Application of chitosan in the treatment of wastewater from agricultural sources

Terence Yep

University of Windsor

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation
https://scholar.uwindsor.ca/etd/5774
Application of chitosan in the treatment of wastewater from agricultural sources

By

Terence Yep

A Thesis
Submitted to the Faculty of Graduate Studies through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

2016

© 2016 Terence Yep
Application of chitosan in the treatment of wastewater from agricultural sources

By

Terence Yep

APPROVED BY:

______________________________
J. Gagnon
Department of Earth and Environmental Sciences

______________________________
K. E. Taylor
Department of Chemistry and Biochemistry

______________________________
B. Mutus, Advisor
Department of Chemistry and Biochemistry

April 18, 2016
DECLARATION OF ORIGINALITY

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

I certify that, to the best of my knowledge, my thesis does not infringe upon anyone’s copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis and have included copies of such copyright clearances to my appendix.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.
ABSTRACT

Modern agricultural practices is dependent on fertilizers, rich in nitrogen, phosphorus, and potassium. However, not all of the nutrients are absorbed and are usually washed away into rivers and streams. These runoffs accumulate in downstream large water bodies and enhance the growth of algae and unwanted plants, which leads to eutrophication. The consequences of eutrophication are the degradation of water quality and destruction of the affected aquatic eco-system. This study primarily examines the efficacy of metal-complexed chitosan composites in the attenuation of phosphates at three field test sites. In addition to this, chitosan was also studied for its potential use in hydrogen sulfide removal and its application in biological treatment. Metal-chitosan composites used in conjunction with red sand proved most effective in the removal of phosphates reducing it from ~19 µg/ml by 6-30 fold. Furthermore, these composites were capable of attenuating dissolved hydrosulfides from 1mM by 100-fold.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Bulent Mutus, for all of his guidance and more so, his patience during these years as a graduate student. I am truly grateful for having such a great mentor who showed me how to find the best of things even in the worst of situations. I would also like to thank the members of my supervisory committee, Dr. Keith E. Taylor, and Dr. Joel Gagnon, for their helpful ideas and suggestions. I would also like to thank Darryl Finnigan from OMAFRA, Michael Saunders, HMGA and Craig Merkley from UTRCA for providing me access and assistance with the field test sites during the field experimentation process.

I would also like to thank all my friends and family, especially my girlfriend Lucy, for all their support and encouragement that they had provided over the years of my studies without whom none of this would have been possible. A special thanks to Micheal Albu for his expertise in polymer chemistry and showing me the utility of chitosan in the chemical industries, Pablo Martinez-Bulit for his expertise and help he had provided in the X-ray diffraction experiments and Dr. Gauld and Sameer Jafaar for their computational contributions to my work. I would like to thank Marlene Bezaire, Elizabeth Kickham, and Cathy Wilson for all their hard work which is seldom appreciated as it should be.

I truly appreciate all the support provided to me by my fellow lab members. To Artur, Bei, Cody and Kathleen. It was truly been a pleasure working with each of you and I will never forget the joys that we had experienced in the lab.
Table of Contents

DECLARATION OF ORIGINALITY ........................................................................... iii
ABSTRACT ........................................................................................................... iv
ACKNOWLEDGEMENTS ..................................................................................... v

Introduction ......................................................................................................... 1
  Physico-chemical properties of Chitosan .......................................................... 2
  Structure: chitin and chitosan ......................................................................... 2
Molecular weight .................................................................................................. 4
Crystallinity ......................................................................................................... 5
Solubility of chitosan .......................................................................................... 5
Ion exchange ....................................................................................................... 6
Metal complexation and chitosan ...................................................................... 7
Modifications of chitosan for wastewater treatment ......................................... 9
Biological wastewater treatment ...................................................................... 10
Sulfide as an environmental pollutant and current methods of treatment ........... 11
Scope of the project ........................................................................................... 12

Materials and Methods ..................................................................................... 13
  Reagents .......................................................................................................... 13
  Equipment ....................................................................................................... 13
  Methods .......................................................................................................... 13

I. Chitosan bead production and determination of its metal binding capacity ........ 16
  1. Synthesis of chitosan micro-particles ......................................................... 16
  2. Determination of metal sorption capacity of chitosan .............................. 19
  3. Determination of phosphate binding capacity of chitosan and its derivatives 22

II. Field testing of chitosan as a potential bio-filter for phosphate wastewater treatment 24
  Site sampling methods: .................................................................................. 24
    1. Application of chitosan packed column for phosphate removal .............. 25
      1.1. Luckharts truck wash station ............................................................... 26
      1.1.1. Design and implementation of new filter unit ................................. 31
    1.2. Application of chitosan at Phillip Gardens vegetable wash station ........ 35
    2. Application of filter unit at Big Bay Point Road ....................................... 40

III. Hydrogen sulfide abatement using metal-complexed chitosan .................... 42
    1. Filter synthesis and reactor setup ............................................................. 42
    2. Application of metal complexed chitosan in the removal of hydrosulfide 43
IV. Application of Chitosan-immobilized cyanobacteria in water purification .................. 48
   1. Bioreactor design ........................................................................................................ 48
   2. Application of cyanobacteria chitosan-flake based bioreactor in phosphate removal ...... 51
Future directions ................................................................................................................... 54
References ............................................................................................................................. 55
Appendix ................................................................................................................................. 60
VITA AUCTORIS .................................................................................................................... 61
Introduction

Modern agricultural practice today is dependent on fertilizers, rich in phosphates, nitrates and potassium.[1] However, most of the nutrients are not absorbed by the crops, and are either precipitated by the metals present in the soil or, are washed away into rivers and streams.[2] The phosphates and nitrates from the run-offs accumulate in the larger bodies of water, which results in uncontrolled proliferation of unwanted plants, and algal growth. Eutrophication is the enrichment of fresh water bodies with inorganic plant nutrients like nitrates and phosphates. Eutrophication is a natural process that occurs over a period of time and is particularly evident in shallow lakes and slow-moving rivers. However, the process has been accelerated lately by anthropogenic input of inorganic nutrients. This leads to the depletion of dissolved oxygen, and depending on the strain of algae, toxins may be released into the water body. This results in the degradation of water quality for human consumption as well as the eco-system dependent on the affected water body for its survival.[3-5]

There has been a lot of research done on the treatment of wastewater for phosphorous. The conventional method for attenuating nutrients in wastewater is biological treatment, with the help of anaerobic and aerobic bacteria to assimilate phosphates and nitrates into their biomass.[6-8] This process removes ~90% of the phosphate from the influent wastewater with the resulting sludge disposed into designated landfill sites.[9] Other forms of treatment are chemical precipitation using metal salts and by ion-exchange columns that depend on physical interactions between the column and the ion to be removed.[10] These processes are the traditional methods used for wastewater treatment and are usually expensive due to various aspects ranging from setting up the infrastructure to the chemicals required for the treatment itself. Certain wastewater treatment plants (WWTP) attempted to utilize algae in their bio-reactors as an alternative method.
for the treatment of phosphate rich wastewater. The reactor requires a steady supply of light, heat and carbon dioxide for the optimal growth of the algae layers which in turn ensures the efficient removal of nutrients in the water.[11, 12]

Treatment by conventional wastewater treatment plants requires an established infrastructure in place to direct the influent into the system. However, most agricultural run-offs do not enter this stream, so alternative methods are required for its treatment.

**Physico-chemical properties of Chitosan**

**Structure: chitin and chitosan**

Chitin was first discovered in mushrooms by Braconnot in 1811. This polymer was found to be the most naturally abundant occurring fiber after cellulose and is structurally similar to cellulose. It contains 2-acetamido-2-deoxy-β-D-glucose units that are linked together via β-(1→4) linkage as shown in Fig. 1.[13] Chitin has about 5 – 8 % (w/v) nitrogen content, which is mostly in the form of primary aliphatic amino groups as found in chitosan.[14]

![Chitin to Chitosan](image)

**Figure 1:** Modification of chitin into chitosan was performed at a high temperature in concentrated sodium hydroxide in order to deacetylate the polymer. The arrow indicates the location of the β-(1→4) linkage.

The most common source of chitin is the shells of crustaceans like shrimps and crabs. Chitosan was reportedly first discovered by Rouget in 1859, when he boiled chitin in concentrated potassium hydroxide solution which gave rise to de-acetylated chitin.[15]
Commercially, chitosan is obtained from waste crab and shrimp shells. It is first treated with acids to remove calcium carbonate, and then treated with alkaline solutions to deacetylate the polymer as well as remove any minerals that are associated with it.[16] Chitosan has been shown to have excellent metal coordination properties due to the availability of free amines and hydroxyl groups present on the polymeric chain.[17, 18] Chitosan is usually further processed into flakes and ultimately into fine powders.

