

5-15-2015

# The Efficacy of Utilizing Chitosan as an Antiviral Agent in Water Treatment

Jessica Wilson

Follow this and additional works at: [http://scholarworks.gsu.edu/iph\\_theses](http://scholarworks.gsu.edu/iph_theses)

---

## Recommended Citation

Wilson, Jessica, "The Efficacy of Utilizing Chitosan as an Antiviral Agent in Water Treatment." Thesis, Georgia State University, 2015.  
[http://scholarworks.gsu.edu/iph\\_theses/402](http://scholarworks.gsu.edu/iph_theses/402)

This Thesis is brought to you for free and open access by the School of Public Health at ScholarWorks @ Georgia State University. It has been accepted for inclusion in Public Health Theses by an authorized administrator of ScholarWorks @ Georgia State University. For more information, please contact [scholarworks@gsu.edu](mailto:scholarworks@gsu.edu).

# The Efficacy of Utilizing Chitosan as an Antiviral Agent in Water Treatment

By

Jessica Wilson

B.S. in Anthropology  
EMORY UNIVERSITY

A Thesis Submitted to the Graduate Faculty  
of Georgia State University in Partial Fulfillment  
of the  
Requirements for the Degree

MASTER OF PUBLIC HEALTH

ATLANTA, GEORGIA  
30303

## TABLE OF CONTENTS

ACKNOWLEDGMENTS .....	3
LIST OF TABLES.....	4
LIST OF FIGURES.....	5
ABSTRACT.....	7
INTRODUCTION.....	10
1.1 Current Options for Water treatment.....	11
1.2 Chitosan Properties.....	12
1.3 Why Chitosan?.....	13
METHODS AND PROCEDURES.....	15
2.1 Preparation of Chitosan Samples.....	15
2.2 MS2 Titer.....	15
2.3 Chitosan Antimicrobial Assay.....	16
2.4 Spot Plates.....	17
RESULTS.....	18
3.1 Chitosan effectiveness in viral removal .....	18
3.1.1 Modified Chitosan effects of viral removal.....	18
3.1.2 <i>Chitosan DD effects of viral removal</i> .....	19
3.1.3. Molecular weight effects on viral removal.....	19
3.2 Efficacy of chitosan viral removal across sample types.....	20
DISCUSSION.....	21
4.1 Effectiveness of Modified Chitosan on Viral Removal.....	21
4.2 Effectiveness of Degrees of Deacetylation of Chitosan on Viral Removal.....	22
4.3 Effectiveness of Molecular Weight on Viral Removal.....	23
4.4 Justification for Methodology .....	24
4.5 A Comparison of Chitosan Effectiveness By Comparing Antimicrobial Data and Coagulation Data .....	25
4.5.1 Chitosan Effectiveness of Bacterial Reduction in Coagulation.....	25
4.5.2 Chitosan Effectiveness of Viral Reduction in Coagulation.....	28
CONCLUSION.....	31
REFERENCES.....	32

## Acknowledgements

Dr. Casanova, I cannot articulate enough how I appreciate your constant support and guidance throughout this process. Your contributions to this project and my growth as a researcher have been invaluable.

I would like to thank Dr. Stauber for feedback and support on this project as my committee member.

To Ampai, your patience and assistance helped make this project possible, thank you.

## List of Tables

Table 1: Chitosan Samples.....	15
Table 2: Modified Chitosan Samples.....	18
Table 3: Chitosan DD.....	19
Table 4: Molecular Weight Chitosan.....	19

## List of Figures

Figure 1: Structure of Chitin and Chitosan.....	12
Figure 2: A Comparison of MIC Across Chitosan Samples.....	20
Figure 3: <i>E. Coli</i> inhibition by chitosan.....	26
Figure 4: MS2 inhibition by chitosan.....	28

APPROVAL PAGE

The Efficacy of Utilizing Chitosan as an Antiviral Agent in Water Treatment

By

Jessica Wilson

Approved:

\_\_\_\_\_ Approved \_\_\_\_\_

Committee Chair

\_\_\_\_\_ Dr. Lisa Casanova \_\_\_\_\_

Committee Member

\_\_\_\_\_ Dr. Christine Stauber \_\_\_\_\_

Date: April 23, 2015

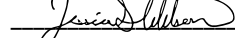
## Abstract

Chitosan is naturally occurring polysaccharide that is related to the molecule chitin and has been shown to act as an antifungal and antibacterial agent, as well as a coagulant for microbial removal from water. However, little research has explored the antiviral properties of chitosan, particularly as an antimicrobial agent to treat drinking water. In this study we investigated the efficacy of utilizing chitosan as an antiviral agent for the purposes of water treatment by measuring the minimum inhibitory concentrations (MIC) of seven chitosan samples- chitosan lactate, chitosan acetate, chitosan HCl, 70DD, 85DD, 95DD, and 100K. An antimicrobial assay was conducted utilizing seven chitosan samples with varying degrees of deacetylation, different molecular weights, and different added functional groups. We determined that amongst all chitosan samples only three samples- 85DD, 95DD, and 100K Da had the lowest MIC, or the concentration at which there were no detectable viral components at a concentration of 5,000  $\mu\text{g}/\text{ml}$ . The results attained in this experiment add to the body of knowledge about the potential benefits of utilizing chitosan as an antimicrobial agent in areas with limited water and sanitation infrastructure.



### Author's Statement Page

In presenting this thesis as a partial fulfillment of the requirements for an advanced degree from Georgia State University, I agree that the Library of the University shall make it available for inspection and circulation in accordance with its regulations governing materials of this type. I agree that permission to quote from, to copy from, or to publish this thesis may be granted by the author or, in his/her absence, by the professor under whose direction it was written, or in his/her absence, by the Associate Dean, College of Health and Human Sciences. Such quoting, copying, or publishing must be solely for scholarly purposes and will not involve potential financial gain. It is understood that any copying from or publication of this dissertation which involves potential financial gain will not be allowed without written permission of the author.

  
\_\_\_\_\_  
Signature of Author

Notice to Borrowers Page

All theses deposited in the Georgia State University Library must be used in accordance with the stipulations prescribed by the author in the preceding statement.

