher chitosan dose of 10 mg/L. The log_{10} reductions
of MS2 by coagulation at dose 3 mg/L were also somewhat higher than the reduction at dose 10 mg/L. Detection of infectious MS2 in flocs was also evaluated. The settled flocs were harvested after coagulation and tested for infectivity of MS2 by the enrichment-spot plate MPN method on *E. coli Famp* host bacteria. High concentrations of infectious MS2 were detected in flocs as shown in Table 6.4. Similar to bacteria results, at the same chitosan doses, more viruses were found in recovered flocs than in the treated water.

Table 6.4 Infectivity of bacteriophage MS2 in treated water and flocs after coagulation with chitosan MW 100,000 daltons by enrichment-spot plate MPN procedure (3-tubes MPN: 10-1-0.1 ml)

<table>
<thead>
<tr>
<th>Trials</th>
<th>Chitosan doses (mg/L)</th>
<th>Initial MS2 concentration in test water (PFU/100 ml)</th>
<th>Log$_{10}$ reductions of MS2 by coagulation</th>
<th>MPN (MS2 in treated water per 100 ml)</th>
<th>MPN (MS2 in flocs per 100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>0</td>
<td>1.50 x 10$^8$</td>
<td>-0.04</td>
<td>&gt; 110000</td>
<td>&gt; 110000</td>
</tr>
<tr>
<td>Control 2</td>
<td>0</td>
<td>1.50 x 10$^8$</td>
<td>-0.04</td>
<td>&gt; 110000</td>
<td>&gt; 110000</td>
</tr>
<tr>
<td>Control 3</td>
<td>0</td>
<td>1.99 x 10$^8$</td>
<td>-0.35</td>
<td>&gt; 110000</td>
<td>46000</td>
</tr>
<tr>
<td>Control 4</td>
<td>0</td>
<td>1.99 x 10$^8$</td>
<td>-0.35</td>
<td>&gt; 110000</td>
<td>46000</td>
</tr>
<tr>
<td>Water 3-1</td>
<td>3</td>
<td>1.50 x 10$^8$</td>
<td>3.84</td>
<td>&gt; 110000</td>
<td>&gt; 110000</td>
</tr>
<tr>
<td>Water 3-2</td>
<td>3</td>
<td>1.50 x 10$^8$</td>
<td>3.92</td>
<td>&gt; 110000</td>
<td>&gt; 110000</td>
</tr>
<tr>
<td>Water 3-3</td>
<td>3</td>
<td>1.99 x 10$^8$</td>
<td>4.18</td>
<td>3800</td>
<td>&gt; 110000</td>
</tr>
<tr>
<td>Water 3-4</td>
<td>3</td>
<td>1.99 x 10$^8$</td>
<td>3.83</td>
<td>46000</td>
<td>&gt; 110000</td>
</tr>
<tr>
<td>Water 10-1</td>
<td>10</td>
<td>1.50 x 10$^8$</td>
<td>3.36</td>
<td>&gt; 110000</td>
<td>&gt; 110000</td>
</tr>
<tr>
<td>Water 10-2</td>
<td>10</td>
<td>1.50 x 10$^8$</td>
<td>3.35</td>
<td>2300</td>
<td>&gt; 110000</td>
</tr>
<tr>
<td>Water 10-3</td>
<td>10</td>
<td>1.99 x 10$^8$</td>
<td>3.68</td>
<td>3800</td>
<td>9300</td>
</tr>
<tr>
<td>Water 10-4</td>
<td>10</td>
<td>1.99 x 10$^8$</td>
<td>3.62</td>
<td>&lt; 300</td>
<td>24000</td>
</tr>
</tbody>
</table>

Note: average Log$_{10}$ reduction at chitosan 3 mg/L = 3.94 ± 0.26 (95%CI)
average Log$_{10}$ reduction at chitosan 10 mg/L = 3.5 ± 0.27 (95%CI)
average initial MS2 concentration in test water = 1.74 x 10$^{10}$ PFU/ml
6.4 Discussion

Antibacterial effects

Based on the results obtained, the MIC of chitosan against *E. coli* bacteria was higher at higher MW of chitosan. The MIC of chitosan MW 50,000 Da was 250 mg/L, indicating the greatest antimicrobial effect against *E. coli*, while the largest MW chitosan (1,000,000 Da) showed weaker antimicrobial effects with an MIC 1,000 mg/L. Liu et al (2006) tested chitosan with MWs from 55,000 to 155,000 daltons against *E. coli* and reported that antibacterial activity was higher at low MW. They found that at the same chitosan concentration (50-100 mg/L), there were less viable bacteria with decreasing MW of chitosan. Our results also agree with those of Qin et al (2006) who studied chitosans MW 1,400 to 400,000 daltons against *Staphylococcus aureus*, *E. coli*, and *Candida albicans* and found that mid-range MW of approximately 50,000 daltons had higher inhibitory effects on the test microbes. Zheng & Zhu (2003) tested antibacterial activity of chitosans MW between 5,000 to 166,000 daltons against *E. coli* and found that antibacterial activity was higher for lower MW chitosan. They reported that inhibition rates of chitosan on *E. coli* were 0 and 50 % for chitosan MWs of 16,000 and < 5,000 Da, respectively. The authors proposed that lower MW chitosan possibly entered bacterial cells to cause cell damage more easily than higher MW chitosan. However, more studies are needed to obtain conclusive proof.

Chitosan DD showed no impact on antibacterial activity. Different DDs of chitosans (70%, 85% and 95%) exhibited the same MIC of 500 mg/L against *E. coli*. This was quite different from what might be expected because the antimicrobial property of chitosan is proposed to relate to the amino groups on the polymer chains (Fahnestock et al, 2011), suggesting stronger antimicrobial effects with higher DD. Liu et al (2001) reported increased
antibacterial activity with increasing DD chitosans. They tested antimicrobial effects of chitosan with different DD from 74 to 96% and found that antibacterial effects of chitosans were higher for higher DD chitosans. The highest antibacterial effects were obtained with chitosan 96%DD.

Modified chitosans had varying antibacterial properties depending on type of derivatives. Based on MIC, chitosan HCl and chitosan lactate were more effective than chitosan acetate for inhibiting *E. coli*, with MICs of 500 mg/L and 1,000, respectively. However, their MICs were in the range similar to chitosan polymers. Results of experiments on antibacterial effects and microbial coagulation (in chapter 4) from this research indicate that modified chitosans such as chitosan HCl, chitosan lactate and chitosan acetate are comparable to non-modified chitosan polymer.

Based on the results of this study, the antibacterial effect of a chitosan coagulant at optimum doses for bacterial coagulation were likely low because concentrations required for inhibiting bacterial growth were much higher than the coagulation optimum doses regardless of the MW or DD. Culturability tests for *E. coli* after coagulation by MPN method with Colilert Quantitray showed culturable *E. coli* both in treated water and in settled flocs. These results suggested that antibacterial activity is not a crucial mechanism during bacterial coagulation. The presence of culturable bacteria in the flocs suggests that physical removal, not inactivation, is likely the dominant mechanism of chitosan action leading to microbial reduction.

**Antiviral effects**

MICs for MS2 virus were also much higher (5,000 – 20,000 mg/L) than the optimum dose for water coagulation (3 – 10 mg/L). Overall, the MICs against virus are much higher than the MICs against bacteria.
Chitosan DD showed impacts on antiviral activity. Greater antiviral activity was observed in higher DD chitosans, with MICs against MS2 going from 20,000 mg/L to 5,000 mg/L as chitosan DD went from 70% to 95%. The polycationic nature of chitosan is believed to play a critical role in its antimicrobial activity. Higher DD chitosans have more amino groups on polymer chain thereby having more positive charges. This characteristic improves interaction between negatively charged microbial cells and viruses and positively charged chitosan molecules. It is expected that there will be greater antimicrobial activity by higher DD chitosans. It is hypothesized that DD exhibits greater antimicrobial effects on viruses than bacteria because viruses possess lower negative surface charges than bacteria. Therefore viruses can be more approachable and easier attach to high DD chitosans than can bacteria.

The antiviral activities of modified chitosans also varied depending on chitosan types. Unlike the antibacterial property, chitosan acetate was more effective than chitosan lactate for inhibiting bacteriophage MS2, with an MIC of 10,000 mg/L. Similar to antibacterial results, the MICs against viruses by modified chitosans were in the range of MICs of non-modified chitosan polymers. It was found that both antiviral effects and viral coagulation of modified chitosans in this research are comparable to those of chitosan polymers.

Only chitosan MW 100,000 Da was tested for antiviral MIC; this chitosan was more effective than modified chitosans and low DD chitosans with an MIC of 7,500 mg/L compared to 10,000-20,000 mg/L for most of the other chitosans tested.

Similar to antibacterial results, the antiviral effects of chitosan at optimum doses for coagulation were likely low because concentrations required to inhibit viral infectivity were much higher than the optimum doses for viral coagulation. Infectivity tests after chitosan coagulation, based on the enrichment-spot plate MPN procedure, showed high amounts of
infectious bacteriophage MS2 present both in treated water and in flocs. These results suggested that antiviral activity was not the crucial mechanism responsible for viral coagulation.

Although not conclusive, antimicrobial activity was unlikely a contributing mechanism for microbial coagulation using chitosan because the concentrations required for inhibiting growth of bacteria and viruses were much higher than concentrations needed for coagulation (as optimum doses).

6.5 Conclusions

Chitosan is effective as a coagulant for removal of turbidity and microbes in water but it has weak antimicrobial effects. Therefore, it can be used effectively as a water coagulant but it cannot be used for water disinfection.

- The antimicrobial property of chitosan was not an important mechanism for coagulation of bacteria and viruses from water, in the scope of this study.
- The antimicrobial property of chitosan can be observed at much higher doses than the optimum doses of chitosans for coagulation of microbes from water.
- The minimum inhibitory concentrations against *E. coli* bacteria were between 250 – 1,000 mg/L and cultural *E. coli* were detectable in treated water and flocs after chitosan coagulation.
- The minimum inhibitory concentrations against bacteriophage MS2 were much higher than MIC against bacteria, ranging from 5,000 – 20,000 mg/L, and infectious MS2 were detectable in treated water and flocs after chitosan coagulation at optimum doses.
• More studies are required to further investigate factors possibly influencing the antimicrobial activity of chitosan in water treatment such as water pH and salinity.
CHAPTER 7
SUMMARY AND CONCLUSIONS

The results from this study showed that chitosans, biopolymers from crustacean shells with demonstrated coagulant properties, have potential to serve as alternative coagulants for improving water quality alone or in combination with other treatment methods of water at household and community levels.