Chitosan is usually characterized by its degree of deacetylation (DOD %). This represents the total number of N-deacetylated sites that are present on the biopolymer. In order to determine this number, there are several methods that can be used, however the use of Fourier transform- Infrared (FT-IR) spectroscopy can aid in the rapid characterization of the polymer.[19]

DOD% of chitosan affects all the physico-chemical properties of chitosan such as viscosity, solubility, and others, which makes this one of the most important parameters.
Molecular weight

The definition of average molecular weight (MW) of polysaccharides and the understanding of its consequences on their physico-chemical behavior have presented a real challenge to chemists for a number of years. In the case of chitosan, information regarding this data is necessary for applications in various industries and future research.

Although the primary structure of chitosan is a backbone of β(1→4)-D-glucosamine residues randomly acetylated to various extents, the name chitosan is in fact a collective term for de-acetylated chitins differing in terms of crystallinity, optical characteristics, DOD%, impurity content, and average molecular weights.

Production methods and origins are mainly responsible for the above differences, which are encountered in various chitosan batches. Methods currently used for molecular weight determination are based on viscometric measurements.[20] and recently size exclusion chromatography and gel permeation/filtration chromatography has been applied to study the MW and MW distribution of synthetic polymers, biopolymers and natural polymers.[21] MW can also be determined by using Infra-red spectroscopy (IR) and multi-angle light scattering (MALLS).

The molecular weight can be determined with the help of standard curves generated with polymers of well-defined molecular weight.[22, 23]

Chitosan has a very high molecular weight which ranges between 50kDa to 200kDa. The DOD % and the degree of polymerization (DP) are the parameters that decide the molecular weight of chitosan. These parameters affect other important physico-chemical properties such as solubility and crystallinity as well as determine its reactivity towards chemical modifications and metal chelation.
Crystallinity

Researchers have studied the three structural forms of chitosan, i.e., hydrated, anhydrous crystal and amorphous with the help of powder X-ray diffraction (p-XRD). They had discovered that the hydrated form showed a strong reflection at an angle (2θ) of 10.4° and the other peaks at 20° and 22°. The anhydrous crystal exhibited a strong peak at 2θ of 15° and a peak at 20°.[24] The amorphous form of chitosan does not show any reflectivity, but it exhibits a broad halo at 2θ = 20°. The presence of crystallinity in those forms of chitosan can be attributed to the presence of α-chitin present in the chitosan matrix. By comparing partially de-acetylated chitin and partially acetylated chitosan, they had concluded that chitosan with a DOD% of 100 is purely crystalline, and any N-acetylation present in the polymer contributed to the decrease in crystallinity of the polymer.[25] Chitosan in its semi-crystalline form is currently being used for various applications in a variety of fields ranging from medical to wastewater treatment applications.

Solubility of chitosan

Chitosan in its native form, is insoluble in water with a pH near neutrality. This can be attributed to the rigid crystalline structure of chitin and chitosan as well as the acetamido and primary amino groups of the bio-polymer participating in intra- as well as inter-molecular hydrogen bonding which gave rise to its peculiar conformational features.[26, 27] The solubility parameters of chitin and chitosan were determined by group contribution methods (GCM) and the values were compared with the values determined from maximum intrinsic viscosity, surface tension, the Flory–Huggins interaction parameter and dielectric constant values.[28] The polymer dissolves very easily in acidic solutions with pH < 6 and certain organic solvents like dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF).[27, 29] In the case of acidic solutions, the protonation of the C-2 primary amine makes it positively charged. This changes chitosan’s nature, and makes
it a water soluble cationic polyelectrolyte.[30] The solubility transition occurs at its logarithmic acid dissociation constant (pKa) value around pH between 6 and 6.5. As the pKa value is highly dependent on the degree of N-acetylation, the solubility of chitosan is dependent on the DOD % and the method of deacetylation used.[31]

**Ion exchange**

Ion exchange (IE) has been used for several years in wastewater treatment to carry out the removal of undesirable anions and cations. These ions can be exchanged for a stoichiometrically equivalent amount of other ions that are of the same sign when the ion exchanger is in contact with the wastewater solution.[32] IE is popularly utilized as a tertiary water treatment technique. The IE is carried out by materials such as zeolites (sodium aluminosilicate) that are naturally occurring porous materials or by polymeric resins that are usually synthesized from the polymerization of organic compounds.[33, 34] These organic compounds have functional groups that define the nature of the ion exchange resin, i.e., cationic or anionic. Cationic exchange resins have acidic functional groups such as the sulfonic (-SO$_3^-$) group which allows it to exchange positively charged ions. On the other hand, anionic exchange resins contain basic groups such as amines (-NH$_2$) and are capable of exchanging anionic contaminants like phosphates.[35, 36]

Ion exchange is a reversible process and hence the spent resin can be regenerated by passing a concentrated counter ion solution to it. For example, if a cation exchanger has exchanged all of its Na$^+$ ions with Ca$^{2+}$ ions in a water softening process, it can be regenerated by the addition of a sodium salts like sodium chloride (NaCl). This reconverts the ion exchanger back into its Na$^+$ form.[37]

This is an essential step during the ion exchange process as it enables the reuse of the ion exchange column that would help lower the cost of its operation, which makes it an economically viable
option. The recovered ions can then be used for other applications such as the production of fertilizers.

**Metal complexation and chitosan**

Chitosan’s ability to chelate transition metals can be attributed to the free amines and/or hydroxyl groups present on the polymer, which are forming coordinate bonds with the metals in aqueous solutions. Complexation of metals to the polymer induces conformational changes in the polymer. Studies performed by Terreux et al. (2006) demonstrated various conformations in which metal ions, specifically copper (Cu(II)), can be chelated by the functional groups present on chitosan (Fig. 2).[38] Crystallographic as well as mass spectrometry experiments to support this argument have been performed in the past by several researchers.[39, 40]
Figure 2: Terreux et al. (2006) had studied the various conformations in which chitosan monomers can bind one Cu (II) ion with their corresponding binding energies. For the B ligands, only amide sites were considered. The calculations were performed using DFT and ab-initio program to determine the approximate energies.
Modifications of chitosan for wastewater treatment

Extensive research on the modifications of chitosan which has garnered great interest from pharmaceutical companies due to its applications as a drug delivery medium because of the non-toxic nature of the compound as well as its bio-compatibility and bio-degradability.[41] Chemically-modified chitosan has also been used for a variety of environmental applications and can be potentially implemented as a tertiary method of treatment in processes like polymer-assisted ultrafiltration. Chitosan has been used in various applications such as the removal of dyes, polychlorinated biphenyls (PCBs), and also in flocculation/coagulation, desalination and filtration applications.[42-45] Chitosan possesses many reactive hydroxyl and primary amine groups, and hence most of the chemical modifications are made with respect to these functional groups of the biopolymer. It can modified by chemical substituents that have functional groups in a process known as “grafting”. Some of the substituents that are added to the polymer contain carboxylic acid, or thionyl groups onto the hydroxyls or the free amines. In addition to these modifications, crosslinking reagents such as glutaraldehyde, ethylene glycol diglycidyl ether (EGDE), and epichlorohydrin (ECH) are some modifications used to improve the chemical resistance of the polymer in acidic media. These modifications enhance the polymer’s metal removal properties and improves polymer stability in acidic environments. Some examples of modifications used for wastewater treatment are N-carboxymethyl chitosan which have been used in the removal of cobalt and copper from sodium fluoride and sodium chloride brine[46], Weltrowski et al. (1996) had demonstrated the application of N-benzyl sulphonated chitosan in metal ion removal from acidic medium.[47] Chitosan can be molded into various shapes such as beads or fibres by neutralizing the acidified chitosan in a caustic solution such as sodium hydroxide.[48] These modified forms of chitosan can potentially be used as materials for ion exchange due to their increased affinity for
transition metals, such as cadmium, copper, lead, iron, mercury and chromium over rare earth metals.[49, 50] This property of chitosan has proven to be useful in the capture of anionic pollutants such as phosphates, fluorides, sulfides, etc. Metal-chitosan composites are capable of acting as anionic exchangers due to the ability of the coordinated metal to form a coordinate bond with negatively charged ions.[51]

**Biological wastewater treatment**

The use of algae as a means of removing contaminants from wastewater is slowly gaining popularity due to its advantages over conventional methods such as the absorption of carbon dioxide and its potential applications for the biomass after the water treatment process.[52] Biological treatment can potentially be used as an alternative method for nutrient removal from wastewater due to their ability to assimilate inorganic phosphate and nitrogen into the microbe’s biomass. However, the problem that is currently being faced in these reactors is the efficient collection of the biomass, as conventional techniques such as centrifugation, are expensive. Therefore, research into growing algae on bio-polymeric supports are underway in order to improve the settling properties of the algae because of its effectiveness in the collection of the biomass hence reducing operational costs.