The author of this thesis is:

Student's Name: Jessica Wilson

Street Address: 6011 Tate Drive

City, State, and Zip Code: Austell, GA 30106

The Chair of the committee for this thesis is: Dr. Lisa Casanova

Department: School of Public Health

College: Health and Human Sciences

Georgia State University  
P.O. Box 3995  
Atlanta, Georgia 30302-3995

Users of this thesis who not regularly enrolled as students at Georgia State University are required to attest acceptance of the preceding stipulation by signing below. Libraries borrowing this thesis for the use of their patrons are required to see that each user records here the information requested.

<b>NAME OF USER</b>	<b>ADDRESS</b>	<b>DATE</b>	<b>TYPE OF USE (EXAMINATION ONLY OR COPY)</b>

## ***Chapter 1***

### **Introduction**

Limited access to improved drinking water still remains a global problem and exacerbates the global burden of diarrheal morbidity and mortality. Improved drinking water is the access to a water source that has been built to be protected from contamination. In 2000, The United Nations developed the Millennium Development Goals, which included targets to reduce the number of individuals who lack access to improved drinking water and basic sanitation by one half by the year 2015 [1-3]. According to the World Health Organization's 2014 Progress on Sanitation and Drinking Water report, approximately 748 million people still remain without access to improved drinking water and 2.5 billion also live without access to improved sanitation [2]. Collectively, individuals residing within households without access to improved sanitation and limited access to drinking water are more vulnerable to acquiring diarrheal disease [1-4].

The global burden of diarrheal diseases remains high with mortality rates around 760,000 children annually, and with the morbidity reaching almost one billion each year [2,5]. Given the multitude of individuals within the world who lack access to treated piped water and sewage infrastructure, it is imperative that alternatives for accessing improved drinking water for these individuals be identified that are both inexpensive and easy to use [2, 3, 5]. Due to the overwhelming impact of diarrheal mortality in global populations, with children disproportionately affected, it is necessary to develop water treatment solutions for developing countries to reduce global diarrheal mortality [5]. Strategic plans regarding water treatment in developing countries becomes challenging when faced with the lack of infrastructure and

resources within these nations to develop and maintain large- scale piped water facilities and sanitation systems, which developed countries built during the 20<sup>th</sup> century to decrease the morbidity and mortality of diarrheal diseases[3, 5]. Most of the infectious diarrhea worldwide is transmitted fecal-orally via contaminated food and water by viral or bacterial pathogens[1, 3].

### **1.1 Current Options for Water treatment**

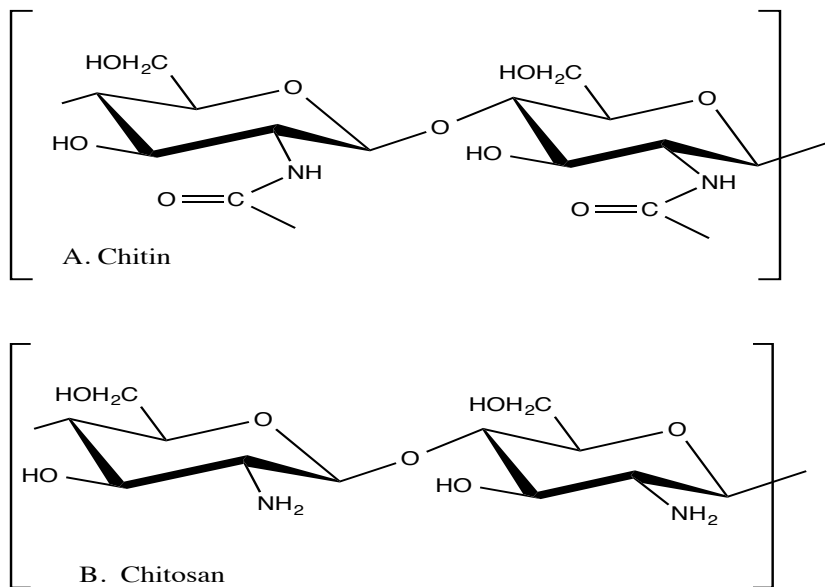
Improved drinking water interventions have been developed that attempt to provide individual household technology, referred to as point of use (POU) systems [3-5]. For individuals without access to sufficient and sustained water sources, treating water at the household level to make it safe from contaminants becomes imperative for access to clean water [4]. In the developing world water treatment at the household level or POU systems are able to reduce the risk of contamination from the source and during transport of the water, by treating the water in the home for those who have limited access to improved piped water [4-5]. Point of use water treatment systems and behavior changes are considered ideal in countries where many inhabitants either live on farms, in rural areas, or live in villages.[3].

The most effective POU systems that have been shown reduce the household and community burden of diarrheal disease and provide improved water quality were: free chlorination, coagulation/ chlorination, solar disinfection, biosand and ceramic filtration [5]. Consumable products such as free chlorination and coagulation/ chlorination, while shown to reduce the incidence of diarrheal disease are not sustainable both financially because the chemicals must be repurchased post-interventions, and also these methods often require behavioral changes to accommodate access to improved water sources [5]. Solar disinfection and other coagulant-disinfection products lack the ability to provide large-scale quantities of

water needed for household uses on a daily basis. The utilization of ceramic filters and biosand overcome the many challenges presented by coagulant disinfection and solar disinfection, in that only the initial purchasing is required and they are able to provide a large enough quantity of water appropriate for household use have been correlated with reduction of diarrheal disease. Biodand and ceramic filters seem promising for household large scale production of water and the wide-spread utilization amongst individuals worldwide [5].

Despite the variety of POU products available, more investigation is needed to find POU systems that provide improved water, are also economically sustainable, and widely available in the developing world. Chitosan, a close chemical relative of chitin, a molecule found in the exoskeleton of arthropods, has shown promise as useful POU system.