This research first screened 17 chitosans (both acid-soluble and water-soluble chitosans) for their efficacy for turbidity removal. For acid-soluble chitosan, two key properties of chitosans, molecular weight (MW) and degree of deacetylation (DD), were tested for their impacts on removal of water turbidity. Two types of clays, bentonite and kaolinite, were used to simulate natural water turbidity. In these experiments, the coagulant properties of chitosan can be observed visually; the formation of flocs during jar tests and after sedimentation is clearly visible. Molecular weight (MW) and degree of deacetylation (DD) influenced chitosan performance for turbidity removal. Generally, chitosan with higher MW showed higher turbidity removal compared to lower MW chitosans. However, the highest MW tested (1,000,000 Da) was not the optimum chitosan for turbidity removal. Degree of deacetylation of chitosans had lesser impacts on turbidity removal than molecular weight and at optimum doses, degree of deacetylation did not affect turbidity removal significantly. Chitosan doses were tested between 1 – 30 mg/L and results showed significant effects of dose on turbidity reduction. Chitosan affects kaolinite and bentonite differently. Physical properties such as cationic exchange capacity (CEC), zeta potential and specific surface area of both bentonite and kaolinite clays may cause
this difference in coagulation response. Bentonite reacts to chitosan faster than kaolinite. Bentonite flocs can be observed from the beginning of rapid mixing while kaolinite flocs usually can be observed only during slow mixing and sedimentation. Also, optimum doses for bentonite removals were broader than the optimum doses for kaolinite removal, although similar doses of chitosan were needed for removal of kaolinite and bentonite turbidity. Chitosan doses for achieving ≥ 80% turbidity removal ranged between 1 – 10 mg/L and highest removals of kaolinite (93%) and bentonite (99%) occurred in this dose ranges. Generally, chitosan doses higher than 10 mg/L were less effective and provided poorer turbidity removals than lower doses. Water-soluble (modified) chitosans showed comparable efficacy to acid-soluble chitosans for turbidity removal. Optimum dose range of water-soluble (modified) chitosans was as broad as that of acid-soluble chitosans for turbidity removal; bentonite and kaolinite removals of ≥ 85% were obtained between chitosan doses of 1-10 mg/L. Empirical dose testing was reinforced by the results of zeta potential measurements; the measured isoelectric points of chitosans on bentonite and kaolinite turbidity coagulation from water were close to the optimum chitosan doses obtained from jar test coagulation-flocculation experiments.

Of zeta potential measurements for all 17 chitosans, 16 showed varying positive zeta potential from 6.61 – 88.93 mV. Chitosans with positive zeta potential were effective for bentonite and kaolinite turbidity coagulation from water and many of them provided ≥ 90% removal for both clays. However, there was no clear trend of an effect of magnitude of chitosan positive charge on bentonite and kaolinite turbidity removal from test water. Chitosans with negative zeta potential (e.g. carboxymethyl chitosan) were not effective for bentonite and kaolinite turbidity removal from water. These results suggest that interparticle bridging mechanisms have a crucial role in bentonite and kaolinite turbidity coagulation using chitosans,
as turbidity removal increased with increasing chitosan molecular weight. Charge neutralization was also found to play a role in bentonite and kaolinite turbidity coagulation by chitosans because only positively charged chitosans but not a negatively charged one exhibited removals.

Nine chitosans which showed extensive bentonite and kaolinite turbidity removals were evaluated for their efficacy on bacteria and virus coagulation. *E. coli* bacteria and bacteriophage MS2 were used as test microorganisms. Chitosans of molecular weight (MW) from low to high (50,000, 100,000, and 1,000,000 Da) and with degrees of deacetylation of 70, 85, and 95% were used to coagulate *E. coli* and MS2 in test water. From this study, chitosans efficiently removed bacteria (2.0 – 4.5 \( \log_{10} \)) and virus (2.0 – 4.1 \( \log_{10} \)) from water at doses of 3 and 10 mg/L. Both molecular weight (MW) and degree of deacetylation (DD) influenced chitosan performance for bacteria and virus removal from water. Generally, chitosan with higher MW and higher DD showed higher removals compared to lower MW and lower DD chitosans. However, the highest MW tested (1,000,000 Da) was not the best chitosan for bacteria and virus removal but the medium molecular weight chitosan (100,000 Da) showed better microbial removal than the lower or higher molecular weight chitosan polymers starting at a low dose of 3 mg/L with 2.27 \( \log_{10} \) *E. coli* reductions and 3.56 \( \log_{10} \) MS2 reductions.

Degree of deacetylation (DD) of chitosans influenced microbial removal. Higher DD chitosans produced higher removals for both bacteria and viruses. Higher DD chitosan exhibited higher bacterial removals in jar test experiments. The highest DD chitosan (95%) gave the highest bacterial removals, with 3 – 4 \( \log_{10} \) reduction at doses 3, 10 and 30 mg/L. These removals were significantly better than *E. coli* removals by 70% DD chitosans (1 -2 \( \log_{10} \)) at the same doses. For virus coagulation at the same chitosan dose, the degree of deacetylation (DD) had significant impacts on virus removal. Chitosan with higher DD exhibited higher virus
removals than lower DD chitosans. The 95% DD and 85% DD chitosans produced similar virus removals at all chitosan doses, with the exception of dose 30 mg/L. Both DD chitosans exhibited high virus removals of $4 \log_{10}$ at 10 mg/L dose. Removals with the lowest chitosan DD tested of 70% were not dose dependent and exhibited approximately $1 \log_{10}$ virus removals across doses of 1 – 30 mg/L.

Results from this study illustrated that modified (water-soluble) chitosans removed bacteria and viruses from water as efficiently as acid-soluble chitosan polymers. Types and doses of water-soluble (modified) chitosans influenced removals of viruses from water but different types gave similar bacteria removals. Chitosan hydrochloride exhibited higher removals of bacteria by coagulation-flocculation only at dose 10 mg/L, with $4.28 \log_{10}$ reduction compared to 3.45 and $3.0 \log_{10}$ by chitosan acetate and chitosan lactate, respectively. Chitosan acetate was the most effective for virus removal with $> 3 \log_{10}$ reduction at doses of 3-30 mg/L.

Chitosan dose had significant effects on microbial removals and its impacts on bacteria removals were somewhat different from virus removals. Generally, chitosan showed a wider range of effective doses for virus removals but a narrow range for bacteria removals. Low doses of chitosans of 3 – 10 mg/L were needed to achieve $2 – 4 \log_{10}$ reductions of bacteria and viruses. Increasing chitosan dose from 1 to 10 mg/L generally increased bacteria removals from $\leq 1 \log$ to $4 \log_{10}$. Most chitosans exhibited the highest bacteria removals at the 10 mg/L dose. At the chitosan dose of 30 mg/L, bacteria removal decreased. In contrast, removals of viruses started at the low chitosan dose of 3 mg/L with as high as $3 \log_{10}$ virus reduction. Most of chitosans tested exhibited the highest virus removal at doses of 3 and 10 mg/L, and their efficiency decreased slightly at dose 30 mg/L. Based on results from this study, optimum
chitosan dose for bacteria removal was 10 mg/L and the optimum doses for virus removal were 3 and 10 mg/L, overlapping with optimum doses for turbidity removal.

It can be concluded from effects of molecular weight (MW) of chitosans on bacteria and virus removals that the bridging mechanism had a crucial role in microbial coagulation using chitosans. Moreover, charge neutralization played a role in removals by microbial coagulation because degree of deacetylation, which indicates the positive charge properties of chitosan, also significantly affected bacteria and virus removals.

Based on overall results from this study, the most effective 3 chitosans for bentonite, kaolinite, bacteria and virus removals are shown in the table below:

**Table 7-1** Ranking of chitosans based on the overall result for removal of each pollutant

<table>
<thead>
<tr>
<th>No.</th>
<th>Bentonite</th>
<th>Kaolinite</th>
<th>Bacteria</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chitosan MW 600,000 Da (~90%DD) 92-99% removal</td>
<td>Chitosan MW 50,000 Da (~90%DD) 87-91% removal</td>
<td>Chitosan MW 100,000 Da (~90%DD) 2.3-4.5 log&lt;sub&gt;10&lt;/sub&gt;</td>
<td>Chitosan MW 100,000 Da (~90%DD) 3.5-3.6 log&lt;sub&gt;10&lt;/sub&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Chitosan MW 100,000 Da (~90%DD) 79-98% removal</td>
<td>Chitosan HCl (80-95% DD) 85-91% removal</td>
<td>Chitosan HCl (80-95% DD) 1.2-4.3 log&lt;sub&gt;10&lt;/sub&gt;</td>
<td>Chitosan acetate (80-95% DD) 3.3-3.7 log&lt;sub&gt;10&lt;/sub&gt;</td>
</tr>
<tr>
<td>3</td>
<td>Chitosan acetate (<em>StormKlear</em>) 81-98% removal</td>
<td>Chitosan MW 100,000 Da (~90%DD) 87-90% removal</td>
<td>Chitosan 95%DD (MW &lt;50,000 Da) 2.9-3.0 log&lt;sub&gt;10&lt;/sub&gt;</td>
<td>Chitosan 95%DD (MW&lt;50,000 Da) 2.8-4.1 log&lt;sub&gt;10&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