Cyanobacteria are prokaryotic autotrophs that are found in almost every habitat on this planet. They exist in various morphologies including unicellular and filamentous forms. The unicellular types exist as single cells, suspended or benthic, or aggregates, whereas filamentous types can be thin or thick, single trichrome or bundles either with or without a sheath.[53] All cyanobacteria are capable of oxygenic photosynthesis but some species are capable of switching to the typical bacterial anoxygenic photosynthesis using sulfide as an electron donor.[54] Under anoxic conditions and during dark conditions, cyanobacteria are also capable of carrying out
Certain cyanobacteria are even capable of fixing nitrogen due to the formation of heterocysts.[56] Certain species of cyanobacteria are capable of forming bio-films or microbial mats. These structures are formed due to the ability of these filamentous bacteria secreting extra polymeric substances (EPS). These secretions allow the microbes to adhere to various types of surfaces such as plastics like polystyrene, or natural polymers like wood, and cotton, as well as rocks. Cell surface hydrophobicity and presence of amyloid adhesins have been suggested to be crucial factors that are necessary for adhering the bacteria initially and allows for the subsequent biofilm development.[57] Mat-forming filamentous cyanobacterial species are useful in wastewater treatment applications due to their ability to immobilize themselves on to various kinds of materials.

Certain *Phormidium* cyanobacterial species are capable of adapting to low temperatures, this property can be useful for wastewater treatment in the colder months of North America where the efficiency of conventional bioreactors would require heat input in order to maintain optimal removal rates.[58] In addition to this, there is a potential use for the cyanobacterial culture as a source of biodiesel.[59]

**Sulfide as an environmental pollutant and current methods of treatment**

Hydrogen sulfide (H₂S) is colorless gas that is heavier than air and is the predominant contaminant of natural gas. Hydrogen sulfide poisoning is still an issue of widespread environmental and occupational exposure from various industrial activities such as farming, and paper-pulp mills. At lower concentrations, it has an offensive odor that smells similar to rotten eggs. At higher concentrations, the human body reacts to it by causing irritation of the eyes and respiratory tract to loss of smell, and olfactory paralysis. Exposure to concentrations higher than 500 parts per
million (ppm) leads to unconsciousness, to pulmonary edema and, if the concentration is high enough, neural and respiratory paralysis, which can often lead to death. The tissues that are most susceptible to hydrogen sulfide toxicity are those with exposed mucous membranes and those with high oxygen demands. H₂S spontaneously hydrolyzes into HS⁻ ions, with one-third remaining protonated at physiological pH (7.4). Gaseous H₂S is capable of crossing the lipid bilayer membrane and can inactivate a number of metal-containing enzymes.[60, 61] Sulfide (S²⁻) is the most reduced form of sulfur and has been known to have a high oxygen demand of 2 mol O₂ per mol S²⁻. This results in the depletion of oxygen where sour wastewater is discharged and therefore, is one of the major reasons for mass fish mortality in aquaculture systems.[62]

The most common methods used for the removal of sulfide involve direct air stripping, oxidation as well as chemical precipitation.[63] The Claus process is also another significant desulfurizing process in which hydrogen sulfide gas is recovered in the form of elemental sulfur. It is a multi-step process that has now become the standard way of attenuating hydrogen sulfide gas.[64] Some researchers have experimented on the simultaneous removal of sulfides and the generation of power in microbial fuel cells.[65]

Scope of the project

The work being presented in this thesis is focused on developing and field-testing of the effectiveness and feasibility of the biopolymer, chitosan, as a potential large scale filter to treat anionic pollutants like phosphates and nitrates in agricultural wastewater samples. In addition to that, chitosan was also tested for its efficiency in the removal of hydrogen sulfide from water.


**Materials and Methods**

**Reagents**

Chitosan flakes (DOD% ~65) (Dungeness environmental), red sand (Hutcheson Sand and Gravel, Bracebridge ON) chitosan powder (DOD% ~65) (Sigma Aldrich), sodium hydroxide (Sigma Aldrich), copper (II) sulfate (Sigma Aldrich), acetic acid, ammonium molybdate tetrahydrate (Sigma Aldrich), ascorbic acid (Sigma Aldrich), 5,5’-Dithio-bis-(2-Nitrobenzoic acid) (Ellman’s reagent) (Sigma Aldrich), sulfuric acid (EMD), malachite green carbinol hydrochloride (Sigma Aldrich), Poly(vinyl alcohol) (Sigma), *Phormidium tenue* (Canadian Phycological Culture Centre (CPCC)), BG-11 medium (CPCC), potassium phosphate dibasic (Fisher Scientific), Iron-complexed chitosan flakes (Chemfil Canada Ltd.)

**Equipment**

Spectramax 384 Plus Absorbance plate reader (Molecular devices); SpectrAA 55B (Varian/Agilent technologies); Syringe pumps (New era syringe pumps Inc), inline pumps (Eheim), peristaltic pumps (Ismatec), Tygon clear rubber tubing (Fisher Scientific); Scout pro balance (Ohaus); AJ100 analytical balance (Mettler Toledo).

**Methods**

The methods that were employed for the detection of the various anions are standard approaches to the spectrophotometric detection method of the anionic pollutants studied in this document, namely phosphates, nitrates, nitrites, and hydrogen sulfide. Molecular dynamics simulations were performed by Sameer Jafaar, under the supervision of Dr. Gauld.

**Malachite green phosphate assay:**

Reagent 1: 1 mM Malachite green carbinol hydrochloride, 1% Polyvinyl alcohol, 0.6M H2SO4;
Reagent 2: 50 mM ammonium molybdate tetrahydrate, 3 M H$_2$SO$_4$

All reagents are prepared with distilled water (dH$_2$O). Solutions are stable at room temperature for ~2 months.

130 µL of reagent 1 was added to 0.7 mL of sample and after 2 minutes 70 µL of reagent 2 was added. The resulting solution is mixed and then added to a 96 well plate. The absorbance of the samples were measured at 630 nm in a UV-Vis spectrophotometer.

**Ascorbic acid-molybdate phosphate quantification assay:**

Stock solutions:

Reagent 1: 2.5% (w/v) ammonium molybdate tetrahydrate solution (stored at 4°C)

Reagent 2: 10% (w/v) ascorbic acid solution (stored at 4°C)

Reagent 3: 3 M H$_2$SO$_4$ (stable at room temp.)

1mM K$_2$HPO$_4$ in dH$_2$O (stable at room temp.)

The stock solutions are added in the following ratio to make working reagent:

2 parts of dH$_2$O; 1 part Reagent 3; 1 part Reagent 2; 1 Part Reagent 1

Working reagent was prepared fresh before the assay is performed. Standards from 10 µM to 150 µM were prepared in advance and stored at room temperature. Working reagent was added in a 1:1 ratio with phosphate samples. Absorbance of the samples were measured at 820 nm in a UV-Vis spectrophotometer.

**Sulfide quantification using Ellman’s reagent:**

Working reagent: 1mM Ellman’s reagent (5,5’-Dithio-bis-(2-Nitrobenzoic acid)) solution prepared in 0.1M Phosphate buffer pH=8.
Working reagent was prepared fresh on the day of the assay. The working reagent was added in a 1:1 ratio with the sample and the absorbance of the samples were measured at 412 nm in a UV-Vis spectrophotometer.

**Nitrite and Nitrate quantification using modified griess reagent:**

Stock solutions:

Stock 1: 2% sulfanilimide in 5% HCl (store in the dark at 4°C)

Stock 2: 0.1% N-(1-Naphthyl) ethylenediamine in dH₂O (store in the dark at 4°C)

Stock 3: 0.051 M Vanadium chloride (VCl₃) in 1 M HCl

1mM sodium nitrate solution (store in dark at -20°C)

To 100 µL of sample, 100 µL of stock 3 was added after which 50 µL of stock 1 and stock 2 were subsequently added. The resulting solution was incubated at 37°C for 30 minutes after which the absorbance was measured at 540 nm in a UV-Vis spectrophotometer.

**Molecular Dynamics (MD) Simulations:**

Four different chitosan-copper complexes were optimized. Two with copper having 4 coordination sites, and another two with copper having 6 coordination sites. Within each couple of 4/6 coordination complexes, one was bound to 1 chitosan monomer and four waters, whereas the other was bound to 2 chitosan monomers and two waters. Based on the M06/6-31+g(d,p) level of theory, each model had a multiplicity of two and a formal charge of zero. The most favourable structure was Cu62C.
I. Chitosan bead production and determination of its metal binding capacity

1. Synthesis of chitosan micro-particles

Chitosan beads have been prepared using various methods such as ionotropic gelation/crosslinking, extrusion-spheronization, spray drying, and precipitation/coacervation. [66] Chitosan powder was solubilized in solutions prepared from acetic acid. This produced chitosan acetate, a golden viscous liquid that was then squeezed through a syringe that added this solution drop-wise into a 1 M sodium hydroxide (NaOH) solution. The sodium hydroxide solution was gently stirred at 150 – 250 revolutions per minute (rpm) in order to prevent aggregation between the unreacted droplets in the solution. This process precipitated chitosan and formed white beads.