### 1.2 Chitosan Properties



**Figure 1: Structures of Chitin and Chitosan**

Chitin (Figure 1A) is a polymeric carbohydrate that is found within nature in both arthropods and other eukaryotic organisms. Chitosan (Figure 1B) has various properties that

have recently been of great interest in biomedical engineering, cosmetics, agriculture, and biopharmaceutics. Pharmacologically, chitosan has been shown to enhance the absorbance of drugs transdermally and orally [6, 7]. The partially deacetylated form it takes is most stable derivatives of chitosan and this form has shown to have both antimicrobial and coagulant properties [6, 8-10]. Interestingly, chitosan has been shown to be useful in agriculture by inhibiting bacteria and viral growth in crops. In waste and water treatment chitosan has coagulant- flocculant activity, and can achieve removal of metal ions, and odor reduction. The potential properties of flocculation and antimicrobial activity in chitosan, as well as the wide availability of chitosan, make it a potential candidate for water treatment [6-8, 11], both as a method for microbial removal and as a method for removing turbidity to make downstream disinfection processes more effective

### ***1.3 Why Chitosan?***

The antibacterial and antifungal properties of chitosan have been widely studied for its benefits to agriculture and pharmacology, however, less information is available relating to chitosans antiviral properties and the potential utilization of chitosan in water treatment. The aim of this project is to fill in the gap of chitosan research by determining if chitosan, which is both easy to use and can be produced inexpensively, can be utilized as an effective method for households to attain improved drinking water. Previous work has demonstrated that chitosan acts as a coagulant to remove viruses from water. Using MS2 as a model for human enteric viruses, the purpose of this work is to determine whether chitosan also has antiviral properties in water. Given the significant global burden of diarrheal disease which result from the consumption of unsafe drinking water, this research proposes to explore the possibility that

chitosan's antimicrobial property, and more specifically its antiviral properties, may work along with coagulant properties to enhance its potential to serve as an effective POU treatment.

## Chapter 2

### **Methods:**

Table 1: Chitosan Samples
Acetate
HCl
Lactate
70DD
85DD
95DD
MW 100K

### **2.1 Preparation of Chitosan Samples**

The seven chitosan molecules utilized in this experiment are listed in Table 1 above were chosen due to previous research conducted by this lab, which indicated that these specific chitosan molecules may be more effective for viral removal by coagulation compared to other chitosan types [25]. Modified chitosan was prepared by the addition of 10 grams of chitosan per every 1L of deionized water. Solutions of Chitosan 70DD, 85DD, and 95 DD and chitosans of different molecular weights were made by adding 10 grams per 1L of 0.5% acetic acid. Chitosan samples were prepared as needed in concentrations of 1 gram per 100mL – 2 grams per 200mL for each assay.

### **2.2 MS2 Titer**

An overnight host was prepared using *E. coli* Famp-host and allowed to incubate for a period of 18-24 hours in TSB. After the overnight period, the host sample was removed from the incubator and the log phase was prepared by taking 100 µm per 25mL of TSB. The log phase host sample was then incubated over a period of 1.5- 2 hours. Following the incubation period



the log-phase growth was confirmed by measuring absorbance of which was between 0.3-1.0 to indicate exponential growth phase.

The bacteriophage MS2 stock, which was being stored at -80 °C was allowed to thaw. After the MS2 was sufficiently thawed the 1mL MS2 sample was added to 19mL of phosphate buffered saline (PBS) (NaCl, KCl, Na<sub>2</sub>HPO<sub>4</sub>, and KH<sub>2</sub>PO<sub>4</sub>). After this initial dilution an additional 3 dilutions were conducted with 1 mL of the previous solution being added to 19mL of PBS. After the dilution of the original frozen stock in 19mL of PBS four times, the MS2 solution was then considered the “stock” solution, with a target concentration of 10<sup>6</sup> PFU/mL.

An additional dilution series was conducted with the stock solution. One milliliter of the stock was added to 9mL of PBS. From this solution four additional dilutions were done, utilizing 1mL of the previous dilution tube. After each addition or removal of each 1mL the dilution tubes were vortexed. It was determined by previous experimentation with this MS2 stock that by proceeding with the aforementioned steps, the frozen stock would yield a concentration of 10<sup>6</sup> PFU/mL. Following each the stock dilution each tube from this series was plated utilizing double agar layer technique [12]. These plates were incubated 18-24 hours at 37°C. After incubation the plates were counted for the number of plaque forming units and was confirmed to be at a value of 10<sup>6</sup> PFU/mL for each MS2 assay.

### ***2.3 Chitosan Antimicrobial Assay***

A total of seven types of chitosan (Table 1) were tested at varying concentrations to determine the most effective chitosan type and concentration, which would yield the lowest minimum inhibitory concentration (MIC). Chitosan was prepared in the method described above by beginning with the higher chitosan concentrations of 20,000 µg/ml and diluting two

fold. The above chitosan preparation method provided concentrations of 10,000 µg/ml. From the chitosan sample prepared in the method described above 1mL of each sample represented 10,000 µg/ml of chitosan, thus a chitosan concentration of 20,000 µg/ml were 2 mL of the chitosan sample. The value of the MIC was the lowest concentration at which no viral replication was visible by spot plate assay.

The appropriate concentrations were added to 10ml sterile tubes along with the addition of PBS as needed. From the stock solution of MS2 1mL was added to every chitosan sample. All experiments were performed in triplicate. The negative control contained virus and acetic acid only, with no chitosan. The positive contained chitosan without virus.

Each chitosan sample and MS2 mixture was allowed to interact for a period of an hour. After an hour had passed 0.5 mL of *E. coli* and 0.5mL of the chitosan/ MS2 mixture was added to 9 mL of TSB for overnight enrichment and incubated 18-24 hours.

#### **2.4 Spot Plates**

Following an 18- 24-hour incubation period the samples were removed from the incubator and assayed using the spot plate method [13]. After all samples had been spotted on the plates the plates were then incubated for an additional 18-24 hours. After the incubation period the presence of virus is indicated by the presence of a clear zone of lysis. Samples were scored as positive or negative, from the lowest sample without virus the MIC was determined. The MIC is the value attained by determining which chitosan sample concentration has no detectable viral lysis present. Thus at this concentration chitosan has been able to completely inhibit viral replication.

## Chapter 3

### Results

#### **3.1 Chitosan effectiveness in viral removal**

##### *3.1.1 Modified Chitosan effects of viral removal*

An antimicrobial assay was utilized to determine the degree of viral inhibition by modified chitosan based on comparing three different chitosan samples from Table 1: chitosan acetate, chitosan HCl, and chitosan lactate. The degree of viral inhibition was measured by the minimum inhibitory concentration (MIC) measured in  $\mu\text{g/ml}$ . The MIC measured for each modified chitosan sample is given in Table 2.