*Chitosans were selected based on overall removals at doses 1-30 mg/L (see Appendix 6: Table A6-4 and Table A6-5 and Appendix 8: Table A8-6 and Table A8-7)
Chitosan 100,000 Da (≈90%DD), which exhibited effective removals of turbidity, bacteria and viruses, was selected to study impacts of water quality parameters on performance of chitosan coagulation. Three water parameters (i.e. pH, turbidity, and salinity) were evaluated for their influences on turbidity and microbial removals. According to overall results from this study, water qualities influenced chitosan efficacies differently for removals of bentonite, kaolinite, bacteria, and viruses. Water pH was found to alter optimum chitosan doses for bentonite and kaolinite coagulation but removals of > 85% were achieved at all water pH levels of 6-9. Generally, optimum doses of chitosan for bentonite and kaolinite removal increased with increasing pH of water and the same trend had been observed for low to high water turbidities of 5-300 NTU. However, pH of water influenced microbial removal differently from turbidity removal. According to the results, water pH had minimal effects on bacterial removal. Chitosan produced high bacterial removal from > 3 log_{10} to 5 log_{10} between chitosan doses of 3-30 mg/L across all test water pH levels, although higher chitosan doses were needed to initiate effective microbial coagulation at high pH 9. Virus removal by chitosan was significantly influenced by water pH and virus removal was greater at acidic pH than removal at basic pH. However, virus removal was also extensive (≥ 3-5 log_{10} between chitosan doses of 3-30 mg/L) at all test water pH levels, similar to bacteria removals. Based on the results of this study, water pH level influences effectiveness of chitosan coagulation because it influences protonation of chitosan (H^+) at different water pH levels. At acidic pH, protonation of chitosan occurs extensively so that there are more positive charges to react to negatively charged microbes. In contrast, protonation of chitosan is minimal at basic pH such as pH 9, with less positive charges produced. Therefore, higher amounts of chitosan are needed at the basic pH compared to acidic pH of water for effective coagulation.
Chitosan was found to be less effective to treat water with a low initial turbidity ≤ 5 NTU and the impact was more prominent for kaolinite (< 15% removal) than bentonite (79 – 85% removal). Therefore, coagulation of source water having turbidity lower than 5 NTU may need a coagulant aid to improve turbidity removal. However, initial water turbidity exhibited less impact when the water turbidity level was higher at 30 and 300 NTU. At optimum coagulant dose of 3 mg/L, kaolinite and bentonite removals were not significantly different between turbidity 30 and 300 NTU (> 90% removal). The effect of initial water turbidity level on microbial removal was tested between turbidities of 5 – 30 NTU and based on the results, higher initial water turbidity of 30 NTU did not impair bacteria and virus removal statistically compared to water of 5 NTU. Bacteria and virus reductions of 3-5 log_{10} were achieved at chitosan doses of 3-30 mg/L for all turbidity levels tested. Also higher initial turbidity levels of test water did not increase the optimum chitosan doses for coagulation of all test pollutants: kaolinite, bentonite, *E. coli* bacteria and bacteriophage MS2.

Water salinity in a range that is acceptable for drinking water (0.1-1.0 ppt (100 -1,000 mg/L) was evaluated for chitosan coagulation effectiveness. Based on the results, the salinity of test water did not statistically influence removals of kaolinite and bentonite from low turbidity (5 NTU) water by chitosan coagulation. At higher salinity of 1.0 ppt, kaolinite removals by coagulation were not improved compared to control. For microbial coagulation in 5 NTU turbidity test water, water salinity influenced chitosan performance for virus removal but not bacteria removal. Overall, virus removal increased with increasing water salinity from 0.1 to 1.0 ppt. Moreover, chitosan still achieved 3 to 5 log_{10} removal of *E. coli* bacteria and indicator virus MS2 at doses of 3–10 mg/L at varying levels of water salinity and at 5 NTU water turbidity.
Antimicrobial effect is another property of chitosan that possibly helps in reducing the number of culturable or infectious microbes from water by inactivation in addition to physical removal. Chitosans which had been used for microbial coagulation in this study were evaluated for their abilities to inhibit or kill bacteria and viruses. However, antibacterial properties of chitosans were observed only at much higher doses than the optimum doses for removal by bacterial coagulation. The minimum inhibitory concentrations (MIC) against *E. coli* bacteria were between 250 – 1,000 mg/L. These results were supported by the presence of culturable *E. coli* bacteria found in treated water and settled flocs after coagulation using chitosan coagulant. Likewise, antiviral properties of chitosans were observed only at much higher doses than the optimum doses for virus coagulation for removal and were also much higher than the MICs against *E. coli* bacteria. The minimum inhibitory concentrations (MIC) against bacteriophage MS2 were in the range of 5,000 – 20,000 mg/L and infectious MS2 were found in treated water and flocs after coagulation by chitosan. Results from this study suggested that inactivation by the antimicrobial properties of chitosan were not a strong contributing mechanism for removal of bacteria and viruses from water using chitosan as a coagulant. However, additional studies are required for more conclusive outcomes of antimicrobial effects of chitosan in water treatment and the identification of factors influencing the antimicrobial activities of chitosans in water treatment, such as pH, salinity or cations in water.

From this study it is concluded that chitosan is an effective natural coagulant to use for water treatment. The effective action of chitosan resides in the amino groups on the chitosan chains which protonate when chitosan dissolves. The most likely coagulation mechanisms are interparticle bridging and charge neutralization. Antimicrobial activity, as suggested by the
results of this study, is not a strong contributing mechanism for microbial coagulation by chitosan. Between initial water pH 6 - 9, the optimum dose of chitosan for bentonite and kaolinite coagulation increases with initial water pH but does not significantly compromise percent turbidity removal or microbial log_{10} reduction. Also chitosan hardly affects the pH of water after treatment, suggesting that adjustment of water pH before and after coagulation is not necessary, making chitosan easier to use. High initial water turbidity and salinity minimally affects chitosan removal efficacy. Chitosan does not inactivate microbes (E. coli and bacteriophage MS2) during the coagulation process, suggesting that regrowth in treated water may be an issue if chitosan is used alone.

Chitosan can be used for water treatment in two forms—acid-soluble and water-soluble chitosans. However, acid-soluble chitosans may be more economical for household water treatment. Results from this study suggest that acid-soluble chitosan with MW 100,000 daltons and DD ≥ 85% is a preferred potential candidate for coagulation-flocculation of kaolinite and bentonite turbidity, bacteria and virus from water as it produced substantial removal for all these pollutants. Although chitosans with MW 100,000 Da and DD 85% are not the best chitosans that exhibited consistently the highest removals for all pollutants tested, it provide high removals as overall results between doses of 1-10 mg/L. Turbidity removals produced by chitosan MW 100,000 Da and DD 85% are as follow: kaolinite 87.4-91.5%, bentonite 79-99.2%, bacteria 2.2-4.5 log_{10}, and virus 3.4-4.0 log_{10}. There is no one chitosan which is best for every turbidity type and microbe tested. Therefore, the intermediate MW ≈100,000 Da and DD ≥ 85% chitosan is selected as the preferred candidate for chitosan coagulation. Chitosan MW100,000 Da was tested to coagulate E. coli and bacteriophage MS2 in natural surface water; water samples were from University Lake, NC and Chattahoochee River, GA. Chitosan MW100,000 Da exhibited
equivalent bacteria removal in natural surface water with the lab test water (3-4 log$_{10}$), however virus removals were slightly lower in natural water (2-3 log$_{10}$) compared to laboratory water (3-4 log$_{10}$). These results suggest that chitosan has potential to improve quality of water from natural sources for drinking and for household consumption.

In summary, chitosans are potential coagulants for household water treatment as they are effective at low doses to achieve improved quality of drinking water and thereby possibly reduce health risks from water. Coagulation using chitosan has minimal of over- and under-dosing risks; this character of chitosan coagulant will benefit household water treatment with non-skilled users. Moreover, chitosans exhibit high efficacy across a range of raw water qualities for drinking water and at a wide range of chitosan doses from 3 to 30 mg/L, which makes chitosan a promising coagulant to use for household water treatment without pre-treatment of raw water such as pH adjustment. Chitosan can be used together with other technology such as filtration after coagulation and flocculation as a multiple barrier system to produce safe drinking water at home.

**Recommendations for future research**

To assess further chitosan efficacy for household water treatment, field studies on coagulation with chitosan are required. Efficacy of chitosan in field studies with various drinking water sources (i.e. surface water, rainwater, deep well water and shallow well water) will provide better information and help in deciding whether chitosan is practical to use at the household level. Field studies of impacts of chitosan on appearance, taste and odor of treated water which relate to acceptance of chitosan coagulants should be done.
The results of microbial coagulation from this study used indicator bacteria *E. coli* and surrogate virus bacteriophage MS2, but protozoa parasites and other important pathogens causing waterborne diseases, were not tested in this study. It is possible that effects of chitosan on removal of protozoa such as Cryptosporidium oocysts or Giardia cysts will be similar to removal of bacteria, because these organisms tend to settle better when attached to particles in water (WHO, 2004). However, the actual impacts of chitosan coagulant on protozoan removal needs further study.

The evidence presented here on antibacterial and antiviral properties of chitosans is the first comprehensive fundamental study of this effect. Further study of the effects of water quality such as pH, alkalinity, turbidity, salinity, cations, dissolved organic carbon (DOC) and natural organic matter (NOM) on chitosan coagulant properties are needed to determine whether the advantages demonstrated in this study of high microbial removal across a range of coagulant doses and water qualities holds for different water types. If they do, chitosan may present a flexible, low cost, easy to use option for improving household water at the point of use.

Moreover, using of chitosan as one step in a multi-barrier approach for household water treatment needs to be studied. For example, chitosan combines with solar disinfection technology by treating raw water with chitosan then followed by solar disinfection. Chitosan can be used with water filters such as the mineral pot filter or the ceramic filter, which are point of use water filters (popular in Southeast Asia). These types of studies will give information whether chitosan can improve the quality of drinking water either as a stand-alone or as a multi-barrier system in the real world.
**APPENDIX 1: WORLD HEALTH ORGANIZATION GUIDELINES**

*Table A1.1* WHO guidelines for *E. coli* in drinking water (number of *E. coli* per 100 mL of water)

<table>
<thead>
<tr>
<th>Number of <em>E. coli</em> per 100 mL of water</th>
<th>Risk level</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Conforms to WHO guidelines</td>
</tr>
<tr>
<td>1-10</td>
<td>low risk</td>
</tr>
<tr>
<td>11-100</td>
<td>intermediate risk</td>
</tr>
<tr>
<td>101-1000</td>
<td>high risk</td>
</tr>
<tr>
<td>&gt;1000</td>
<td>very high risk</td>
</tr>
</tbody>
</table>
Table A2.1 Survey of drinking water quality by Department of Health and Provincial Office of Public Health in rural area of Thailand between 1997-8, showed as percent of samples that do not meet WHO guidelines

<table>
<thead>
<tr>
<th>Samples (number)</th>
<th>Fecal coliforms</th>
<th>Fe</th>
<th>Hardness</th>
<th>Chloride</th>
<th>Nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deep well (613)</td>
<td>13.9</td>
<td>24.5</td>
<td>5.4</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>Stored rainwater (436)</td>
<td>15.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Shallow well (424)</td>
<td>16.5</td>
<td>10.1</td>
<td>-</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Village pipe water (4390)</td>
<td>44.5</td>
<td>17.3</td>
<td>2.3</td>
<td>2.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Sources: Provincial Waterworks Authority (Thailand)
## APPENDIX 3: CHARACTERISTICS OF TEST WATER FOR POU TECHNOLOGY