In order to produce a large amount of these beads for our experiments, chitosan acetate was pumped using a syringe pump at a rate of 0.1 mL/min with the needles at a height of 3 – 4 cm from the surface of the 1 M NaOH solution in order to produce spherically shaped beads as shown in Fig. 3. The beads were 3.11 ± 0.44 mm in diameter.

Beads that formed were washed with water in order to remove any excess NaOH. Once rinsed, the beads were crosslinked in an aqueous solution of 5% ethylene glycol diglycidyl ether (EGDE) for 3 hours. [67, 68]
Figure 3: The chitosan beads were produced using syringe pumps. In the image shown above, 2% chitosan-acetate solution was neutralized in a 1 M sodium hydroxide solution, where chitosan regenerated itself in the form of beads.

Metal sorption kinetics of the chitosan beads were to be determined. The metal binding capacity of the chitosan beads were determined indirectly. Dried crosslinked beads (1 g) were added to 50 mL of metal ion solutions (100 ppm). The solutions were gently mixed using a nutator and were sampled at various time intervals. In order to determine the metal ion removal rate, samples were taken every 15 minutes and were analyzed using an atomic absorption spectrometer (AAS). The metal ions tested were copper (Cu²⁺), iron (Fe³⁺), manganese (Mn²⁺), and zinc (Zn²⁺) as shown in Fig. 4 (A – D).
Figure 4 (A – D): Metal ion concentration remaining in solution over different periods of exposure to dried chitosan beads (1 g). The beads were then incubated in 50 mL of 100 ppm metal solutions for 4 hours.

The results for A (Iron), B (Copper) and C (Manganese) indicated that there was rapid removal of ~90% of metal ions present in the wastewater within an hour of incubation as indicated in Fig. 4 (A-D). The initial readings (t = 0 min.) shown in Fig. 4 (A-D) can be a combination of reasons. One possibility can be attributed to the precipitation of the metals upon addition to the chitosan beads. This may be due to residual sodium hydroxide that was still present on the surface and was not washed off the beads. Another possibility is that chitosan chelates the metal ions too rapidly to be determined via the methods that were currently being employed.
2. Determination of metal sorption capacity of chitosan

As described earlier, chitosan can theoretically act as a tetradentate ligand due to the presence of reactive amine and hydroxyl groups, since it is capable of forming coordinate bonds with transition metals. The current application of chitosan requires the determination of the maximum sorption capacity of the metal ion on the polymer. The metal binding capacity for copper and iron were determined for the various derivatives of chitosan that were going to be used in future applications and results are presented in Table 1.

The sorption capacity was determined by using indirect method of determination, i.e, metal-saturated polymers were treated with competing chelating agents or a direct method where the polymer was treated with acidic solutions, which would depolymerize the polymer. In both the methods, the resulting solution’s metal concentration is determined using AAS.

The various forms of chitosan were incubated in 20 mL of 0.1 M copper sulfate or iron sulfate for 4 hours. The metal-saturated polymers were then treated with 20 mL of 0.1 M EDTA (pH 7.4) and 20 mL of 1 M acid solution in separate experiments. The choice of acid was dependent on the sample preparation methods that were specific each metal ion, which in this case was hydrochloric acid (HCl) for iron samples and nitric acid (HNO₃) for copper samples. The amounts of the desorbed metal ions for each desorption experiment are presented in Table 1.

Ethylenediamine tetraacetic acid (EDTA) is known to act as a weak acid, and at pH 7 all carboxylic groups and one amine group are deprotonated. At this pH, EDTA can act as a hexadentate ligand and is capable of forming dative bonds with cupric (Cu²⁺) ions and competes with the biopolymer’s amines and hydroxyl groups. The experiments showed close correlation between the amounts of metal ions desorbed by acid as well as EDTA for cupric ions from EGDE crosslinked chitosan as well as chitosan powder for cupric ions however, there were some discrepancies in the
values for the flakes and beads as shown in Table 1. Ferric (Fe$^{3+}$) ions desorbed in the same way had shown inconsistencies in the amount of iron desorbed for all the forms of chitosan that were used. The most probable reason for the discrepancy in the values may be due to chitosan being able to form more favorable chelation complexes with cupric ions in the flake and bead forms and whereas with ferric ions it had far more stable complexes than EDTA in all its forms. [69-72]

An MD simulation was performed in order to obtain a thermodynamically stable model of the chelated metal ion’s position that has been held in place by chitosan monomers as shown in Figure 5. The metal ion formed 6 coordinate bonds and their average distances between the different functional groups and the metal ion are also shown in Fig. 5. The relative energy of the diagram shown in Figure 4 was -85.17 kJ/mol. The results here demonstrates that chitosan was capable of forming fairly stable coordinate bonds with a divalent cation and relates very closely to the experimental characterization of the metal-ligand interactions in chitosan performed by Rhazi, et al. (2002).72
Table 1: Maximum sorption capacity of chitosan for each of the ions were determined by incubating the polymer (1 g dry weight) in a solution of the desired metal ion (20 mL). Different forms of the polymer were tested due to difference in surface area as well as the availability of reactive amines and hydroxyl groups. The metal-saturated polymers were treated with acid solutions (1 M, 20 mL) ((HNO₃) or Hydrochloric acid (HCl) depending on the metal being analyzed). A separate desorption experiment was performed with the help of EDTA (0.1 M, 20 mL). In both experiments, the solutions were analyzed for metal content via AAS.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Form of native chitosan used</th>
<th>Mⁿ⁺ desorbed by acids (mgMⁿ⁺/gChitosan)</th>
<th>Mⁿ⁺ desorbed by EDTA (mgMⁿ⁺/gChitosan)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Copper</strong> (λ=244.2nm)</td>
<td>Flakes</td>
<td>121 ± 3</td>
<td>46.3 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>Beads</td>
<td>71.1 ± 0.5</td>
<td>22.8 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>EGDE Crosslinked-beads</td>
<td>30.7 ± 0.3</td>
<td>28.6 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Powder</td>
<td>48.9 ± 1.0</td>
<td>45.5 ± 1.6</td>
</tr>
<tr>
<td><strong>Iron</strong> (λ=372.0nm)</td>
<td>Flakes</td>
<td>45.9 ± 1.9</td>
<td>4.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Beads</td>
<td>67.0 ± 2.2</td>
<td>25.4 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>EGDE Crosslinked-beads</td>
<td>79.6 ± 3.2</td>
<td>48.3 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Powder</td>
<td>63.6 ± 3.6</td>
<td>40.4 ± 0.6</td>
</tr>
</tbody>
</table>
Figure 5: Representative structure taken from Molecular Dynamics simulation (MD) showing the chelation of copper (II) to chitosan monomers (de-acetylated). Blue labeled moieties depict free amines of the polymer while red moieties represent hydroxyl groups. The average distance of the bond between the amine and metal is ~1.99 Å, between hydroxyl groups and metal ~2.17 Å, and finally, between water molecules and metal ~2.31 Å. Simulations of complexation of copper by one and two chitosan monomers were performed by Sameer Jafar of Dr. Gauld’s group.

3. Determination of phosphate binding capacity of chitosan and its derivatives

Metal-complexed chitosan has been shown to be capable of sorbing phosphate ions onto the polymer. Several researchers have suggested that the metal complexed chitosan can electrostatically interact with ortho-phosphate ions (Fig. 6). The sorption capacities of native chitosan, copper- and iron-complexed chitosan are indicated in Table 3. Based on the results shown in Table 3, copper-complexed chitosan flakes were determined to have the highest phosphate sorption capacity ~40 mg PO$_4^{3-}$ and would be ideal for phosphate-laden wastewater treatment applications. The low phosphate binding capacity in the EGDE crosslinked-chitosan beads could be due to the absence of sorption sites for the phosphate molecules.
Table 3: Metal-complexed (Copper and Iron) and native chitosan were incubated in 20 mL of 0.1 M Phosphate buffer (pH 7). The beads are washed with distilled water and then treated with 20 mL of 1 M sodium chloride solution to elute phosphates from the chitosan derivatives.