Chitosan lactate yielded the highest MIC of 15,000  $\mu\text{g/ml}$  compared to the other samples, chitosan HCl and chitosan acetate. Table 2 shows that the ability of chitosan HCl to cause viral inhibition was gained at a concentration of 10,000  $\mu\text{g/ml}$ . Chitosan acetate also had a MIC of 10,000  $\mu\text{g/ml}$ . From these antimicrobial assays of modified chitosan, the chitosan HCl and chitosan acetate are more effective at viral inhibition than chitosan lactate.

<b>Table 2: Modified Chitosan Samples</b>	
<b>Sample</b>	<b>Minimum Inhibitory Concentration (MIC) <math>\mu\text{g/ml}</math></b>
HCl	10,000 $\mu\text{g/ml}$
Acetate	10,000 $\mu\text{g/ml}$
Lactate	15,000 $\mu\text{g/ml}$

##### *3.1.2. Chitosan DD effects of viral removal*

Chitosans with varying degrees of deacetylation (chitosan DD) were prepared utilizing acetic acid and measured in the antimicrobial assay. Three DD chitosans were measured in the

antimicrobial assay: 70DD, 85DD, and 95DD. For each of the DD samples measured the MIC values are provided in the Table 3 below.

Beginning from the lowest level of degrees of deacetylation, 70DD yielded the highest MIC of 20,000 µg/ml. From the two remaining chitosan DD samples, 85DD and 95DD, the minimum inhibitory concentration was found to be 10,000 µg/ml and 5,000 µg/ml, respectively. Thus at 5,000 µg/ml 95DD has the best degree of viral inhibition of the DD chitosans tested.

<b>Table 3: Chitosan DD</b>	
<b>Sample</b>	<b>MIC</b>
70DD	20,000 µg/ml
85DD	10,000 µg/ml
95DD	5,000 µg/ml

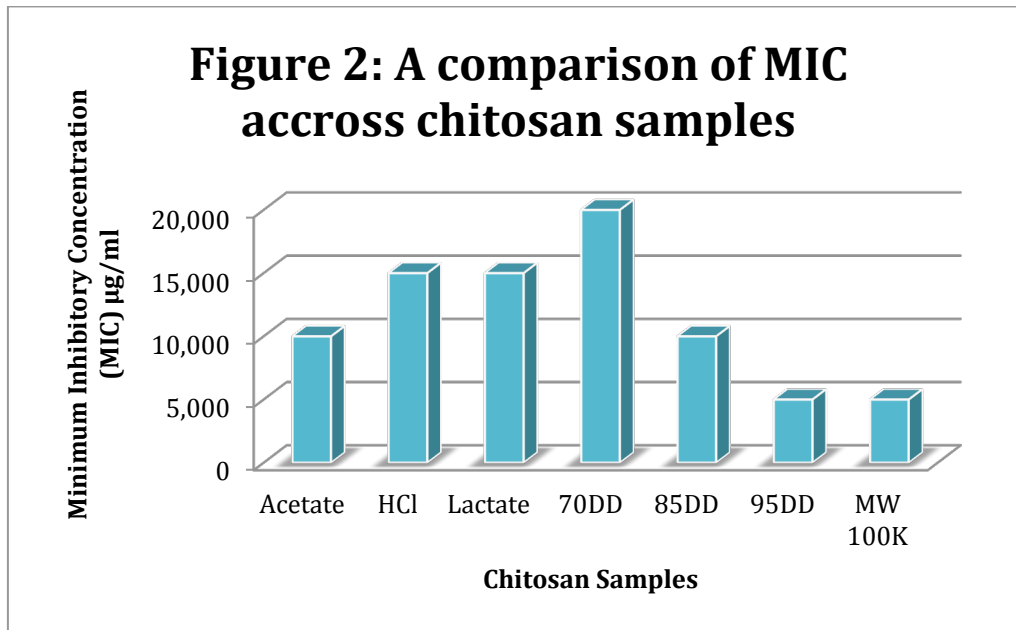
### 3.1.3. Molecular weight effects on viral removal

Chitosan with the molecular weight 100,000 Daltons (Da) was tested to determine its capacity for viral removal. Chitosan with a molecular weight of 100,000 Da had an MIC of 5,000 µg/ml, shown in table 4 below.

<b>Table 4: Molecular Weight</b>	
<b>Sample</b>	<b>MIC</b>
100 kDa	5,000 µg/ml

### 3.2 Efficacy of chitosan viral removal across sample types

After comparing chitosan samples within chitosan categories denoted in this study, the differences between chitosan groups MIC were compared and compiled into Figure 2. Across all chitosan samples three out of seven samples of chitosan have the lowest minimum inhibitory concentrations of 5,000 µg/ml - 95DD and the MW of 100 kDa.



## **Chapter 4**

### **Discussion**

This project provides evidence to support the notion that chitosan has antimicrobial properties and has the potential serve as an inexpensive and easy-to-use solution for safe household drinking water in low-income countries. According to the UN's 2014 Millennium Development Goal report, it is still estimated that approximately 748 million people still remain without access to safe drinking water [2]. Point of use (POU) water treatment has been available for use in low- income countries for many years. POU treatment systems have included boiling and other technologies such as chlorination, combined coagulant–chlorine disinfection systems, and biosand are available but have not be very widely used due to there lack of sustainability in cost and effectiveness in low-income settings [ 4,5]. In this experiment seven chitosans were divided into three categories were tested for their antiviral removal efficacy-modified chitosan, degrees of deacetylation, and a polymeric chitosan with the molecular weight of 100, 000 Da. The determination of chitosan's antimicrobial properties was determined by the MIC of each sample.

#### ***4.1 Effectiveness of Modified Chitosan on Viral Removal***

In order to determine the efficacy of modified chitosan- chitosan HCl, chitosan acetate, and chitosan lactate were studied. Each chitosan sample was prepared by dissolving the solid chitosan samples in deionized water. The results of the antimicrobial assays for the modified chitosans are given in Table 2. Table 2 describes the minimum concentration at which MS2 was removed from the sample, and thus the minimum concentration of chitosan's antiviral properties. The results of these assays provide support for chitosan HCl and chitosan acetate

being the most effective chitosan from the group of the three modified chitosans that were tested at an MIC of 10,000 µg/ml.