### Table A3.1 Guidelines of test water for testing POU technology

<table>
<thead>
<tr>
<th>Constituents</th>
<th>EPA’s general test water</th>
<th>NSF’s general test water</th>
<th>WHO’s test water type 1</th>
<th>WHO’s test water type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine (mg/L)</td>
<td>Free</td>
<td>&lt; 0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>6.5 - 8.5</td>
<td>7 ± 0.5</td>
<td>7.0 - 9.0</td>
<td>6.0 – 10.0</td>
</tr>
<tr>
<td>TOC (mg/L)</td>
<td>0.1 – 5.0</td>
<td>0.1 -2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>0.1 – 5</td>
<td>&lt; 1</td>
<td>&lt; 5</td>
<td>&gt; 30</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>20 ± 5</td>
<td>20 ± 5</td>
<td>20 ± 5</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>TDS (mg/L)</td>
<td>50 - 500</td>
<td>50 – 500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkalinity (mg/L as CaCO₃)</td>
<td>-</td>
<td>100 ± 20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Background bacteria (CFU/100 ml)</td>
<td>-</td>
<td>≥ 10³</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(EPA, 1987; NSF, 2008; WHO 2011)

### Table A3.2 Characteristics of test water used in this research

<table>
<thead>
<tr>
<th>Constituents</th>
<th>For turbidity removal test</th>
<th>For microbial removal test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine (mg/L)</td>
<td>Free</td>
<td>Free</td>
</tr>
<tr>
<td>pH</td>
<td>7.0 – 7.5</td>
<td>7.0 – 7.5</td>
</tr>
<tr>
<td></td>
<td>(6, 7, 9 for effects of water pH)</td>
<td>(6, 7, 9 for effects of water pH)</td>
</tr>
<tr>
<td>TOC (mg/L)</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(5, 30, 300 for effects of turbidity level)</td>
<td>(5, 10, 30 for effects of turbidity level)</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>20 ± 5</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>TDS (mg/L)</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>(100, 300, 1000 for effects of water salinity)</td>
<td>(100, 300, 1000 for effects of water salinity)</td>
</tr>
<tr>
<td>E. coli B bacteria initially seeded into water (CFU/100 ml)</td>
<td>-</td>
<td>10⁹</td>
</tr>
<tr>
<td>Bacteriophage MS2 initially seeded into water (PFU/100 ml)</td>
<td>-</td>
<td>10⁹</td>
</tr>
</tbody>
</table>
APPENDIX 4: PROTOCOL FOR BACTERIA REDUCTION

Turbidity and Microbial coagulation experiment with chitosan

The purpose of this experiment is to determine the optimal dose of chitosan for removal of turbidity, *E. coli* and bacteriophage MS2 from test water by coagulation. The coagulant used in this experiment will be various types of chitosans and modified chitosans. The doses used will be 1, 3, 10, and 30 mg/L. The test organism will be *E. coli* B, and bacteriophage MS2. Turbidity material will be kaolinite and bentonite. The test water will be artificial surface water adapted from recommendation of USEPA, NSF, and WHO as shown in Appendix 3.

*For turbidity removal, the procedure is the same but exclude all step involve microbes.*

Day before experiment:
1. Prepare test water, leave at room temperature and keep stirring overnight.
2. Start overnight culture of *E. coli* B and/or *E. coli* Famp (as host of MS2) separately with 25 mL TSB in a shaker flask.
3. Incubate on shaker platform at 100 rpm for 16-24 hours at 37°C in incubator.

Day of experiment:
4. Add 0.1 mL (100 µl) of *E. coli* and/or *E. coli* Famp to 50 mL fresh TSB in a new shaker flask
5. Incubate on shaker platform at 100 rpm for 2-2.5 hours at 37°C in walk-in incubator
6. Measure OD520 using the spectrophotometer. Record.
7. Keep a flask with *E. coli* Famp in a beaker with ice until starting MS2 assay
8. Thaw 4 ml of frozen MS2 stock
9. Split the 50 mL *E. coli* into 2 50 mL-centrifuge tubes
10. Centrifuge at 3000 rpm for 10 minutes at 4 °C
11. Pour off the TSB from both tubes into a waste beaker.
12. Pipette 2 mL of test water into the first tube
13. Vortex until the *E. coli* pellet is resuspended in the water
14. Pipette 2 mL of test water into the second tube
15. Vortex until the *E. coli* pellet is resuspended in the water
16. Combine the 2 tubes into a single tube (total volume 4 mL)
17. Measure turbidity of a sample of test water.
18. Measure pH from a sample of test water
19. Transfer 5 liters of water into a bucket with a stir bar.
20. Add the 4 ml of *E. coli* B (~10⁹ CFU/ml) and/or 4 ml of MS2 (~10¹⁰ PFU/ml) to the 5 liters of test water. Stir on a stir plate for 30 minutes.
21. To determine the initial concentration of *E. coli* B:
   a. Make a dilution series to 10⁻⁷ by transferring 2 ml of sample into 18 ml PBS.
   b. Membrane filter (0.45 µM, 47 mm) 9 ml of each dilution in duplicate, beginning with the most dilute sample. Rinse filter funnels with sterile PBS.
   c. Place filters on MacConkey agar plates.
   d. Incubate at 37°C for 18 – 24 hours.
22. To determine the initial concentration of MS2:
a. Make a dilution series to $10^{-8}$ by transferring 1 ml of sample into 9 ml PBS.
b. Do double agar layer plaque assay of each dilution in duplicate, beginning with the most dilute sample.
c. Incubate at 37°C for 18 – 24 hours.

23. Separate water into five 1 L volumes. One is the control; the other four are chitosan experiments (1, 3, 10, and 30 mg/L).
24. Weigh out the chitosan and add it to each beaker except a control beaker.
25. Stir each beaker for 30 seconds with a plastic rod.
26. Place beakers under the jar test apparatus.
27. Stir each beaker at 100 rpm for 1 minute, then 25 rpm for 15 minutes. Remove beakers from the jar test apparatus and allow settling for 30 minutes.

28. When removing samples, remember to always sample from midway down the water column, being very careful not to disturb flocs that has formed.

31. To determine concentrations after settling for *E. coli* B:
   a. Make a dilution series to $10^{-6}$ by transferring 2 ml into 18 ml PBS.
   b. Membrane filter (0.45 µM, 47 mm) 9 ml of each dilution in duplicate, beginning with the most dilute sample. Rinse filter funnels with sterile PBS.
   c. Place filters on MacConkey agar plates.
   d. Incubate at 37°C for 18 – 24 hours.

32. To determine concentrations after settling for MS2:
   a. Make a dilution series to $10^{-8}$ by transferring 1 ml of sample into 9 ml PBS.
   b. Do double agar layer plaque assay of each dilution in duplicate, beginning with the most dilute sample.
   c. Incubate at 37°C for 18 – 24 hours.

33. At 18 – 24 hours, read plates and determine concentrations and log reductions.
APPENDIX 5: JAR TEST AND PLAQUE ASSAY FOR VIRUS REDUCTION

Microbes: Bacteriophage MS2, E. coli F<sub>amp</sub>

1. **Top agar tubes:** (without antibiotics)
   Need 10 ml top agar per plate of a standard 100mm petri dish

**Material needed (per liter)**
- Tryptic soy broth (TSB) 30g
- Bacto agar 7.5g

Add TSB and Bacto agar to water in an Erlenmeyer flask. Add a stir bar. Heat the flask to boiling on a hot plate with stirring until the agar is completely dissolved. Set up test tubes in racks. When agar is completely dissolved, dispense into test tubes, 10 mL per tube. Cap with white caps. Store at 4°C until be used. When ready to use, remove from the refrigerator and autoclave on a liquid cycle.

2. **Bottom agar plates: Tryptic soil agar**
   Need 10 ml per plate of a standard 100mm petri dish

**Material needed (per liter)**
- Tryptic soy broth (TSB) 30g
- Bacto agar 15g
  (Or used TSA premade powder)

   Add the powder to water in an autoclave bottle then autoclave, cool and dispense into 100-mm petri dishes.

3. **Preparation of the overnight culture:**
   Place 25 mL of warm (at least room temperature) TSB in a sterile shaker flask. Remove frozen stock of host culture from the -80 freezer. DO NOT THAW. Using a sterile stick, scrape a small amount of bacteria from the top of the frozen stock tube and place it in the TSB. Immediately put the stock bacteria back in the freezer. Place the cap on the shaker flask loosely. Incubate on a shaking platform at ~120 rpm at 37°C for 18-24 hours.

4. **Preparation of the log phase culture**
   This should be done a few hours before the assay. To start the culture; place warm (at least room temperature) TSB in a sterile shaker flask. You will need at least 1 mL of log phase host for every plate you plan to do. You will need about 100μL of overnight culture for every 25 mL of TSB in the flask. Pipette the appropriate amount of overnight culture into the new shaker flask. Incubate on a shaking platform at ~120 rpm at 37°C for 2-4 hours. When the culture becomes cloudy, remove and measure the OD<sub>520</sub> using the spectrophotometer. The OD reading should be somewhere between 0.3 and 1. Once it reaches this level, it is ready for use. If the assay is not started when the culture is ready, place the shaker flask on ice until ready for use.
5. Preparation before starting assay 2-3 hours
- Start the log phase culture
- Make sure you have a waterbath set to 45°C
- Autoclave top agar
- Warm bottom agar plates to room temperature
- Put media for making dilution tubes in a 37°C waterbath to warm
- Set up required materials in the hood and turn on the UV light.
- When the top agar is ready, place it in the 45°C waterbath.
- Label bottom agar plates with appropriate dilutions. Don’t forget negative controls.