<table>
<thead>
<tr>
<th>Type</th>
<th>Form of chitosan</th>
<th>[Soluble phosphates] recovered (mM)</th>
<th>Sorption capacity (mgPhosphates/g Chitosan)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native chitosan</td>
<td>Flakes</td>
<td>5.59 ± 0.57</td>
<td>10.83 ± 1.07</td>
</tr>
<tr>
<td></td>
<td>EGDE-Crosslinked beads</td>
<td>1.9 ± 0.47</td>
<td>4.88 ± 0.97</td>
</tr>
<tr>
<td>Iron chitosan</td>
<td>Flakes</td>
<td>8.79 ± 0.23</td>
<td>17.04 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>EGDE-Crosslinked beads</td>
<td>0.31 ± 0.13</td>
<td>6.3 ± 0.77</td>
</tr>
<tr>
<td>Copper chitosan</td>
<td>Flakes</td>
<td>20.49 ± 0.46</td>
<td>39.74 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>EGDE-Crosslinked beads</td>
<td>3.02 ± 0.45</td>
<td>8.12 ± 0.98</td>
</tr>
</tbody>
</table>

Figure 6: The proposed model shown above describes how chitosan complexed with cupric ions are capable of electrostatically interacting with the orthophosphate ions in an aqueous solution. Crosslinking the polymer reduces the number of sites at which the cupric ion can be coordinated and thus reduces the phosphate sorption capacity.[73]
II. Field testing of chitosan as a potential bio-filter for phosphate wastewater treatment

With the theoretical phosphate binding capacity of metal-chitosan flakes determined, the biopolymer was now ready for scaling up from pilot studies. The sites were chosen by Darryl Finnigan, OMAFRA (Ontario Ministry of Agriculture, Food and Rural Affairs) and Craig Merkley, UTRCA (Upper Thames River Conservation Authority) based on the urgency for phosphate removal from certain point sources.

The sites that will be talked about in this study are:

- Phillip Gardens – a vegetable farm based in Ansnorveldt, Newmarket, ON (near Holland Marsh)
- Luckharts – a truckwash facility based in Sebringville, ON
- Big Bay Point Road – a site adjacent to nearby corn fields, based in Innisfil, ON

Site sampling methods:
The influent was sampled in different ways at each of the sites due to the difference in timing of the wash processes. At Luckhart’s truckwash station, the transport trucks were washed in order to remove any animal waste from the trucks and prepare it for future operations. The water is an initial sample was taken from the wastewater source before every filtration process that was performed and the value at t = 0 hours represented in the data presented in Fig. 10, 11, 15, and 16 was the average concentration of the soluble phosphates that was being introduced into both types of the filtration system (Prototype 1 and 2). A similar approach was used for sampling at Phillip Gardens where the facility’s wash cycles were seasonal and depended on the timing of crop
harvest. Big Bay Point Road also had similar issues, was dependent on how often water was applied to the corn fields.

1. Application of chitosan packed column for phosphate removal

This filter unit was designed based on the initial total suspended solids (TSS) and phosphate concentration of the settling pond. It had one column dedicated to prevent suspended solids from entering the subsequent columns that housed the chitosan filter. A schematic diagram of the filter unit is shown in Fig. 7. The wastewater was introduced into the system with the help of a pump with a flowrate of 5 gallons/min (or 22.7 L/min). Leaching of metals was predicted to be a concern during the operation of the filter unit and hence, iron-complexed chitosan flakes were used instead of copper-complexed flakes. The entire unit was powered by car batteries and were recharged with the help of a solar panel.

![Figure 7: Components and mechanics of chitosan filter unit- prototype 1. Pump is powered by a 12 V battery, unit A was fitted with a replaceable cotton filter, units B and C were filled with metal-complexed chitosan. The unit was designed with the help of Chemfil Canada Ltd. and Ace Manufacturing Inc.](image-url)
Figure 8: Prototype 1 filter unit setup used in initial field tests at Phillip Gardens vegetable wash station (i) and Luckhart’s animal wash station (ii). The units were both equipped with a pre-filter unit (A) that houses a cotton filter and 2 columns (B&C) for the filtering material (~1 kg). The unit is powered by a 12 V battery and is recharged with the help of a solar panel. Sampling was carried out using an autosampler (ISCO model#: 3700). The influent wastewater was pumped into the unit using a motorized pump.

1.1. Luckharts truck wash station

Luckharts trucks are designated to transport pigs to various locations. The animal’s excreted waste was washed away at this facility and were collected at an on-site primary (1°) settling pond (Fig. 9). This was then distributed into the on-site secondary (2°) settling ponds situated nearby (Fig. 9). The wastewater was suspected to contain high concentrations of BOD (organic matter), and to be rich in N, P and S contaminants.

The filter unit prototype employed for the removal of phosphates at this site is shown in Figure 8(ii). The initial tests were performed with copper-complexed chitosan in the filter columns.
Figure 9: Luckharts truck wash station. Left: 1° settling pond for truck wash effluents; Right: 2° settling pond situated downstream of 1° settling pond and site for influent source of wastewater for chitosan filter unit.

The wastewater from the 2° settling pond was introduced to the prototype filter unit. The results from the initial tests with the filter unit revealed promising results as it was capable of reducing soluble phosphates (SP) from ~50 μg/mL by ~2-fold.

The results shown in Fig. 10 indicated that the usage of copper-complexed chitosan was effective in the removal of phosphates from the influent wastewater. However, the filter reaches its sorption limit within 48 hours into the filtration process as the phosphate concentrations in the effluent water were having similar concentrations as that of the influent wastewater. Furthermore, AA analysis was performed in order to check for metal leaching from the chitosan filter, and it was found that 5.22 ± 0.21 ppm (mg/L) (See Appendix 1) of copper was leaching out of the filter unit.

In light of this data, it was decided to utilize iron-complexed chitosan instead of its copper counterpart in order to avoid leaching of copper ions into the treated effluent. The leaching of copper would lead to the breaching of provincial water quality objectives (PWQO) for this contaminant (5 μg/L) and is also an environmental concern due to its toxicity to aquatic life. The effect of copper on fishes for example, if the concentration is >5μg/L, it has been found to impaired
reproduction, and hence disrupts migration. It also alters the blood chemistry due to the increased metallothionein synthesis in hepatocytes, and impairs respiration.[74]

The chitosan filter unit was very effective at removing TSS and soluble phosphates from the influent wastewater. In both the iron- and copper-complexed chitosan filters, removal of ~99% of TSS from the influent was observed, but, at the cost of the filter unit (Fig. 10, 11). Similar phosphate removal capacity was observed with its sorption limit ~24 hours of treatment. This suggests that there may be “membrane fouling” due to the collection of sediments as well as microbes on the metal-complexed chitosan flakes ultimately leading to the degradation of the filter. The accumulation of TSS and degradation of the filter lead to the plugging of the filter unit within 48 hours of operation.
**Figure 10:** Post-treatment phosphate amounts measured from the effluent stream of prototype 1. The initial reading taken at time $t = 0$ hours was sampled directly from the settling pond. These readings were taken during the sampling window from July 9th to July 16th 2014. The filter unit was filled with 500 g of copper-complexed chitosan. The samples were collected every 8 hours and the samples (50 mL) were analyzed spectrophotometrically using ascorbic acid-molybdate phosphate method. TSS values were also analyzed from the same samples and were shown to have drastically decreased in the effluent stream. The error bars represent standard error ($n = 3$).

**Figure 11:** Post-treatment phosphate amounts measured from effluent stream of prototype 1 were monitored during the sampling window October 14th to 27th 2014. The filter unit was filled with 500 g of iron-complexed chitosan. The samples were collected every 8 hours and the samples (50 mL) were analyzed spectrophotometrically using ascorbic acid-molybdate phosphate method. TSS values were also analyzed from the same samples and were shown to have drastically decreased in the effluent stream. The error bars represent standard error ($n = 3$).
The state of the metal-complexed flakes after treating the truck wash effluent for 1 week, resulted in discoloration of the filter (Fig. 12) suggesting the deposition of sediments from the settling pond. However, upon washing the filter with distilled water, the color of the flakes did not change. This suggests that it is highly likely that the filter had also adsorbed dissolved hydrosulfides from the influent wastewater. The inference was made based on the qualitative analysis of the wastewater, i.e., the malodorous smell at the site and the discoloration that was characteristic of iron/copper sulfide.
1.1.1. Design and implementation of new filter unit

In order to improve the flow of water into the filtration system. The schematics of the new system as shown in Fig. 13, the cotton filter has been replaced with red sand as the pre-filter. It utilizes gravity-fed passive filtration to separate the sediments from the initial source of wastewater and then, the pre-filtered water is collected in a well and is ready to be pumped into the filter unit. The new filter unit setup, as shown in Fig. 12, consisted of a 55 gallon water storage barrel that housed the iron-chitosan (Fe-Ch) flake filter with the influent wastewater which was still pumped into the system from the top of the barrel. The wastewater was pumped using a sump pump with the flow-rate of ~3.5 L/min and a detention time for ~10 minutes. In addition to this, a motor with a paint mixer was installed in order to ensure proper mixing of the filter with the wastewater. The water level is maintained with the help of solenoid valves. The treated effluent was allowed to exit from the bottom of the barrel. A small mesh filter was also installed at the outlet in order to keep the filter from escaping the unit.