Chitosan solubility in water plays a major role in its ability to function as an antimicrobial agent [14, 15]. Native chitosan is insoluble in water having a pH below 6, however, when chitosan is dissolved in organic acids such as acetic acid and lactic acid in this case, the solubility of chitosan increases as the pH decreases. It is suspected that much of chitosan's antimicrobial ability is related to its biochemical properties to interact with its side chains [15]. The addition of lactic acid, hydrochloric acid, and acetic acid to chitosan to form the chitosan salts- chitosan acetate, chitosan lactate, and chitosan HCl are the result of the protonation of chitosans side chains leading to more neutral side chain amino groups and increased chitosan solubility[14-16]. The neutralization of the chitosan side chains increases chitosan solubility in water, and decreases chitosans antimicrobial functionality [15, 17]. According to the data found in this experiment that higher MIC values for modified chitosans, which is supported by the literature, chitosans that are modified to be water-soluble have reduced functionality as antimicrobial agents [15-18].

#### ***4.2 Effectiveness of Degrees of Deacetylation of Chitosan on Viral Removal***

The degree to which chitosan is deacetylated depends on a number of factors such as the type of organism that chitosan has been extracted from and the method in which chitosan is prepared for laboratory use [7,8]. The degree of deacetylation refers to the percentage of chitosan molecules in sample in which the acetyl group has been removed [7,8,21]. The efficacies of chitosans with various degrees of deacetylation were measured in this experiment to determine the effect of deacetylation on MIC. Three chitosan DD samples were tested in this

experiment- chitosan 70DD, chitosan 85DD, and chitosan 95DD. Table 3 shows that chitosan 70DD has the highest MIC, which indicates that chitosan 70DD would make a poor candidate for a POU. The reasons that chitosan 70DD would make a less effective POU compared to the other chitosan samples, is that it requires a higher concentration of the chitosan sample is needed to inhibit viral growth compared to the other samples and this will inevitably lead to higher costs to the consumer. As the deacetylation increase from samples 70DD to the 95DD the MIC decreased. The decreasing MIC from 85DD to 95DD indicates that as deacetylation increases, the antiviral properties of the chitosan increase.

Chitosans with varying degrees of deacetylation have a range of antiviral properties that are associated their level of acetylation[19]. Davydova and colleagues found that for chitosan samples, when the molecular weight is held constant and the only difference in the chitosan samples, is the degree of deacetylation, as the degree of deacetylation increase the antiviral activity increases in conjunction. They compared the molecular mass of a chitosan sample that was 17.0 kDa, one sample having a 17% degree of deacetylation and the other sample having a 25% degree of deacetylation. The 17% deacetylated sample had a approximate degree of antiviral activity that was 73%, alternatively the 25% DD sample was found to exhibit approximately 84% antiviral activity [19]. The results observed in this study are supported by the results found in the literature, which indicate that as the degree of chitosan deacetylation is increased the antiviral capacity of chitosans increase [16, 18-21].

#### ***4.3 Effectiveness of Molecular Weight on Viral Removal***

The structure of chitosan confers its functional interaction with microbes. In this experiment to determine the effect of molecular weight on chitosan's antiviral property the



chitosan sample 100,000 Da was utilized. This chitosan sample with the molecular weight of 100,000 Da showed similar effects on viral removal as the deacetylated chitosan samples 85DD and 95DD presented above. Figure 2 shows that the minimum inhibitory concentration for these two samples is 5,000 µg/ml. This indicates that chitosans with high molecular weights could potentially make effective POU agents for water treatment.

Molecular weight is a component of chitosan structure that plays an important role in chitosan's antimicrobial properties. The literature presents controversial data regarding the nature of chitosan's antiviral properties as the molecular weight is increased [20, 22, 23]. Chirkov noted in his review of current literature about the antiviral activity of chitosan, that chitosan having increasing antiviral abilities as the molecular weight is also increased [20]. The contradictory data from the literature that asserts that chitosan's antiviral property decreases with molecular weight may be explained by how the chitosan sample and the source of the chitosan [20]. Chitosans prepared from acid and alkaline hydrolysis may have varying degrees of distribution for groups of acetyl residues that may impact their biological activity [20, 22].

#### ***4.4 Justification for Methodology***

The initial method for conducting this experiment to assess the antiviral properties of chitosan utilizing the MS2 bacteriophage was the double-agar method [12]. However, the double-agar method was determined to provide inconsistent and inaccurate results of chitosans antiviral properties. Our laboratory determined that instead of determining the number of viral colonies remaining after chitosan exposure, a more salient picture of chitosan's antiviral functionality could be determined utilizing the spot plate method [13]. The spot plate

method has provided us with consistent results for the chitosan samples which were conducted in triplicates, and allowed us to determine if virus remained in the sample or not.

#### ***4.5 A Comparison of Chitosan Effectiveness By Comparing Antimicrobial Data and Coagulation Data***

##### ***4.5.1 Chitosan Effectiveness of Bacterial Reduction in Coagulation***

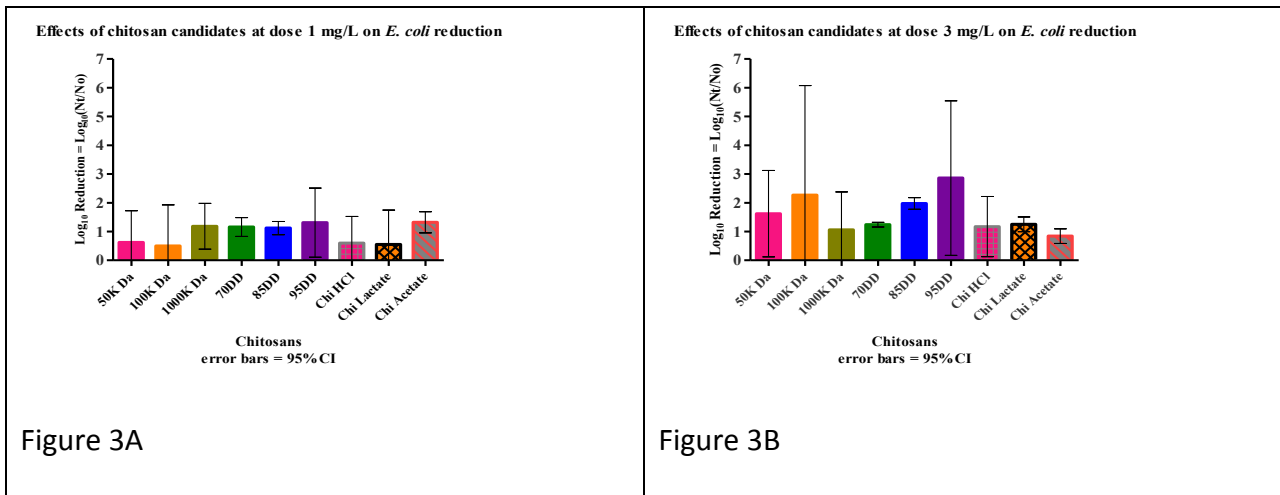
Previous experiments conducted by this laboratory on the effectiveness of chitosan in coagulation have been enlightening for developing a better understanding of the chitosan data collected in this experiment. Coupled together this data makes a compelling argument for the utility of chitosan as an antimicrobial agent and potentially effective POU system due to the joint effects of coagulation and antimicrobial activity.