Plaque assay
1. Make the appropriate dilutions of your virus stock or sample. Each plate can be inoculated with up to 1 mL of sample. Dilutions can be made in sterile water, PBS, TSB, or other diluents.
2. Work with only a few samples at a time, so the top agar does not harden while you are working. Remove 6-8 tubes at a time from the waterbath and place in a rack in front of you. Test against your wrist to ensure agar is not too hot.
3. Add 0.5 mL of host bacteria to each tube
4. Add 0.5 ml of sample to each tube
5. Pour the contents of the tube into a plate.
6. Inoculate negative control tubes with host bacteria only to check for host contamination
7. Once plates solidify, invert and incubate at 37°C 16-24 hours.
8. Count plaques using a light box.
APPENDIX 6: PROTOCOL FOR CHITOSAN ANTIMICROBIAL EFFECTS

**Antibacterial effects: Minimum Inhibitory Concentration (MIC) of chitosans against bacteria**

1. Prepare chitosan stock solution (10 g/L) by dissolving chitosan powder in 0.5% (v/v) acetic acid, (deionized water for water-soluble modified chitosan) and stir at room temperature until totally dissolve
2. Prepare overnight culture of *E. coli* B using 100 µl of frozen stock
3. Transfer 100 µl of overnight culture of *E. coli* B for every 25 ml of TSB to prepare log phase culture (≈ 2-2.30 hours)
4. Prepare chitosan concentration series using sterilized Mueller Hinton broth (MHB) as shown in Table 1 below
5. Dispense 1 ml of *E. coli* B containing 10⁶ CFU/ml into sterile glass test tubes; labels the test tubes parallel to chitosan concentrations
6. Add 1 ml of each chitosan concentration into the parallel glass test tubes with *E. coli* bacteria and mix well. The positive control tubes will not have *E. coli*, only 1 ml of culture media (MHB) and 1 ml of chitosan solution. The negative control will have only *E. coli* and 0.5% acetic acid, no chitosan
7. Incubate the tubes at 37°C for 18-24 hours
8. Turbidity of the test tubes is used as indicator of growth of microbes. The concentration of tubes which show no growth (clear tubes) will be the MIC

**Antiviral effects: Minimum Inhibitory Concentration (MIC) of chitosans against virus**

1. Prepare chitosan stock solution (10 g/L) by dissolving chitosan powder in 0.5% (v/v) acetic acid, (for water-soluble modified chitosan dissolved in deionized water) and stir at room temperature until totally dissolve
2. A day before experiment day, prepare overnight culture of *E. coli F_{amp}* from a frozen stock and incubate at 37°C on a shaker overnight
3. Transfer 100 µl of overnight culture of *E. coli F_{amp}* for each 25 ml TSB to prepare log phase culture (≈ 2-2.30 hours)
4. Thawed MS2 stock at room temperature
5. Prepare chitosan concentration series using sterilized 10X diluted PBS as test media as shown in Table 1 below
6. Dispense 1 ml of MS2 containing 10⁶ PFU/ml into sterile glass test tubes; labels the test tubes parallel to chitosan concentrations
7. Add 1 ml of each chitosan concentration into the parallel test tubes with MS2 and mix well. The positive control tubes will not have MS2, only 1 ml 10X diluted PBS and 1 ml chitosan solution. The negative controls will have only MS2 and 0.5% acetic acid, no chitosan
8. The tubes were incubated at room temperature for 1 hour, then dilute the samples serially 10-fold in TSB
9. Add 100 µl of *E. coli* F<sub>amp</sub> host into the TSB dilution tubes and incubate overnight for coliphage replication and enrichment
10. Centrifuge the overnight TSB dilutions (enriched dilutions) at 3000xG for 15 minutes to remove *E. coli* F<sub>amp</sub> host
11. Recover the supernatants for doing spot plate method on soft TSA agar with *E. coli* F<sub>amp</sub> as bacterial host to determine growth of MS2
12. Incubate plates at 37 °C for 18 - 24 hours
13. Clear zone around each spot indicate growth of MS2
14. The concentration of chitosan with the absence of lysis in the spot was counted as the MIC against MS2.

<table>
<thead>
<tr>
<th>Table A6.1</th>
<th>Chitosan dilution series for testing antimicrobial effects of chitosans using Broth - Susceptibility Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
<td>Chitosan solution (µg/ml)</td>
</tr>
<tr>
<td>1</td>
<td>10,000</td>
</tr>
<tr>
<td>2</td>
<td>1,000</td>
</tr>
<tr>
<td>3</td>
<td>1,000</td>
</tr>
<tr>
<td>4</td>
<td>1,000</td>
</tr>
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<td>5</td>
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<td>6</td>
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<td>7</td>
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</tr>
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<td>8</td>
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<td>12</td>
<td>1.95</td>
</tr>
<tr>
<td>13</td>
<td>1.95</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control tubes</th>
<th>Chitosan control</th>
<th>Microbial control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No chitosan</td>
<td>1 ml of 0.5% (v/v) Acetic acid</td>
</tr>
<tr>
<td></td>
<td>1 ml chitosan 1000 µg/ml</td>
<td>1 ml MHB</td>
</tr>
</tbody>
</table>

Note: 1. *10X* diluted PBS will be used as the test solution for antiviral activity of chitosans
2. This table is adapted from Clinical and Laboratory Standards Institute, 2007. Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement, 1-182.
Table A7.1 Reduction of turbidity as effects of chitosan molecular weight (MW)

<table>
<thead>
<tr>
<th>Chitosan MW (Da)</th>
<th>Turbidity</th>
<th>Reduction (%) at each chitosan dose (± 95%CI)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 mg/L</td>
<td>1 mg/L</td>
<td>3 mg/L</td>
<td>10 mg/L</td>
<td>30 mg/L</td>
</tr>
<tr>
<td>5000</td>
<td>kaolinite</td>
<td>9.33 (±8.92)</td>
<td>-0.94 (±6.00)</td>
<td>0.26 (±1.89)</td>
<td>0.38 (±0.19)</td>
<td>79.76 (±2.46)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>17.31 (±1.13)</td>
<td>10.47 (±5.84)</td>
<td>24.70 (±12.14)</td>
<td>46.23 (±4.61)</td>
<td>41.06 (±22.46)</td>
</tr>
<tr>
<td>50,000</td>
<td>kaolinite</td>
<td>8.85 (±2.22)</td>
<td>87.37 (±0.92)</td>
<td>90.62 (±0.45)</td>
<td>1.56 (±1.06)</td>
<td>2.53 (±1.26)</td>
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<tr>
<td></td>
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<td>6.43 (±2.31)</td>
<td>92.62 (±2.58)</td>
<td>98.82 (±0.01)</td>
<td>94.57 (±0.65)</td>
<td>37.60 (±3.31)</td>
</tr>
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<td>100,000</td>
<td>kaolinite</td>
<td>4.97 (±5.97)</td>
<td>87.37 (±9.03)</td>
<td>90.18 (±12.85)</td>
<td>0.72 (±8.90)</td>
<td>0.89 (±1.22)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>43.89 (±7.24)</td>
<td>98.12 (±0.29)</td>
<td>97.90 (±0.34)</td>
<td>79.02 (±3.63)</td>
<td>2.12 (±15.64)</td>
</tr>
<tr>
<td>600,000</td>
<td>kaolinite</td>
<td>22.93 (±10.27)</td>
<td>20.76 (±3.22)</td>
<td>14.68 (±10.25)</td>
<td>-1.97 (±15.22)</td>
<td>-8.67 (±28.20)</td>
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<tr>
<td></td>
<td>bentonite</td>
<td>16.76 (±15.87)</td>
<td>98.18 (±1.14)</td>
<td>99.05 (±0.45)</td>
<td>92.40 (±2.20)</td>
<td>63.86 (±23.59)</td>
</tr>
<tr>
<td>1,000,000</td>
<td>kaolinite</td>
<td>3.59 (±2.90)</td>
<td>87.45 (±0.08)</td>
<td>88.89 (±0.64)</td>
<td>3.13 (±1.89)</td>
<td>1.69 (±1.86)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>7.85 (±0.98)</td>
<td>96.17 (±1.11)</td>
<td>98.66 (±0.10)</td>
<td>83.44 (±0.76)</td>
<td>56.82 (±0.32)</td>
</tr>
</tbody>
</table>
Figure A7.1 Effects of Molecular Weight on turbidity removal; pictures show the treated water at 30 minutes of settling (initial water turbidity 30 NTU, ≈ pH 7.0-7.5)

Table A7.2 Turbidity reduction as effects of degree of deacetylation (DD) of chitosans

<table>
<thead>
<tr>
<th>DD (%)</th>
<th>Turbidity</th>
<th>Reduction (%) at each chitosan dose (± 95%CI)</th>
<th>0 mg/L</th>
<th>1 mg/L</th>
<th>3 mg/L</th>
<th>10 mg/L</th>
<th>30 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>kaolinite</td>
<td>6.39 (±9.27)</td>
<td>47.34 (±71.85)</td>
<td>92.68 (±1.74)</td>
<td>1.82 (±6.43)</td>
<td>2.17 (±4.25)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>9.51 (±8.64)</td>
<td>90.50 (±2.79)</td>
<td>99.02 (±0.15)</td>
<td>92.94 (±6.47)</td>
<td>22.76 (±27.78)</td>
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</tr>
<tr>
<td>75</td>
<td>kaolinite</td>
<td>3.39 (±8.71)</td>
<td>1.07 (±1.75)</td>
<td>91.43 (±2.25)</td>
<td>6.47 (±20.12)</td>
<td>0.96 (±1.14)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>3.47 (±8.12)</td>
<td>87.70 (±5.65)</td>
<td>98.88 (±0.25)</td>
<td>98.21 (±0.59)</td>
<td>59.00 (±6.04)</td>
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</tr>
<tr>
<td>80</td>
<td>kaolinite</td>
<td>2.74 (±8.38)</td>
<td>68.48 (±37.94)</td>
<td>90.35 (±1.28)</td>
<td>-0.05 (±1.75)</td>
<td>0.70 (±1.54)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>4.68 (±3.79)</td>
<td>90.50 (±3.93)</td>
<td>98.94 (±0.03)</td>
<td>97.68 (±0.23)</td>
<td>62.66 (±9.73)</td>
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</tr>
<tr>
<td>90</td>
<td>kaolinite</td>
<td>-0.59 (±8.12)</td>
<td>29.11 (±111.63)</td>
<td>89.66 (±3.37)</td>
<td>75.01 (±4.83)</td>
<td>2.29 (±3.39)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>11.23 (±8.81)</td>
<td>87.73 (±0.54)</td>
<td>98.84 (±0.18)</td>
<td>98.16 (±0.22)</td>
<td>62.33 (±8.56)</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>kaolinite</td>
<td>2.80 (±10.79)</td>
<td>0.33 (±7.66)</td>
<td>87.59 (±1.97)</td>
<td>62.30 (±8.96)</td>
<td>1.82 (±14.07)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>-0.55 (±2.36)</td>
<td>86.45 (±3.14)</td>
<td>99.03 (±0.23)</td>
<td>98.04 (±0.43)</td>
<td>53.92 (±7.54)</td>
<td></td>
</tr>
</tbody>
</table>
Figure A7.2 Effects of Degree of Deacetylation on turbidity removal; pictures show treated water at 30 minutes of settling (initial water turbidity 30 NTU, ≈ pH 7.0-7.5)