![Prototype 1](image)

**Figure 12:** Current chitosan filter design (Prototype 2) being used for the treatment of phosphates.
Figure 13: Schematics for the new chitosan filter unit, prototype 2’s setup are similar to that of a continuous stirred tank reactor (CSTR). It comes equipped with (A) Motor attached to a 3 ft. paint mixer 8.5 cm wide. The water source was passively passed through (B) red sand. A well was manually dug out in order to make a crude well and with the help of a pump, the water was drawn into the reactor. The water level was adjusted with the help of solenoid water level sensors (C). The solenoid sensors are connected to portable central control panel which controls the activation of the submersible pump. The filter unit was a 55 gallon water storage barrel that had a 0.5 mm mesh filter (D) that would keep the iron chitosan from leaving the unit. Finally, the whole unit is powered by a 12 V battery power source. The battery was recharged using a solar panel (E).

Figure 14: From left to right: Wastewater sample from Luckharts truck wash station’s 2° settling pond (Pre-red sand), after passing through red sand (Post-red sand) and the chitosan treated effluent (Post-chitosan filter). Suspended solids in the wastewater is greatly reduced after it passes through red sand.
During the sampling periods May – July 2015, the influent wastewater was passed through the red sand filter, and the resulting effluent’s phosphate levels are represented with “black bars” in Fig. 15 and 16. This effluent is then introduced into the unit and the “patched bars” indicate the phosphate levels present in the post-chitosan effluent stream. Red sand attenuated SP by about 6 – 30 fold (~500 µg/L) from the settling pond prior to the introduction into the chitosan filtration unit. The chitosan filtration unit was able to lower further to ~200 µg/L. Under these conditions, the iron-chitosan composite did not degrade for up to 2 months of continuous operation.

In conclusion, a hybrid SP filtration system composed of a red sand pre-filter and a Fe-Ch flakes can effectively yield SP-levels of ~0.5 µg/mL from inflows from point sources containing ~10-20 fold larger SP and high levels of suspended solids.
Figure 15: Samples (50 mL) were taken from the effluent pond every 8 hours during the sampling period of May – June 2015. The black bars indicate the influent wastewater/ post-red sand effluent that was being introduced to the filter unit containing iron-complexed chitosan (3 kg). The “patched” data bars are the samples (50 mL) that were taken from the effluent stream. Samples were analyzed for phosphate content using ascorbic acid-molybdate phosphate method. Fresh wastewater was input at time $t = 0$, 48 hours.

Figure 16: Samples (50 mL) were taken from the settling pond and was compared to the post-red sand effluent. The “patched” samples data bars show the amount of phosphates present in the effluent stream after it was treated with iron complexed chitosan. The sampling was performed during the period of June 2015 - July 2015. Sample were analyzed using ascorbic acid-molybdate phosphate method. The error bars represent standard error ($n = 3$).
1.2. Application of chitosan at Phillip Gardens vegetable wash station

The vegetable wash station owned by Phillip Gardens wash harvested produce seasonally and follow an irregular schedule. However, the operation demands a large amount of water (>6000L/wash cycle) and the source of phosphate contamination arises from the organic matter washed away from the produce (Fig. 17). The untreated wash effluents generated was directed into a stream nearby. Due to the high concentration of phosphates and nitrates in these effluents, there were signs of eutrophication present in the slow moving stream. Initially, a wood chip filter was constructed by a different group of researchers for the attenuation of nitrates at this site. In order to decrease the amount of phosphates in their effluents, our filter unit was installed in sequence with the wood chip setup.

![Figure 17: Pictures from inside the vegetable wash station. The size of the operation is fairly large and uses upward of 6000 gallons of water per day. The influent wastewater from this source contained soil and other resinous residues resulting from the wash process.](image)

The iron-complexed chitosan (Fe-Ch.) flake composite bio-filter was first used at the vegetable wash station in October 2014 to November 2014 (Figure 18) and once again during the period 04/2015-05/2015 (Figure 19). The Fe-Ch. flakes were effective in lowering phosphate content in carrot wash effluents ranging from ~14 μg/mL to ~2 μg/mL by ~7-fold. Due to the high
concentration of suspended solids that resulted from the vegetable washes, the influent stream to the bio-filter had experienced reduced flow rate. In addition to this, the Fe-Ch flakes were exposed to this influent water and had degraded due to membrane fouling due to the accumulation of suspended solids from the carrot wash. The degradation of chitosan had resulted in the release of phosphates into the treated effluent stream, after 3 vegetable wash cycles (Fig. 19). In subsequent trials, the wastewater was passed through red sand and a reduction in the vegetable wash SP levels was noticed. It had reduced from ~19 µg/mL to ~1 to 2 µg/mL as shown in Fig. 20, and Fig. 21. The chitosan bio-filter connected in tandem, further lowered PS levels to ~ 0.5 µg/mL, again with no significant degradation of the chitosan matrix. However, regeneration studies were not performed due to the degradation of the filter after the filtration process had completed.

In conclusion, a hybrid filtration system composed of a red sand pre-filter and iron-complexed chitosan flakes can effectively yield SP concentrations of ~ 0.5 µg/mL from inflows from point sources containing ~10-20 fold larger SP and high levels of suspended solids. The hybrid biofiltration system as tested can treat the waste water at flow rates of ~3.5 L/min. After each sampling cycle, the red sand was washed with ammonium acetate and sodium chloride in order to regenerate it. Desorption experiments were performed on red sand samples. In one experiment it was done by washing the barrel of sand (~75 kg) with 5 L of 0.1 M ammonium acetate and in a separate experiment, a sample of the red sand (10 g) was washed with 50 mL of 1 M sodium chloride. The soluble phosphates that were recovered was 3.43 ± 0.21 µg/mL and 4.11 ± 0.71 µg/mL for ammonium acetate and sodium chloride washes, respectively. This indicates that the phosphates may have precipitated with the iron present in the sand.
Figure 18: Prototype 1 filter unit pilot test with Iron-chitosan as the filter material at Phillip Gardens vegetable wash station. The filter unit design being used in this test is shown in figure 6(A). Sampling window October 11th 2014 to November 4th 2014. Solid black data points indicates fresh input of vegetable wash wastewater. These samples (50 mL) were collected during the wash cycles on the stated dates and patched data points indicate samples (50 mL) that were collected from the post-chitosan effluent. The phosphate amounts were determined using ascorbic acid-molybdate phosphate method. The error bars represent standard error (n = 3).

Figure 19: Phosphate attenuation results for the sampling window of April 2015 – May 2015. The solid black data points indicate fresh input of wastewater. These samples (50 mL) were collected at the beginning of vegetable wash cycles every day and “patched” data points indicate samples (50 mL) taken from effluent stream. The data was acquired during the sampling window July 29 to August 7, 2015. Phosphates are being released back into the effluent stream due to degradation of the filter at t = 152 hours. The error bars represent standard error (n = 3).
Figure 20: (A) Comparison of phosphate levels in pre-red sand influent samples (50 mL) with post-red sand and post-chitosan effluent samples (50 mL) acquired at Phillip Gardens using prototype 2 hybrid filtration system during the sampling period of February 2016 – March 2016. (B) Magnified view of the phosphate levels in post-red sand and post-chitosan effluent samples (50 mL). All samples were analyzed spectrophotometrically with the help of malachite green phosphate assay.\[76, 77\] The error bars represent standard error (n = 3).
Figure 21: (A) Comparison of phosphate amounts in pre-red sand influent samples (50 mL) with post-red sand and post-chitosan effluent samples (50 mL) acquired using prototype 2 filtration system during the sampling period of March – April 2016. (B) Magnified view of the amount of phosphates present in post-red sand and post-chitosan effluent samples (50 mL). Samples were analyzed using malachite green phosphate assay. The error bars represent standard error (n = 3).
2. Application of filter unit at Big Bay Point Road

In the initial samples that were analyzed from Big Bay Point Road site, showed that the soluble phosphates were in the range of ~300 – 650 µg/L as well as negligible TSS content which was relatively low compared to the previous sites.

The same filter unit shown in Fig. 8(i) was used for phosphate attenuation at the Big Bay Point Road site. The set up at Big Bay Point is shown in Fig. 22, and had the influent wastewater pumped into the cotton filter in the smaller filter (left) followed by treatment by 3 kg of iron-chitosan in the larger filter (right). As shown in Fig. 23, during the sampling window (November – December 2015) the soluble phosphates and TSS levels were low ranging between ~300 to 640 µg/L and ~13 to 29 µg, respectively. Owing to low suspended solids, the chitosan-iron flakes were able to lower soluble phosphate levels to ~180 to ~350 µg/L, with no apparent degradation of the bio-filtration matrix.
Figure 22: Big Bay Point Road site filtration (prototype 1) unit setup.