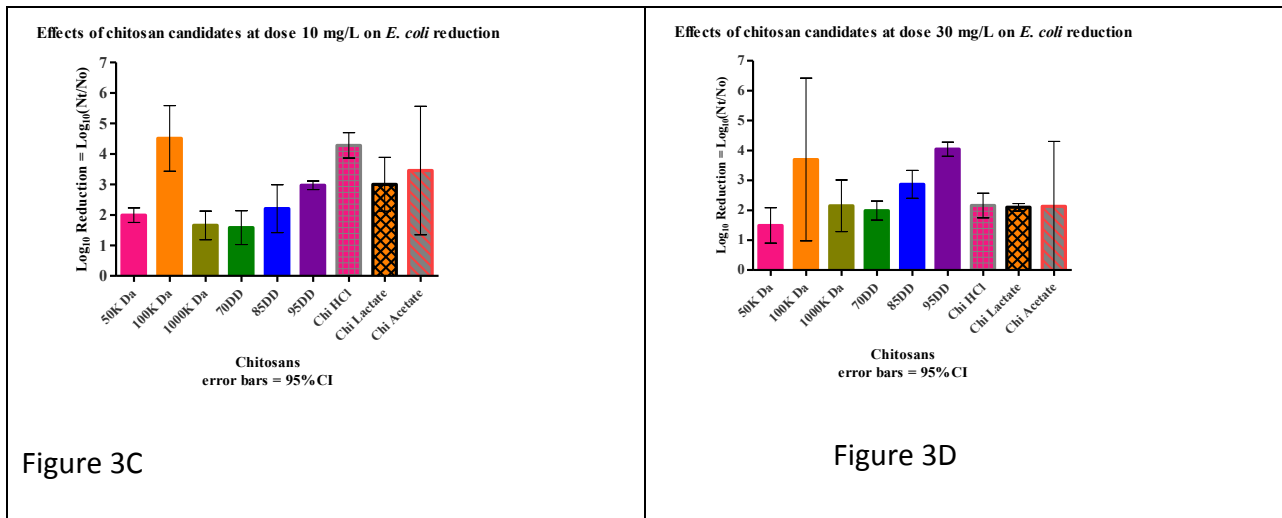
It is widely accepted in microbiological research that the concentrations of disinfectants are much lower for bacteria, than those same disinfection agent concentrations when used on viruses [24]. Therefore, although the assessment of bacterial inhibition was not measured in this experiment, the results for chitosan effectiveness as an antibacterial and antiviral agent in coagulation experiments conducted in our laboratory. These results are presented here to show that chitosan's antimicrobial properties noted here are similar to results measured in other microbiological assays are described in Figure 3 and Figure 4 [25].

Chitosan has been shown to be an effective coagulant to reduce bacterial spread and infection [14, 15, 18, 21]. Figure 3 displays the chitosan samples used in this experiment and an additional two chitosan samples with varying molecular weights- 50K Da and 1000K Da. Figure 3A shows the effective reduction of *E. coli* from chitosan with a concentration of 1 mg/L. At this concentration chitosan acetate, chitosan 70DD, chitosan 85 DD, chitosan 1000k Da, chitosan

95DD were the most effective of all the chitosan samples resulting in a greatest degree of bacterial removal to yield a log reduction value of 1.5. When the concentration of the chitosan is tripled in Figure 2B shows that chitosan 100K Da and chitosan 95DD are the most effective chitosan samples with log reductions of approximately 2 and 3, respectively. The log reduction of chitosan 95DD indicates that it is 99.9% effective at reducing bacteria. In Figures 3C and 3D the chitosan concentration is raised again to 10mg/L and 30mg/L, respectively. The data shown in these two figures is consistent with the data in 3B, and shows that with increasing concentrations all chitosans become more effective at bacterial reduction, however, chitosan 100k and chitosan 95DD present the most promising results with log reductions from 4-4.5. Thus, these chitosans are 99.99%-99.999% effective at bacterial reduction, respectively.