Table A7.3 Effects of modified chitosans on turbidity removal

<table>
<thead>
<tr>
<th>Chitosan type</th>
<th>Turbidity</th>
<th>0 mg/L</th>
<th>1 mg/L</th>
<th>3 mg/L</th>
<th>10 mg/L</th>
<th>30 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxymethyl chitosan</td>
<td>kaolinite</td>
<td>6.72 (±2.07)</td>
<td>0.57 (±4.24)</td>
<td>0.99 (±5.52)</td>
<td>1.46 (±5.42)</td>
<td>1.32 (±7.76)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>8.49 (±5.24)</td>
<td>36.14 (±10.79)</td>
<td>53.38 (±7.53)</td>
<td>58.25 (±4.44)</td>
<td>59.60 (±6.83)</td>
</tr>
<tr>
<td>Chitosan lactate</td>
<td>kaolinite</td>
<td>-0.10 (±0.42)</td>
<td>-1.47 (±9.23)</td>
<td>88.70 (±2.05)</td>
<td>-1.07 (±6.40)</td>
<td>0.62 (±4.49)</td>
</tr>
<tr>
<td></td>
<td>bentonite</td>
<td>5.52 (±4.68)</td>
<td>80.90 (±0.83)</td>
<td>98.55 (±0.18)</td>
<td>97.29 (±1.20)</td>
<td>67.14 (±3.57)</td>
</tr>
<tr>
<td>Chitosan lactate</td>
<td>kaolinite</td>
<td>-1.52 (±6.52)</td>
<td>2.53 (±8.49)</td>
<td>87.89 (±6.97)</td>
<td>1.43 (±5.70)</td>
<td>2.98 (±5.12)</td>
</tr>
</tbody>
</table>

*) StormKlear*
<table>
<thead>
<tr>
<th></th>
<th>Bentonite</th>
<th>Kaolinite</th>
<th>Bentonite</th>
<th>Kaolinite</th>
<th>Bentonite</th>
<th>Kaolinite</th>
<th>Bentonite</th>
<th>Kaolinite</th>
<th>Bentonite</th>
<th>Kaolinite</th>
<th>Bentonite</th>
<th>Kaolinite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan acetate</td>
<td>3.81 (±6.07)</td>
<td>76.86 (±11.73)</td>
<td>97.98 (±1.33)</td>
<td>98.06 (±0.59)</td>
<td>79.35 (±1.46)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kaolinite</td>
<td>1.72 (±6.19)</td>
<td>1.41 (±7.03)</td>
<td>16.00 (±44.76)</td>
<td>2.89 (±0.96)</td>
<td>-0.48 (±3.73)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Bentonite</td>
<td>3.74 (±17.22)</td>
<td>89.83 (±3.14)</td>
<td>98.77 (±0.33)</td>
<td>96.42 (±2.77)</td>
<td>77.81 (±4.79)</td>
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<td></td>
</tr>
<tr>
<td>Chitosan acetate (StormKlear)</td>
<td>4.33 (±8.28)</td>
<td>82.13 (±30.42)</td>
<td>60.82 (±20.05)</td>
<td>0.95 (±2.44)</td>
<td>2.96 (±2.65)</td>
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</tr>
<tr>
<td>Bentonite</td>
<td>4.00 (±0.60)</td>
<td>97.14 (±2.47)</td>
<td>98.84 (±0.17)</td>
<td>81.04 (±2.44)</td>
<td>12.06 (±3.58)</td>
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</tr>
<tr>
<td>Chitosan HCl</td>
<td>1.29 (±5.54)</td>
<td>2.00 (±6.30)</td>
<td>91.29 (±4.08)</td>
<td>85.08 (±9.08)</td>
<td>2.37 (±1.16)</td>
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<tr>
<td>Bentonite</td>
<td>6.06 (±6.61)</td>
<td>87.39 (±0.85)</td>
<td>98.67 (±0.38)</td>
<td>98.54 (±0.35)</td>
<td>89.01 (±11.34)</td>
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<td></td>
</tr>
</tbody>
</table>
Chitosan acetate on kaolinite
Chitosan acetate on bentonite
Chitosan acetate-SK on kaolinite
Chitosan acetate-SK on bentonite
Chitosan lactate on kaolinite
Chitosan lactate on bentonite
Figure A7.3 Effects of modified chitosans on turbidity removal; pictures show treated water at 30 minutes of settling (initial water turbidity 30 NTU, ≈ pH 7.0-7.5)
Table A7.4 The most effective chitosans for kaolinite turbidity removals

<table>
<thead>
<tr>
<th>No.</th>
<th>Chitosan</th>
<th>Turbidity</th>
<th>Reduction (%) at each chitosan dose (± 95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 mg/L</td>
</tr>
<tr>
<td>1</td>
<td>MW50,000</td>
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<td>8.85</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(±2.22)</td>
</tr>
<tr>
<td>2</td>
<td>Chitosan HCl</td>
<td>kaolinite</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±5.54)</td>
</tr>
<tr>
<td>3</td>
<td>MW100,000</td>
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<td></td>
<td>(±5.97)</td>
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<td>kaolinite</td>
<td>3.59</td>
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<tr>
<td></td>
<td></td>
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<td>(±2.90)</td>
</tr>
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<td>(±8.12)</td>
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<td>2.74</td>
</tr>
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<td></td>
<td></td>
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<td>(±8.38)</td>
</tr>
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<td>(±9.27)</td>
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<td>(±9.27)</td>
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</tr>
<tr>
<td></td>
<td>StormKlear</td>
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<td>(±8.28)</td>
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<td>(±8.71)</td>
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<td>-0.10</td>
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<td></td>
<td>StormKlear</td>
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<td>(±0.42)</td>
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<td>-1.52</td>
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<tr>
<td></td>
<td>StormKlear</td>
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<td>(±6.52)</td>
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<td></td>
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<td>(±10.27)</td>
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<td>Chitosan acetate</td>
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<td>(±6.19)</td>
</tr>
<tr>
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<td>chitosan</td>
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<td>(±2.07)</td>
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<td>(±8.92)</td>
</tr>
<tr>
<td>No.</td>
<td>Chitosan</td>
<td>Turbidity</td>
<td>Reduction (%) at each chitosan dose (± 95%CI)</td>
</tr>
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<td>------------------</td>
<td>-----------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>0 mg/L</td>
</tr>
<tr>
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<td>MW600,000</td>
<td>bentonite</td>
<td>16.76 (±15.87)</td>
</tr>
<tr>
<td>2</td>
<td>MW100,000</td>
<td>bentonite</td>
<td>43.89 (±7.24)</td>
</tr>
<tr>
<td>3</td>
<td>Chitosan acetate</td>
<td>bentonite</td>
<td>4.00 (±0.60)</td>
</tr>
<tr>
<td>4</td>
<td>MW1,000,000</td>
<td>bentonite</td>
<td>7.85 (±0.98)</td>
</tr>
<tr>
<td>5</td>
<td>MW50,000</td>
<td>bentonite</td>
<td>6.43 (±2.31)</td>
</tr>
<tr>
<td>6</td>
<td>DD80</td>
<td>bentonite</td>
<td>4.68 (±3.79)</td>
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<td>7</td>
<td>DD75</td>
<td>bentonite</td>
<td>9.51 (±8.64)</td>
</tr>
<tr>
<td>8</td>
<td>Chitosan acetate</td>
<td>bentonite</td>
<td>3.74 (±17.22)</td>
</tr>
<tr>
<td>9</td>
<td>DD90</td>
<td>bentonite</td>
<td>11.23 (±8.81)</td>
</tr>
<tr>
<td>10</td>
<td>DD75</td>
<td>bentonite</td>
<td>3.47 (±8.12)</td>
</tr>
<tr>
<td>11</td>
<td>Chitosan HCl</td>
<td>bentonite</td>
<td>6.06 (±6.61)</td>
</tr>
<tr>
<td>12</td>
<td>DD95</td>
<td>bentonite</td>
<td>-0.55 (±2.36)</td>
</tr>
<tr>
<td>13</td>
<td>DD85</td>
<td>bentonite</td>
<td>3.44 (±4.09)</td>
</tr>
<tr>
<td>14</td>
<td>Chitosan lactate</td>
<td>bentonite</td>
<td>5.52 (±4.68)</td>
</tr>
<tr>
<td>15</td>
<td>Chitosan lactate</td>
<td>bentonite</td>
<td>3.81 (±6.07)</td>
</tr>
<tr>
<td>16</td>
<td>Carboxymethyl</td>
<td>bentonite</td>
<td>8.49 (±5.24)</td>
</tr>
<tr>
<td>17</td>
<td>MW5,000</td>
<td>bentonite</td>
<td>17.31 (±1.13)</td>
</tr>
</tbody>
</table>
# APPENDIX 8: MICROBIAL REDUCTION

## Table A8.1 Log$_{10}$ Reduction of *E. coli* B after coagulation by different chitosan molecular weight (MW), degree of deacetylation (DD) and modified chitosans

<table>
<thead>
<tr>
<th>Chitosans</th>
<th>Log$_{10}$ Reduction of <em>E. coli</em> B at each chitosan dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mg/L</td>
</tr>
<tr>
<td>50,000 Da</td>
<td>0.62 (±0.70)</td>
</tr>
<tr>
<td>100,000 Da</td>
<td>0.50 (±0.92)</td>
</tr>
<tr>
<td>1,000,000 Da</td>
<td>1.18 (±0.51)</td>
</tr>
<tr>
<td>70 % DD</td>
<td>1.16 (±0.33)</td>
</tr>
<tr>
<td>85 % DD</td>
<td>1.12 (±0.23)</td>
</tr>
<tr>
<td>95 % DD</td>
<td>1.31 (±1.20)</td>
</tr>
<tr>
<td>Chitosan acetate</td>
<td>1.32 (±0.37)</td>
</tr>
<tr>
<td>Chitosan HCl</td>
<td>0.60 (±0.93)</td>
</tr>
<tr>
<td>Chitosan lactate</td>
<td>0.55 (±1.20)</td>
</tr>
</tbody>
</table>