Figure 23: Phosphate levels of pre- and post-filter samples (250 mL) taken at Big Bay Point Road during the sampling period of October 29 – December 7, 2015. Samples were analyzed using malachite green phosphate assay. The error bars represent standard error (n = 3).
III. Hydrogen sulfide abatement using metal-complexed chitosan

1. Filter synthesis and reactor setup

Iron chitosan powder was prepared by incubating chitosan in 20 mL of 0.1 M iron sulfate (FeSO$_4$.7H$_2$O) solution for 4 hours. The iron chitosan powder was air dried at room temperature.

For all sulfide removal experiments, sodium hydrosulfide (NaHS) prepared in 0.1 M phosphate buffer at pH 7.4 was used. The reactions were carried out in a 22 mL vial with a PTFE/Silicone septa in order to emulate a closed system and prevent loss of volatile gases from the system (Fig. 24). Samples were taken using a syringe every 5 minutes until the sulfide concentration were no longer changing. Upon completion of one experiment, the buffer volume was regenerated and a fresh injection of NaHS was added to the reactor.

![Figure 24: Filtration unit setup. (Left) Iron-complexed chitosan powder reacts almost instantaneously with hydrogen sulfide upon its injection into the system. The filter turned black (Middle) once the filter was saturated. Upon saturation, the excess hydrosulfides in solution start reacting with the iron sulfide precipitate and the suspension slowly changes color to a wheat-like color.](image)
2. Application of metal complexed chitosan in the removal of hydrosulfide

Chitosan is capable of adsorbing hydrosulfide ions onto its surface, however the rate of sorption is not quick enough for wastewater treatment applications (Fig. 25). There is an observable change in color on the surface of the polymer upon the sorption of hydrosulfides. Chitosan-metal complexes instantly reacts with sulfides to form metal sulfide precipitates. The precipitation of sulfide is suspected to be in the form of $M_xS_y$ where $x = 1 – 7$, $y = 1 – 8$. This can be noted from the formation of black colored precipitates in the reactor upon addition of hydrogen sulfide.

![Figure 25](image1.png)

*Figure 25: Native chitosan by itself has poor sulfide removal capabilities. At time $t = 1$, 31 minutes, indicated by blue arrows, a new injection of 200 µL of 0.1 M NaHS into 19.8 mL of phosphate buffer. This was added in order to bring the solutions concentration back up to 1 mM NaHS. There is noticeable change in color as chitosan sorbs more HS$^-$ ions. The error bars represent standard error ($n = 3$).*

Iron chitosan was very effective in reducing the concentration of dissolved hydrosulfides (20 mL) from 1 mM (20 µmol) to ~0.01 mM (~0.2 µmol), a 100-fold decrease as depicted in Fig. 26. The addition of sulfides into the solution could have desorbed some of the iron due to the formation of iron-sulfide precipitate along-side the sorption of hydrosulfide ions onto the iron-chitosan filter. The iron sulfide formation is very rapid forming a black precipitate which remains in the supernatant of the reaction mixture for some time. After some time, it undergoes a change in chemical composition and the black precipitate starts dissipating and changes to a pale yellow hue,
suggesting the formation of a different metal-sulfide complex. The formation of elemental sulfur was also suspected due to the deposition of yellowish substance staining the sides of the glassware. The most likely reaction that may have occurred is represented by the mechanism represented below [78-81]:

\[
\text{NaHS(s) + H}_2\text{O (l)} \rightleftharpoons \text{H}_2\text{S (aq) + Na}^+ + \text{OH}^- \quad \text{--- (1)}
\]

\[
\text{H}_2\text{S (aq)} \rightleftharpoons \text{HS}^- + \text{H}^+ \quad \text{--- (2)}
\]

\[
\text{H}_2\text{S (aq) + Fe}^{3+} (\text{OH}^-) \text{Chelant}^n \rightleftharpoons \text{Fe}^{3+}(\text{SH})\text{Chelant}^n + \text{H}_2\text{O} \quad \text{--- (3)}
\]

Followed by the formation of a sulfido bridged dimer complex

\[
\text{Fe}^{3+}(\text{SH})\text{Chelant}^n + \text{Fe}^{3+}(\text{OH})\text{Chelant}^n \rightleftharpoons (\text{Fe}^{3+}\text{Chelant}^n)_2 \text{S}^2^- + \text{H}_2\text{O} \quad \text{--- (4)}
\]

This leads to the precipitation of S\(^0\) in the aqueous system

\[
(\text{Fe}^{3+}\text{Chelant}^n)_2\text{S}^2^- \rightarrow 2\text{Fe}^{2+}\text{Chelant}^n + \text{S} \downarrow \quad \text{--- (5)}
\]

The excess hydrosulfide starts reacting with the iron sulfide suspension

\[
\text{FeS} + \text{H}_2\text{S (aq)} \rightarrow \text{FeS}_2 + \text{H}_2 (\text{g}) \quad \text{--- (6)}
\]

Regeneration of Iron (III) chitosan from Iron (II)

\[
\text{O}_2(\text{g}) + \text{H}_2\text{O (l)} \rightleftharpoons \text{O}_2(\text{aq}) \quad \text{--- (7)}
\]

\[
\text{O}_2 (\text{aq}) + 4\text{Fe}^{2+}\text{-Chelant}^n + 2\text{H}_2\text{O} \rightleftharpoons 4\text{Fe}^{3+}\text{-Chelant}^n + 4\text{OH}^- \quad \text{--- (8)}
\]
Figure 26: (A) Iron-complexed chitosan powder was effective at adsorbing sulfide ions from solution. The remaining dissolved sulfides in solution are a result of unreacted sulfides and a mixture of unreacted sulfides and iron sulfide at higher concentrations as the experiment proceeded. The volume was adjusted for the sampled volumes and a fresh volume (200 µL) of 0.1 M NaHS was injected into the closed vial reactor containing 19.8 mL of phosphate buffer in order to introduce 1 mM of NaHS in the solution. This was repeated several times over as shown by the blue arrows in (A). (B) The same approach was used with higher concentrations of hydrogen sulfide yielded similar results to (A). All samples were analyzed spectrophotometrically using Ellman’s reagent.[82] The error bars represent standard error (n = 3).

After the filtration process, a layer of pale-yellowish precipitate was found on the surface of the filter, which was analyzed using X-ray powder diffraction (Fig. 28-A). The identity of the compound was confirmed after the results indicated that the products matched the crystal patterns of Arcanite (K$_2$SO$_4$) as well as iron (Fe). [83-86]
**Figure 28**: X-ray powder diffraction pattern of the material developing as a “floating” surface layer on top of the iron-complexed chitosan powder. The experiment was performed at 40 kV-40 mA, and the selected (CuKα) X-ray wavelength was 1.541 Å. The blue arrows indicate the peaks that corresponded to the theoretical peak of arcanite with an figure of merit (FOM) of 0.87 and the red arrow is for the theoretical peak that matched iron with an FOM of 0.99.

The production of sulfates in the system can be attributed to the presence of oxidants like dissolved oxygen and Chitosan-Fe(III) present in the reaction vessel. This can give rise to the formation of a number of sulfoxyl intermediates such as polythionates \( S_nO_6^{2-} \) where \( n = 4, 5, 6 \), thiosulfates \( S_2O_3^{2-} \), bisulfites \( HSO_3^- \) and sulfites \( SO_3^{2-} \). The mixture of sulfoxyl intermediates are in equilibrium and can be expressed by the Wackenroder reaction represented in equation 9.[87]
\[ S_{n}O_{6}^{2-} + S_{2}O_{3}^{2-} \rightleftharpoons S_{n+1}O_{6}^{2-} + SO_{3}^{2-} \quad --- (9) \]

It involves a bimolecular nucleophilic displacement (SN2) reaction mechanism[88] and the reaction will proceed left if the pH > 7, and to the right if pH < 7. However, recent studies suggest that stirring the solution at high speeds brings the pyrite particles close proximity to each other, which allows the surface of pyrite to act as a catalyst in the oxidation of sulfoxy intermediates by positioning them in a stereochemically favorable position or by the conduction of electrons away from the immediate to a cathodic reaction site where an oxidant is reduced.[89] Another proposed model for pyrite oxidation suggests that the initial step of oxidation is the transfer of hydroxyl ions and the dehydration reactions that follows. The Fe-S bonds weaken until the nascent sulfoxy anion is more stable in solution than bonded to the pyrite ion.[90] This suggests that the possibility of the sulfides reacting with free iron present in the solution that may have leached off the metal-chitosan composite to form iron sulfide. Since the iron sulfide is free in solution, it was possible that there was the formation of iron polysulfides being produced in solution. However, due to the constant introduction of dissolved O2 into the system, these polysulfides may have undergone oxidation and formed sulfates. In conclusion, iron-complexed chitosan is capable of removing aqueous hydrogen sulfide convert the sulfides into sulfates in the presence of oxygen.
IV. Application of Chitosan-immobilized cyanobacteria in water purification

1. Bioreactor design

For all the biological phosphate removal experiments, mat-forming cyanobacteria (CB) species were used due to their unique ability to attach themselves to rough surfaces with the help of extracellular polymeric substances (EPS). *Phormidium tenue* (*P.*tenue) was chosen due to the fact they were mat-forming cyanobacteria, and had fewer microbes that grow along with it. In addition to this, the culture was known to not produce any toxic by-products during its growth phase.