Figure 3: *E. Coli* inhibition by chitosan\*



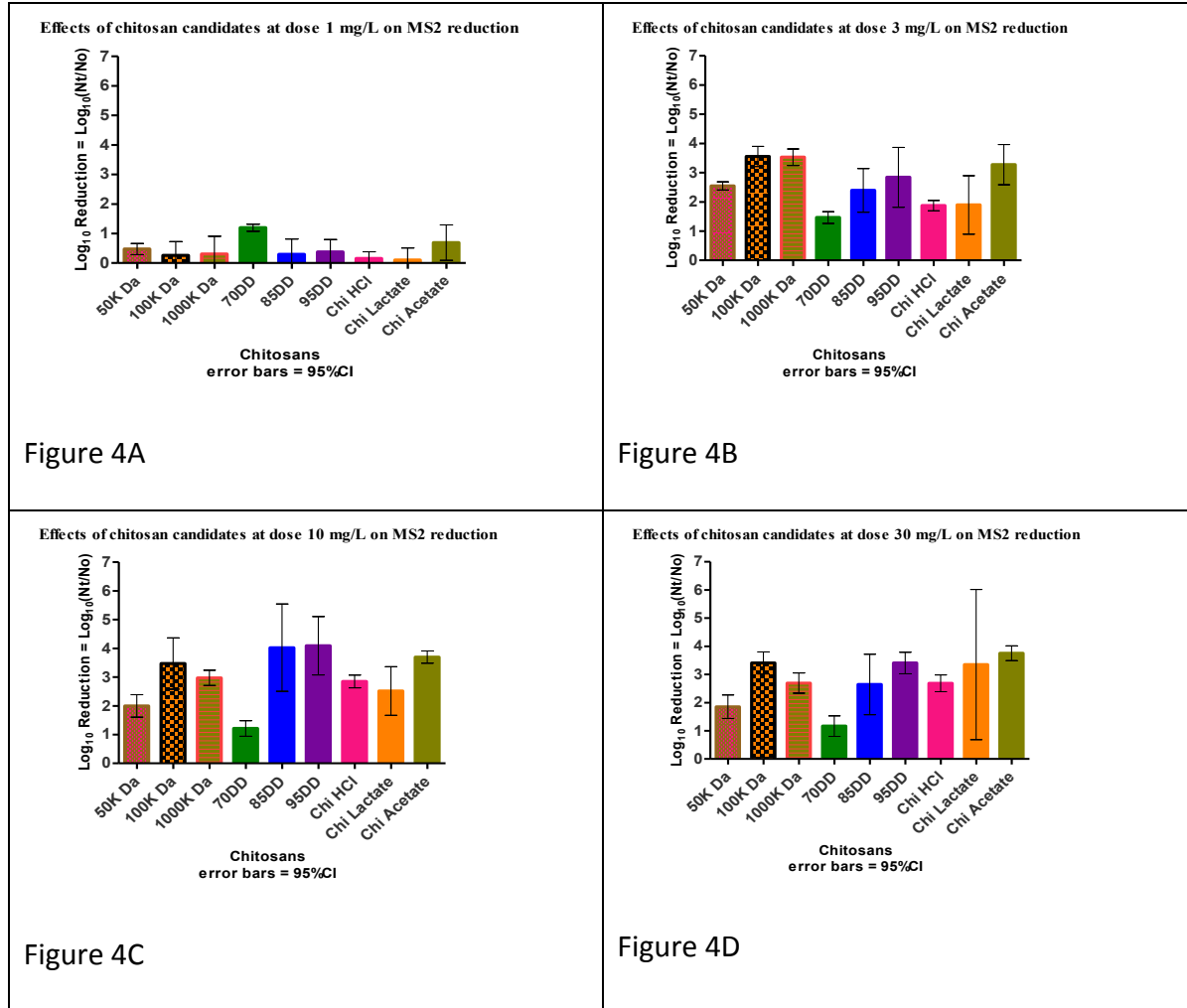


\* [25]

The data presented in Figure 3 above concerning the comparison of nine chitosan samples in various concentrations during coagulation experiments corresponds to the results found in this experiment. From the antimicrobial assays we observed that chitosan samples 95DD and chitosan 100k Da were the most effective chitosans to yield the minimum concentration of chitosan needed for bacterial growth inhibition. Chitosan has capacity to serve as an antibacterial agent, against *E. coli* and many other bacterial sources [14, 15, 21]. The data displayed in Figure 3A-D seems to indicated that as we have already observed, both the degree of deacetylation and molecular weight of the chitosan sample are key components of its antimicrobial properties [14, 15, 19].

#### 4.5.2 Chitosan Effectiveness of Viral Reduction in Coagulation

Figure 4: MS2 inhibition by chitosan



Viral reduction by chitosan is dose dependent according to the data presented in Figure 4A-D [25]. In Figure 4A at a concentration of 1 mg/L the only chitosan sample to reach a log reduction of 1 and over was 70DD, all other chitosan samples had log reductions around 0.5 mg/L or lower. When the concentration of chitosan is tripled in Figure 4B there is a dramatic change in the chitosan antiviral activity. The higher molecular weight chitosans- 100k Da and 1000K Da have around a 3.5 log reduction. While chitosan 85 DD and chitosan 95DD have log

reductions 2.5 and 3 respectively. Of the modified chitosans, chitosan acetate presents a log reduction at around 3.5. In Figure 4C the antiviral effectiveness of chitosan 100K Da remains around 3.5, chitosan 85DD and chitosan 95DD had a log reduction of 4, and chitosan lactate also had a log reduction of 4. After the chitosan concentration is raised again from 10 mg/L to 30 mg/ L, chitosan 100K Da, chitosan 95DD, and chitosan acetate have log reduction values around 3.5. Interestingly, throughout all chitosan concentrations the 70DD chitosan sample maintains the same log reduction value, which is around a log reduction of 1.

Both the bacterial and viral results from the coagulation agree with the results found in this experiment, which indicates that the most effective chitosans amongst both bacterial and viral interactions of chitosans are chitosan 100K Da and chitosan 95DD. This data aligns with the notion that properties of chitosan that lead to bacterial deactivation may also lead removal the ability viral infectivity, and a chitosan sample exhibiting antibacterial properties, may also have antiviral properties worth exploring [14, 15, 21, 23, 26].

Taken together the results from both the antimicrobial assay conducted in this experiment and other coagulation experiments conducted by our laboratory and presented in this paper highlight the effectiveness of chitosan as an antimicrobial agent. Chitosans with larger molecular weights such as 100K Da and other chitosans with varying degrees of deacetylation such as 85DD and 95DD, these chitosans were found to be most effective for both bacterial and viral reduction overall. The reason for molecular weight and the degrees of deacetylation play an essential role in chitosan antimicrobial activity, may be linked to the biochemical interactions related to chitosans molecular structure [15, 23, 26].

While the specific mechanism of chitosan viral and bacterial inhibition still remains unknown a number of theories of inactivation have been proposed. Preparation of chitosan under acidic conditions, such as the utilization of acetic acid which was conducted in this experiment, leads to the protonation of the amino groups leaving an overall positive charge on the chitosan [20]. The positive charged chitosan then is able to interact with a negatively charged bacterial wall or viral coat [14, 19, 20]. Larger molecular weight chitosans have a greater surface area and thus may have an increased interaction with its side chain and microbial structures. Few mechanisms that occur on the biochemical level that may explain the antimicrobial properties of chitosan are widely known.

