THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF CHEMICAL ENGINEERING

CONFIRMATION OF ADHERENCE OF MORINGA OLEIFERA CATIONIC PROTEIN TO SAND AND STORABILITY OF FUNCTIONALIZED SAND

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A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Chemical Engineering
with honors in Chemical Engineering

Reviewed and approved* by the following:

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ABSTRACT

Moringa oleifera (Moringa) seeds contain a natural cationic protein (MOCP) which has flocculant and antimicrobial properties. As a result, it is used for water clarification. However, use of Moringa seeds to clarify water does not produce potable water. Although the water is initially clarified and drinkable, dissolved organic matter (DOM) is left over in the water. DOM contributes to growth of any pathogens that come into contact with the stored water. This results in water that, once clarified, must be used immediately and cannot be stored for any amount of time. A new way of using Moringa seeds to clarify drinking water has been developed. This new method allows the flocculant and antimicrobial properties of the MOCP to be maintained and the DOM to be rinsed away. This is achieved through adsorption of the MOCP to the surface of sand. The result is MOCP functionalized sand, or *f*-sand. It has been shown that the protein remains adsorbed onto the sand and the ability of the antimicrobial functionalized sand (*f*-sand) to clarify turbidity and kill bacteria is maintained[i]. The presence of MOCP on the surface of the sand was confirmed through SDS-PAGE and mass spectrometry and the adsorption of the protein was studied briefly using absorbance at 280 nm. The DOM was shown to be significantly reduced in solution treated by measuring biochemical oxygen demand (BOD) and chemical oxygen demand (COD). The functionalized sand was shown to be effective after being stored wet, as well as after being dehydrated and stored dry. This confirms *f*-sand's potential as a locally sustainable water treatment option for developing communities.

TABLE OF CONTENTS

INTRODUCTION

Moringa oleifera, or the "miracle tree" has many beneficial properties. Its leaves and seed pods contain high levels of nutrients and protein and are harvested and eaten. The dried seed cake can be pressed to produce high quality oil for use in cooking and cosmetics and is a potential avenue for economic prosperity.[2] The areas of the world in which the tree grows (mostly equatorial regions) [3], coincide with the parts of the world that are most in need of the advantages that *Moringa oleifera* (Moringa) has to offer.[4] Most notably, the seeds of the Moringa tree can be crushed and used to treat drinking water. Treating drinking water is a problem that many developing communities face.[5] This paper will focus on studying a new method for using the seeds of Moringa to treat drinking water.

The seeds of the Moringa tree (Figure 1) contain a cationic, antimicrobial protein (MOCP). This protein removes negatively charged particles from solution resulting in a reduction in turbidity. Since most bacteria are negatively charged, the protein also removes bacteria from solution.[6] MOCP also has antimicrobial functionality which results from a "molecular knife" structure. This contains a hydrophobic proline within a helix-loop-helix motif surrounded by positively charged portions of the protein. Bacteria are electrostatically attracted to the positive sections allowing the hydrophobic proline loop to disrupt the cell wall. These properties are discussed thoroughly in the literature.[7,8,9,10,11,12]

Figure 1. Whole, dried Moringa seeds from ECHO seed bank.

A significant problem with using Moringa oleifera to clean drinking water is the presence of dissolved organic matter in the clarified water [2]. This organic matter contributes to re-growth of pathogens and prevents the clarified water from being potable. In order for the water to be stored and used at a later time, the water must be further treated to remove the organic matter which costs additional time and money and complicates the procedure.

In previous studies, MOCP was electrostatically adsorbed onto sand to form MOCP-functionalized sand, or "*f*-sand." The *f*-sand could be rinsed to remove excess organic matter in solution, while the MOCP remained adhered to the sand surface. It was proven that the immobilized protein retained its flocculant and antimicrobial activity while the resulting treated water contained significantly less dissolved organic material. This initial research confirmed that *f*-sand is very effective at removing turbidity from solution and is an extremely promising technology for implementation in developing communities. Use of Moringa, specifically *f*-sand, could enable a community to become more prosperous. The entire process can be sustained within one community and requires no outside expertise, expensive supplies, electricity, or environmentally unsafe chemicals[1].

In this work, I examine two research objectives:

- **Storability.** I examine whether seed stored under both wet and dry conditions for two months retains its effectiveness at clarifying turbid solutions and **optimization** of *f*-sand, and the
- **Identity of the protein on sand.** I adsorb the Moringa protein onto sand, desorb it at high salt, and check its composition with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry[1].