**Figure A8.1** Turbidity removal of test water containing 5 NTU kaolinite and *E. coli* bacteria as influenced by different doses of chitosans with different molecular weight (MW), degree of deacetylation (DD) and modified chitosans
Table A8.2 Log$_{10}$ Reduction of bacteriophage MS2 influenced by chitosan molecular weight (MW), degree of deacetylation (DD) and modified chitosans

<table>
<thead>
<tr>
<th>Chitosan</th>
<th>Log$_{10}$ Reduction of bacteriophage MS2 at each chitosan dose</th>
<th>1 mg/L</th>
<th>3 mg/L</th>
<th>10 mg/L</th>
<th>30 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>50,000 Da</td>
<td>0.47 (± 0.19)</td>
<td>2.55 (± 0.14)</td>
<td>2.00 (± 0.39)</td>
<td>1.85 (± 0.42)</td>
<td></td>
</tr>
<tr>
<td>100,000 Da</td>
<td>0.26 (± 0.47)</td>
<td>3.56 (± 0.34)</td>
<td>3.47 (± 0.89)</td>
<td>3.41 (± 0.39)</td>
<td></td>
</tr>
<tr>
<td>1,000,000 Da</td>
<td>0.31 (± 0.60)</td>
<td>3.53 (± 0.28)</td>
<td>2.97 (± 0.26)</td>
<td>2.69 (± 0.36)</td>
<td></td>
</tr>
<tr>
<td>70 % DD</td>
<td>1.20 (± 0.12)</td>
<td>1.46 (± 0.20)</td>
<td>1.21 (± 0.27)</td>
<td>1.17 (± 0.36)</td>
<td></td>
</tr>
<tr>
<td>85 % DD</td>
<td>0.30 (± 0.52)</td>
<td>2.39 (± 0.75)</td>
<td>4.02 (± 1.52)</td>
<td>2.65 (± 1.07)</td>
<td></td>
</tr>
<tr>
<td>95 % DD</td>
<td>0.38 (± 0.42)</td>
<td>2.84 (± 1.02)</td>
<td>4.09 (± 1.01)</td>
<td>3.41 (± 0.38)</td>
<td></td>
</tr>
<tr>
<td>Chitosan acetate</td>
<td>0.69 (± 0.60)</td>
<td>3.27 (±0.69)</td>
<td>3.70 (±0.21)</td>
<td>3.75 (±0.26)</td>
<td></td>
</tr>
<tr>
<td>Chitosan HCl</td>
<td>0.16 (±0.22)</td>
<td>1.87 (±0.18)</td>
<td>2.85 (±0.22)</td>
<td>2.68 (±0.30)</td>
<td></td>
</tr>
<tr>
<td>Chitosan lactate</td>
<td>0.10 (±0.41)</td>
<td>1.90 (±1.00)</td>
<td>2.52 (±0.85)</td>
<td>3.35 (±2.67)</td>
<td></td>
</tr>
</tbody>
</table>

Effects of chitosan candidates at dose 1 mg/L on turbidity removal

Effects of chitosan candidates at dose 3 mg/L on turbidity removal

Effects of chitosan candidates at dose 10 mg/L on turbidity removal

Effects of chitosan candidates at dose 30 mg/L on turbidity removal

Figure A8.2 Turbidity removal of test water containing 5 NTU kaolinite and bacteriophage MS2 as influenced by different doses of chitosans with different molecular weight (MW), degree of deacetylation (DD) and modified chitosans
Table A8.3 Zeta potential of *E. coli* bacteria and bacteriophage MS2 in stock culture and in the test water

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Zeta potential (mV) of <em>E. coli</em> bacteria and bacteriophage MS2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E.coli-TSB stock</td>
</tr>
<tr>
<td>1</td>
<td>-16.3</td>
</tr>
<tr>
<td>2</td>
<td>-16.7</td>
</tr>
<tr>
<td>3</td>
<td>-16.4</td>
</tr>
<tr>
<td>4</td>
<td>-15.5</td>
</tr>
<tr>
<td>5</td>
<td>-16.9</td>
</tr>
<tr>
<td>6</td>
<td>-18.6</td>
</tr>
<tr>
<td>7</td>
<td>-15.9</td>
</tr>
<tr>
<td>8</td>
<td>-15.5</td>
</tr>
<tr>
<td>9</td>
<td>-17.6</td>
</tr>
<tr>
<td>Number of measurement</td>
<td>9</td>
</tr>
</tbody>
</table>

Mean: -16.6 |-38.06 | -6.87 | -19.84

95%CI: 0.78 | 2.72 | 0.332 | 4.14

Lower 95% CI: -17.38 | -40.78 | -7.202 | -23.98

Upper 95% CI: -15.82 | -35.33 | -6.538 | -15.71

Note: statistical value calculate by GraphPad

Table A8.4 *E. coli* coagulation using chitosan MW 100,000 daltons in artificial and natural surface water samples

<table>
<thead>
<tr>
<th>Water</th>
<th>Log(_{10}) Reduction of <em>E. coli</em> B at each chitosan dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 mg/L</td>
</tr>
<tr>
<td>Test water 5NTU</td>
<td>2.27 (±2.44)</td>
</tr>
<tr>
<td>Test water 10NTU</td>
<td>3.15 (±1.55)</td>
</tr>
<tr>
<td>Chattahuchee River</td>
<td>3.35 (±0.07)</td>
</tr>
<tr>
<td>University Lake</td>
<td>3.85 (±0.65)</td>
</tr>
</tbody>
</table>

Table A8.5 MS2 coagulation by chitosan MW 100,000 daltons comparing between natural surface water and test water

<table>
<thead>
<tr>
<th>Water</th>
<th>Log(_{10}) Reduction of MS2 at each chitosan dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 mg/L</td>
</tr>
<tr>
<td>Test water 5NTU</td>
<td>3.56 (±0.34)</td>
</tr>
<tr>
<td>Test water 10NTU</td>
<td>4.89 (±0.88)</td>
</tr>
<tr>
<td>Chattahuchee River</td>
<td>2.34 (±0.09)</td>
</tr>
</tbody>
</table>
Table A8.6 The most effective chitosans for *E. coli* bacteria removals

<table>
<thead>
<tr>
<th>No.</th>
<th>Chitosans</th>
<th>Log₁₀ Reduction of <em>E. coli</em> B at each chitosan dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 mg/L</td>
</tr>
<tr>
<td>1</td>
<td>MW100,000 Da</td>
<td>0.50 (±0.92)</td>
</tr>
<tr>
<td>2</td>
<td>Chitosan HCl</td>
<td>0.60 (±0.93)</td>
</tr>
<tr>
<td>3</td>
<td>DD 95%</td>
<td>1.31 (±1.20)</td>
</tr>
<tr>
<td>4</td>
<td>Chitosan acetate</td>
<td>1.32 (±0.37)</td>
</tr>
<tr>
<td>5</td>
<td>Chitosan lactate</td>
<td>0.55 (±1.20)</td>
</tr>
<tr>
<td>6</td>
<td>DD 85%</td>
<td>1.12 (±0.23)</td>
</tr>
<tr>
<td>7</td>
<td>50,000 Da</td>
<td>0.62 (±0.70)</td>
</tr>
<tr>
<td>8</td>
<td>1,000,000 Da</td>
<td>1.18 (±0.51)</td>
</tr>
<tr>
<td>9</td>
<td>DD 70%</td>
<td>1.16 (±0.33)</td>
</tr>
</tbody>
</table>

Table A8.7 The most effective chitosans for bacteriophage MS2

<table>
<thead>
<tr>
<th>No.</th>
<th>Chitosans</th>
<th>Log₁₀ Reduction of bacteriophage MS2 at each chitosan dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 mg/L</td>
</tr>
<tr>
<td>1</td>
<td>MW100,000 Da</td>
<td>0.26 (±0.47)</td>
</tr>
<tr>
<td>2</td>
<td>Chitosan acetate</td>
<td>0.69 (±0.60)</td>
</tr>
<tr>
<td>3</td>
<td>DD 95%</td>
<td>0.38 (±0.42)</td>
</tr>
<tr>
<td>4</td>
<td>MW500,000 Da</td>
<td>0.31 (±0.60)</td>
</tr>
<tr>
<td>5</td>
<td>Chitosan lactate</td>
<td>0.10 (±0.41)</td>
</tr>
<tr>
<td>6</td>
<td>Chitosan lactate</td>
<td>0.16 (±0.22)</td>
</tr>
<tr>
<td>7</td>
<td>MW50,000 Da</td>
<td>0.47 (±0.19)</td>
</tr>
<tr>
<td>9</td>
<td>DD 70%</td>
<td>1.20 (±0.12)</td>
</tr>
</tbody>
</table>
APPENDIX 9: ZETA POTENTIAL TITRATION GRAPHS

Water sample (5 NTU kaolinite, pH 7-7.5) titrated with different chitosans between doses 0 – 50 mg/L

1. Chitosan 5,000 daltons did not reach IEP; at dose 50 mg/L it was still in negative charge (pH 7.4)

2. Chitosan 100,000 daltons had IEP at 4.61mg/L at ≈ pH 7.2. (Water pH = 6.5, at dose 50 mg/L)
3. Chitosan HCl had IEP at 2.19 mg/L at pH 7.2; the final pH at 50mg/L was pH 6.98.