## **Chapter 5**

### **Conclusion**

The effectiveness of chitosan and its derivatives to be utilized as an antimicrobial agent is related to both the degree of deacetylation and its molecular weight [14]. We assessed the antiviral properties of seven chitosan samples on the MS2 bacteriophage. The results of our antimicrobial assays described the chitosans with the lowest MIC values and therefore best chitosans were- chitosan 95DD and chitosan 100K Da. From these results we estimated that chitosan antiviral properties are dependent on degree of deacetylation and on molecular weight [14, 15, 19]. We then compared our antimicrobial assay results to previous work conducted in our laboratory utilizing these same chitosan samples in coagulation experiments [25]. After assessing the results from both experiments, it was determined that across coagulation and antimicrobial experiments the antimicrobial properties of chitosan remained consistent, both molecular weight and degree of deacetylation were imperative to that antimicrobial properties of chitosan samples. The specific mechanisms that these chitosans implore to inactivate or kill microbes still remain elusive. More research is needed to determine the specific mechanism of action that lead chitosan to have improved antimicrobial properties. The results achieved by these experiments provide evidence to support the use of 100k Da chitosan and chitosan 95DD to serve as POU systems. Diarrheal diseases continue to contribute to the global morbidity and mortality rates, this experiment has shown that chitosan has the potential to serve as a POU system to improve household water quality and decrease the global diarrheal burden.



## References

1. Santosham M, Chandran A, Fitzwater S, Fischer-Walker C, Baqui AH, Black R: **Progress and barriers for the control of diarrhoeal disease.** *The Lancet* 2010, **376**(9734):63-67.
2. World Health O: " **Progress on Drinking-Water and Sanitation–2014 Update**" 2014.
3. Zwane AP, Kremer M: **What works in fighting diarrheal diseases in developing countries? A critical review.** *The World Bank Research Observer* 2007, **22**(1):1-24.
4. Rosa G, Clasen T: **Estimating the scope of household water treatment in low-and medium-income countries.** *The American journal of tropical medicine and hygiene* 2010, **82**(2):289-300 %@ 0002-9637.
5. Sobsey MD, Stauber CE, Casanova LM, Brown JM, Elliott MA: **Point of use household drinking water filtration: a practical, effective solution for providing sustained access to safe drinking water in the developing world.** *Environmental science & technology* 2008, **42**(12):4261-4267.
6. Bhatnagar A, Sillanpää M: **Applications of chitin-and chitosan-derivatives for the detoxification of water and wastewater—a short review.** *Advances in Colloid and Interface Science* 2009, **152**(1):26-38.
7. Rinaudo M: **Chitin and chitosan: properties and applications.** *Progress in polymer science* 2006, **31**(7):603-632.
8. Davydova VN, Nagorskaya VP, Gorbach VI, Kalitnik AA, Reunov AV, Solov'eva TF, Ermak IM: **Chitosan antiviral activity: Dependence on structure and depolymerization method.** *Applied biochemistry and microbiology* 2011, **47**(1):103-108 %@ 0003-6838.
9. Jung EJ, Youn DK, Lee SH, No HK, Ha JG, Prinyawiwatkul W: **Antibacterial activity of chitosans with different degrees of deacetylation and viscosities.** *International journal of food science & technology* 2010, **45**(4):676-682 %@ 1365-2621.
10. Rabea EI, Badawy MET, Stevens CV, Smagghe G, Steurbaut W: **Chitosan as antimicrobial agent: applications and mode of action.** *Biomacromolecules* 2003, **4**(6):1457-1465 %@ 1525-7797.
11. Su X, Zivanovic S, D'Souza DH: **Effect of chitosan on the infectivity of murine norovirus, feline calicivirus, and bacteriophage MS2.** *Journal of Food Protection*® 2009, **72**(12):2623-2628 %@ 0362-2028X.
12. Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP: **Enumeration of bacteriophages by double agar overlay plaque assay.** In: *Bacteriophages.* Springer; 2009: 69-76.
13. Beck N, Callahan K, Nappier S, Kim H, Sobsey M, Meschke J: **DEVELOPMENT OF A SPOT - TITER CULTURE ASSAY FOR QUANTIFYING BACTERIA AND VIRAL INDICATORS.** *Journal of Rapid Methods & Automation in Microbiology* 2009, **17**(4):455-464.
14. Rabea EI, Badawy ME-T, Stevens CV, Smagghe G, Steurbaut W: **Chitosan as antimicrobial agent: applications and mode of action.** *Biomacromolecules* 2003, **4**(6):1457-1465.
15. Mourya V, Inamdar NN: **Chitosan-modifications and applications: opportunities galore.** *Reactive and Functional polymers* 2008, **68**(6):1013-1051.

16. Alves N, Mano J: **Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications.** *International journal of biological macromolecules* 2008, **43**(5):401-414.
17. Qin C, Li H, Xiao Q, Liu Y, Zhu J, Du Y: **Water-solubility of chitosan and its antimicrobial activity.** *Carbohydrate polymers* 2006, **63**(3):367-374.
18. Hwang KT, Kim JT, Jung ST, Cho GS, Park HJ: **Properties of chitosan - based biopolymer films with various degrees of deacetylation and molecular weights.** *Journal of applied polymer science* 2003, **89**(13):3476-3484.
19. Davydova V, Nagorskaya V, Gorbach V, Kalitnik A, Reunov A, Solov'eva T, Ermak I: **Chitosan antiviral activity: Dependence on structure and depolymerization method.** *Applied biochemistry and microbiology* 2011, **47**(1):103-108.
20. Chirkov S: **The antiviral activity of chitosan (review).** *Applied Biochemistry and Microbiology* 2002, **38**(1):1-8.
21. Ohtakara A, Izume M, Mitsutomi M: **Action of microbial chitinases on chitosan with different degrees of deacetylation.** *Agricultural and biological chemistry* 1988, **52**(12):3181-3182.
22. Kulikov S, Chirkov S, Il'ina A, Lopatin S, Varlamov V: **Effect of the molecular weight of chitosan on its antiviral activity in plants.** *Applied Biochemistry and Microbiology* 2006, **42**(2):200-203.
23. Zheng L-Y, Zhu J-F: **Study on antimicrobial activity of chitosan with different molecular weights.** *Carbohydrate Polymers* 2003, **54**(4):527-530.
24. Chaidez C, Moreno M, Rubio W, Angulo M, Valdez B: **Comparison of the disinfection efficacy of chlorine-based products for inactivation of viral indicators and pathogenic bacteria in produce wash water.** *International journal of environmental health research* 2003, **13**(3):295-302.
25. Ampai S: *Forthcoming 2015* 2015.
26. TSAI GJ, SU WH, CHEN HC, PAN CL: **Antimicrobial activity of shrimp chitin and chitosan from different treatments and applications of fish preservation.** *Fisheries Science* 2002, **68**(1):170-177.