The growth rate of *P.*tenue was determined qualitatively, and was grown in BG-11 nutrient media (20 mL). The cells required 14 days to mature in a 10 cm bacterial cell culture plate (Fig. 30-A). However, the addition of chitosan showed pronounced growth of CB. The rough surface of chitosan proved to be useful in the attachment of the CB (Fig. 29). Fig. 30-C shows the enhanced growth of CB on chitosan flakes.

The bioreactor A (Fig. 31-A) for the cyanobacteria-chitosan flake composite (CBCF) was designed to accommodate continuous flow of wastewater into the system. Inflow rate was 0.2 mL/min and the average retention time for the wastewater was 30 minutes. Chitosan flakes (28 g) were packed into the tubing. The CBCF was allowed to acclimate itself to the wastewater for 7 days before adding it to the head of the tubing. CB was distributed onto the latter sections of the chitosan filter due to the formation of fragments from the seed culture. The bioreactor B (Fig. 31-B) used a polyethylene bottle that has been perforated at the 250 mL mark at one of the faces as well as the bottom of the bottle as to allow the effluent to flow out of the system. The unit was designed to
perform nutrient removal in a similar fashion as bioreactor A. The treated effluent would be re-introduced into the influent stream and the process continues until an appreciable amount of phosphates were removed.

Figure 29: SEM photographs of chitosan flakes.[91]

Figure 30: Growth of P.tenue. A) P.tenue was grown in 20 mL of BG-11 media for 2 weeks. P.tenue was not able to grow quickly due to the cyanobacteria being unable to attach itself to the cell culture plate to stimulate growth. B) 20X light microscope image of the morphology of P.tenue depicting its growth pattern. C) Growth of P.tenue on chitosan (1 g). Enhanced growth was noticed due to the attachment of P.tenue onto chitosan flakes.
Figure 30: **Bioreactor A.** Chitosan-supported cyanobacteria inserted into 5 ft. of clear Tygon rubber tubing with inner diameter of 2 cm and outer diameter of 2.7 cm illuminated with white fluorescent light source from 25 cm away in order to maintain constant light exposure at 25 μmol photons/m²/sec. The wastewater (4 L) was pumped from source at 0.2 mL/min. The tubing was fitted with nylon filters at both ends of the tubing in order to prevent cyanobacteria from entering the effluent stream or contamination in case of a backflow. **Bioreactor B.** Left: Picture of bioreactor setup. Right: Flow diagram of the bioreactor design. Chitosan flakes were added to perforated polyethylene bottle along with a sample of CBCF that was grown in wastewater (4 L). Wastewater was recirculated back into the reactor for 5 days.
2. Application of cyanobacteria chitosan-flake based bioreactor in phosphate removal

In each of the bioreactors shown above (Fig. 30), the CBCF was introduced into a reactor that contained BG-11 media (4 L) and was illuminated with fluorescent light with an intensity of 25 μmol photons/m²/sec. The reactors were also maintained at 15°C with the help of an ice-bath. The media was pumped into bioreactor A at 0.2 mL/min, whereas in bioreactor B, the media was recycled with the help of a pump at 5 L/min. Native chitosan flakes are added to the reaction vessel and a seeding sample of *P.tenue* is added to the native chitosan flakes. The microbes are allowed to grow on the chitosan flakes and the water was sampled every 24 hours.

Chitosan-supported cyanobacteria was grown in different bioreactor setups and yielded roughly the same phosphate removal rate. These reactors showed that it can remove soluble phosphates from the wastewater as it grew on the chitosan flakes. The influent wastewater contained ~17 μg/mL phosphates and were reduced to ~5 μg/mL within 5 days as shown in Fig. 30.

During this period, accumulation of nitrates and nitrites were detected in the reactor. Within ~3 days the wastewater nitrate concentration rose from the initial concentration ~25 μg/mL to ~125 μg/mL and nitrites also started emerging in the effluent as the CB grew. However, during the 4th day there was drastic drop in the concentration of both the contaminants. The removal can be explained by the rapid growth of heterotrophic bacteria in the reactor, which is likely due to the accumulation of nitrates and organic matter.[92] This can be confirmed by the foul smell being generated and the increased turbidity in the reactor by the 5th day after which sampling was halted and the reactor was cleaned using 10% bleach and soap and water.
Figure 31: Phosphate amounts present in the water after passing through bioreactor A. Rate of phosphate removal of P.tenue-chitosan flake composite over a period of 5 days. Accumulation and subsequent removal of nitrate and nitrite from influent wastewater in the bioreactor with P.tenue-chitosan flake composite. Samples were analyzed phosphate levels using ascorbic acid-molybdate phosphate method and nitrite and nitrate levels using modified griess reagent.[93] The error bars represent standard error (n = 3).

Figure 32: Phosphate levels in samples (1 mL) taken from bioreactor B. Trials with different weights of chitosan were performed and each batch was seeded with 1 g CBCF. Samples (1 mL) were analyzed using ascorbic acid-molybdate phosphate method. The error bars represent standard error (n = 3).
The bioreactor was able to attenuate soluble phosphates from ~23 µg/mL by ~4-fold. There were observable cyanobacterial sheaths and chitosan flakes outside the filter unit which had passed through the small reactor holes suggesting the need for biocidal treatment of treated effluent. Furthermore, as more of the holes had been blocked by the chitosan-cyanobacteria composite, the flow of the water was restricted due to the blockage of the outlet holes of the reactor. This lead to a backflow of the influent wastewater, which resulted in the contamination of the influent lines with cyanobacterial fragments as well as the spillage of the chitosan composite into the effluent.

In conclusion, chitosan was able to enhance filamentous cyanobacterial growth (P.tenue) as the polymer was able to provide a surface for the attachment of P.tenue. A ~4-fold or ~60% decrease was observed in soluble phosphate levels within 5 days in both bioreactors. P.tenue culture was unable to metabolize the nitrates present in the bioreactor due to its autotrophic nature and the accumulation of nitrates, nitrites and organic matter in the reactor led to a heterotrophic bacterial bloom which attributed to the sharp drop in nitrate and nitrite levels in the bioreactor. The source of organic matter was most probably due to accumulation of dead cyanobacterial cells in the reactor.
Future directions

Studies for the regeneration and reuse of metal-chitosan flakes as well as crosslinked metal-chitosan flakes in the field are required in order to minimize the costs of the process and improve its chances of being an economically viable option for wastewater treatment. In addition to that, investigation into regenerant solutions other than sodium chloride should be performed. One such reagent may be ammonium acetate, which can be used to recover the adsorbed phosphates in a form that can be utilized in the production of fertilizers or controlled precipitation in a form like struvite. Struvite is a phosphate mineral that comprises of ammonia, magnesium and phosphate (NH₄MgPO₄.6H₂O) that can be applied directly to the fields.

Further investigation by elemental analysis is required for the characterization of the yellow precipitate, to confirm the identity of what was being formed after the iron-complexed chitosan had reacted with soluble hydrogen sulfide. This filter has promising applications in sulfide removal, and as such, investigation of anoxic by-products is also carried out as it opens up possibilities for the application of this filter in anoxic reactors. In addition to this, metal-chitosan should also be tested for its sulfide sorption capacity with hydrogen sulfide gas.

In order to scale up the bioreactor, it is necessary to develop a method that can quantify the growth rate of the cyanobacteria. In addition to this, investigating the growth on other cheaper biopolymers than chitosan such as sawdust, should be investigated in order to develop an economically viable technique for the removal of phosphates and nitrates.
References


57


Appendix

1. Readings for copper leaching from chitosan flakes during the sampling at Luckharts with prototype 1.

Table 1: Copper ions were detected in the sampling during sampling window of July 9th to July 16th 2014. Samples (50 mL) were acidified with 2% HNO₃ and analyzed via AAS.

<table>
<thead>
<tr>
<th>Time point of sample (hrs)</th>
<th>[Cu²⁺] (mg/L)</th>
<th>Average [Cu²⁺] (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>5.15</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>5.01</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>5.43</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>5.12</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>5.1</td>
<td>5.22 ± 0.21</td>
</tr>
</tbody>
</table>
NAME: Terence Yep

PLACE OF BIRTH: New Delhi, India

YEAR OF BIRTH: 1987

EDUCATION:
University of Windsor, Windsor, Ontario
2009 – 2012 (Honours B.Sc. Biochemistry)

University of Windsor, Windsor, Ontario
2014 – 2016 (M.Sc. Biochemistry)