4. Chitosan carboxymethyl did not have IEP and water pH increased to pH 8 at 50mg/L chitosan

5. Chitosan 70DD got IEP at 2.15 mg/L at pH 7.2.
The final water pH at 50mg/L chitosan was 6.3
6. Chitosan 95DD had IEP at 1.88 mg/L at pH 7.2, water pH dropped to pH 6.4 at 50mg/L

7. Changes of zeta potential related to water pH during bentonite coagulation using chitosan MW100K

8. Changes of zeta potential related to water pH during kaolinite coagulation using chitosan MW100K
## APPENDIX 10: WATER QUALITY ON TURBIDITY AND MICROBIAL REMOVAL

### Table A10.1 Effects of different water pH on turbidity removal using chitosan coagulant, initial turbidity 5 NTU

<table>
<thead>
<tr>
<th>Doses (mg/L)</th>
<th>Average Percent Reductions (± 95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Effects of water pH on kaolinite removal</td>
</tr>
<tr>
<td></td>
<td>pH 6</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-11.89 (±5.87)</td>
</tr>
<tr>
<td>3</td>
<td>-2.17 (±3.75)</td>
</tr>
<tr>
<td>10</td>
<td>-0.15 (±5.18)</td>
</tr>
<tr>
<td>30</td>
<td>-0.75 (±3.57)</td>
</tr>
</tbody>
</table>

### Table A10.2 Effects of different water pH on turbidity removal using chitosan coagulant, initial turbidity 30 NTU

<table>
<thead>
<tr>
<th>Doses (mg/L)</th>
<th>Average Percent Reductions (± 95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Effects of water pH on kaolinite removal</td>
</tr>
<tr>
<td></td>
<td>pH 6</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>89.72 (±6.25)</td>
</tr>
<tr>
<td>3</td>
<td>3.64 (±37.24)</td>
</tr>
<tr>
<td>10</td>
<td>21.28 (±39.43)</td>
</tr>
<tr>
<td>30</td>
<td>22.79 (±40.74)</td>
</tr>
</tbody>
</table>
Table A10.3 Effects of different water pH on turbidity removal using chitosan coagulant, initial turbidity 300 NTU

<table>
<thead>
<tr>
<th>Doses (mg/L)</th>
<th>Average Percent Reductions (± 95%CI)</th>
<th>Effects of water pH on kaolinite removal</th>
<th>Effects of water pH on bentonite removal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6</td>
<td>pH 7</td>
<td>pH 9</td>
</tr>
<tr>
<td>1</td>
<td>85.14 ± 17.65</td>
<td>73.41 ± 81.25</td>
<td>36.82 ± 16.47</td>
</tr>
<tr>
<td>3</td>
<td>14.29 ± 43.55</td>
<td>94.27 ± 7.16</td>
<td>38.62 ± 17.65</td>
</tr>
<tr>
<td>10</td>
<td>-30.74 ± 74.86</td>
<td>43.01 ± 43.19</td>
<td>89.31 ± 6.69</td>
</tr>
<tr>
<td>30</td>
<td>-36.19 ± 38.85</td>
<td>-15.87 ± 53.44</td>
<td>-35.01 ± 64.21</td>
</tr>
</tbody>
</table>

Table A10.4 Percent removal of kaolinite and bentonite tested at neutral water pH with varying level of water turbidity

<table>
<thead>
<tr>
<th>Dose (mg/L)</th>
<th>Average Percent Reductions (± 95%CI)</th>
<th>Effects of water turbidity on kaolinite removal</th>
<th>Effects of water turbidity on bentonite removal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6</td>
<td>pH 7</td>
<td>pH 9</td>
</tr>
<tr>
<td>1</td>
<td>14.43 ± 39.78</td>
<td>87.37 ± 9.03</td>
<td>77.01 ± 81.25</td>
</tr>
<tr>
<td>3</td>
<td>-22.62 ± 12.04</td>
<td>90.18 ± 12.85</td>
<td>94.70 ± 7.16</td>
</tr>
<tr>
<td>10</td>
<td>-11.09 ± 10.82</td>
<td>0.72 ± 8.90</td>
<td>45.46 ± 43.19</td>
</tr>
<tr>
<td>30</td>
<td>-2.08 ± 2.94</td>
<td>0.89 ± 1.22</td>
<td>-14.54 ± 53.44</td>
</tr>
</tbody>
</table>

*Experiments were performed at water pH 7, water salinity 300 mg/L
Table A10.5 Percent removal of kaolinite and bentonite at different water salinity

<table>
<thead>
<tr>
<th>Dose (mg/L)</th>
<th>Average Percent Reductions (± 95%CI) (Initial turbidity 5 NTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Effects of water salinity on kaolinite removal</td>
</tr>
<tr>
<td></td>
<td>100 mg/L (0.1 ppt)</td>
</tr>
<tr>
<td>1</td>
<td>17.38 (±20.66)</td>
</tr>
<tr>
<td>3</td>
<td>-17.23 (±13.06)</td>
</tr>
<tr>
<td>10</td>
<td>-8.11 (±11.59)</td>
</tr>
<tr>
<td>30</td>
<td>-1.09 (±4.81)</td>
</tr>
</tbody>
</table>

*Experiments were performed at pH7

Table A10.6 Average Log_{10} reduction of *E. coli* bacteria and bacteriophage MS2 after treatment with chitosan biopolymer at different levels of water pH

<table>
<thead>
<tr>
<th>Dose (mg/L)</th>
<th>Average Log_{10} Reduction (± 95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Effects of water pH on bacterial removal</td>
</tr>
<tr>
<td></td>
<td>pH 6</td>
</tr>
<tr>
<td>1</td>
<td>1.01 (±0.47)</td>
</tr>
<tr>
<td>3</td>
<td>3.65 (±2.00)</td>
</tr>
<tr>
<td>10</td>
<td>4.76 (±1.30)</td>
</tr>
<tr>
<td>30</td>
<td>4.82 (±3.23)</td>
</tr>
</tbody>
</table>

Note: each value is the average of 3 replicates

Water salinity was 300 mg/L and water turbidity was 5 NTU
**Table A10.7** Average Log$_{10}$ reduction of *E. coli* bacteria and bacteriophage MS2 after treatment with different doses of chitosan biopolymer with different level of water turbidity

<table>
<thead>
<tr>
<th>Dose (mg/L)</th>
<th>Average Log$_{10}$ Reduction (± 95%CI)</th>
<th>Effects of water turbidity on bacterial removal</th>
<th>Effects of water turbidity on viral removal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 NTU</td>
<td>10 NTU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 NTU</td>
<td>10 NTU</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>1.15 (±0.30)</td>
<td>1.43 (±0.02)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>3.91 (±1.29)</td>
<td>3.15 (±1.55)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>4.08 (±1.03)</td>
<td>4.24 (±0.23)</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>3.20 (±0.15)</td>
<td>4.12 (±1.44)</td>
</tr>
</tbody>
</table>

Note: each value is the average of 3 replicates  
Water pH was pH 7 and water salinity was 300 mg/L (0.3 ppt)

**Table A10.8** Average Log$_{10}$ reduction of *E. coli* bacteria after treatment with different doses of chitosan biopolymer and different levels of water salinity

<table>
<thead>
<tr>
<th>Dose (mg/L)</th>
<th>Average Log$_{10}$ Reduction (± 95%CI)</th>
<th>Effects of water salinity on bacterial removal</th>
<th>Effects of water salinity on viral removal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100 mg/L (0.1 ppt)</td>
<td>300 mg/L (0.3 ppt)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mg/L (0.1 ppt)</td>
<td>300 mg/L (0.3 ppt)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0.04 (±0.10)</td>
<td>1.15 (±0.30)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>4.86 (±1.60)</td>
<td>3.91 (±1.29)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>4.40 (±2.01)</td>
<td>4.08 (±1.03)</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>4.80 (±2.55)</td>
<td>3.20 (±0.15)</td>
</tr>
</tbody>
</table>

Note: each value is the average of 3 replicates  
Tested at pH 7 and 5 NTU water turbidity
# Appendix 11: Characteristics of the Tested Chitosans

**Table A11.1** Physical and chemical characteristics of commercially available chitosans used in this study

Note: These are approximate values which obtained from chitosan vendors; except zeta potential values were measured in laboratory.

<table>
<thead>
<tr>
<th>Chitosans</th>
<th>Molecular weight (Da)</th>
<th>Degree of deacetylation (%)</th>
<th>Viscosity (millipascal-second, mPa·S))</th>
<th>Zeta potential, mV (± 95% CI, n = 9)</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW 5,000</td>
<td>5,000</td>
<td>90</td>
<td>≤ 5</td>
<td>6.61 (±5.28)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td>MW 50,000</td>
<td>50,000−80,000</td>
<td>90</td>
<td>16-30</td>
<td>19.79 (±3.24)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td>MW 100,000</td>
<td>100,000−300,000</td>
<td>90</td>
<td>200-500</td>
<td>88.93 (±10.88)</td>
<td>Acros Organics, Bridgewater, NJ, USA</td>
</tr>
<tr>
<td>MW 1,000,000</td>
<td>≥ 1,000,000</td>
<td>90</td>
<td>5,501-12,500</td>
<td>38.41 (±6.28)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td>70% DD</td>
<td>n/a</td>
<td>68-73</td>
<td>≤ 7</td>
<td>41.98 (±2.73)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td>75% DD</td>
<td>n/a</td>
<td>73-78</td>
<td>≤ 7</td>
<td>31.86 (±8.08)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td>80% DD</td>
<td>n/a</td>
<td>78-83</td>
<td>≤ 7</td>
<td>38.32 (±8.29)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td>85% DD</td>
<td>n/a</td>
<td>83-88</td>
<td>≤ 7</td>
<td>24.43 (±5.98)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-------</td>
<td>---------</td>
<td>-----</td>
<td>-----------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td><strong>90% DD</strong></td>
<td>n/a</td>
<td>88-93</td>
<td>≤ 7</td>
<td>36.84 (±9.09)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td><strong>95% DD</strong></td>
<td>n/a</td>
<td>≥ 93</td>
<td>≤ 7</td>
<td>19.84 (±12.00)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td><strong>Chitosan acetate</strong></td>
<td>n/a</td>
<td>80-95</td>
<td>≥ 5</td>
<td>43.08 (±20.00)</td>
<td>HaloSource, Bothell, WA, USA</td>
</tr>
<tr>
<td><strong>Chitosan acetate-SK</strong></td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>39.02 (±6.42)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td><strong>Carboxymethyl chitosan</strong></td>
<td>n/a</td>
<td>80-95</td>
<td>5-300</td>
<td>-29.64 (±2.20)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td><strong>Chitosan HCl</strong></td>
<td>n/a</td>
<td>80-95</td>
<td>2-200</td>
<td>30.37 (±10.07)</td>
<td>Heppe Medical Chitosan Gmbh, Halle, Germany</td>
</tr>
<tr>
<td><strong>Chitosan lactate</strong></td>
<td>n/a</td>
<td>80-95</td>
<td>≥ 5</td>
<td>16.50 (±9.67)</td>
<td>HaloSource, Bothell, WA, USA</td>
</tr>
<tr>
<td><strong>Chitosan lactate-SK</strong></td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>64.74 (±13.21)</td>
<td>HaloSource, Bothell, WA, USA</td>
</tr>
</tbody>
</table>
REFERENCES


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