MATERIALS AND METHODS

***f*-sand Production:** We make *f*-sand by incubating clean sand with Moringa extract containing MOCP. To make Moringa extract, whole Moringa seeds (including shell and wings) were crushed using mortar and pestle. Approximately 1.0 gram or about 4 seeds were added to 40 mL of deionized water. The solution was agitated by rolling in a 50 mL centrifuge tube for one hour. At the end of one hour, the centrifuge tubes were stood upright for 40 minutes to allow the large particles within the serum to settle to the bottom. After settling, the top 30 mL of serum were pulled off. For each batch of *f*-sand approximately 2.0 grams of sand was used. The amount of Moringa serum added to the sand was varied depending on the purpose of the experiment. Initially, 10 mL of serum was used for each 2.0 grams of sand, corresponding to about 0.5 seeds per gram of sand. For the optimization studies, the serum was diluted with deionized water before adding it to the *f*-sand. After optimization it was found that 0.25 mL of serum could be used for 2.0 grams of sand. This corresponds to about 0.125 seeds per gram of sand. Following incubation, each sample of sand was rinsed at least ten times with approximately 10 mL of deionized water. Wet stored samples were stored in approximately 10 mL of deionized water.

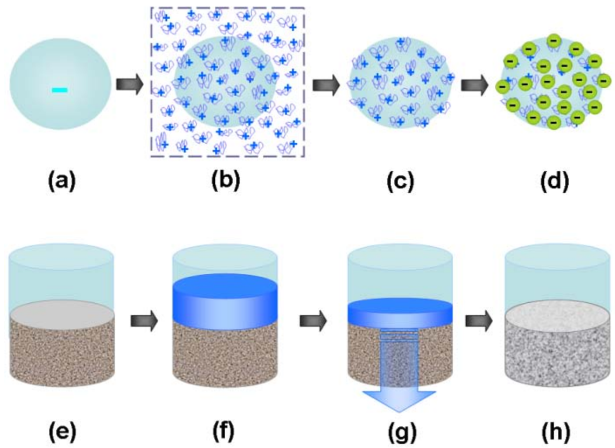

Figure 2. Process for production of *f*-sand. The process is shown on the individual sand grain (a-d) and for the batch process (e-h). The anionic sand grains (a, e) are soaked with Moringa serum containing MOCP (b, f). After removal of the Moringa serum, the MOCP remains adhered to the surface of the sand grains (c, g). Anionic particles, including bacteria, can then adhere to the cationic functionalized sites on the sand grains (d, h) [i].

Turbidity Reduction Studies: To determine if *f*-sand retained its functionality after optimization, dehydration, or storage, a turbidity reduction study was done using UV-Vis. A kaolin clay solution was used as a model turbid solution for all studies and the efficacy of the *f*-sand was determined based on how well it removed kaolin from solution as measured with absorbance. The kaolin suspension was prepared by adding 5.0 grams of dry kaolin powder to 1.0 L of deionized water. The solution was stirred for one hour and then was allowed to settle for 24 hours. The supernatant was removed and stored as a stock kaolin solution. A calibration curve of absorbance versus concentration was prepared for each stock solution. Before each experiment the solution was sonicated for 30 minutes and the absorbance was then measured at 650 nm using a Helios ThermoSpectronic UV-Vis spectrophotometer. If the absorbance was above 1.0, the solution was diluted appropriately and tested again to ensure absorbance below 1.0. To test the *f*-sand's ability to reduce turbidity, 2.0 grams of *f*-sand were added to a 15 mL centrifuge tube along with 6.0 mL of kaolin solution. The solution was agitated by gentle rolling and absorbance was measured at 650 nm every 10 minutes for the duration of the experiment (at least one hour and up to three hours). To test the absorbance, the tubes were set upright and the sand was allowed to settle. Each sample was taken with a disposable pipette and measured in a disposable cuvette. Each absorbance sample was returned to the centrifuge tube to continue rolling until the next time point [i].

Optimization, Dehydration, and Storage: To optimize the amount of Moringa seed needed to functionalize sand, Moringa serum was prepared and diluted with deionized water. It was diluted to 50% (approximately 2 seeds per 10mL serum), 25% (approximately 1 seed per 10mL of serum) and 12.5% (approximately 0.5 seeds per 10 mL of serum) of its initial concentration. The diluted serum was used to make *f*-sand following the original process described. Each dilution was tested for effectiveness using a turbidity removal test as previously described. To test the storability of *f*-sand, sand was prepared and stored in approximately 10 mL of deionized water for varying lengths of time (up to 22 days). The *f*-sand was then used in turbidity removal tests to determine whether it retained its ability to remove turbidity from solution. To determine whether the sand could be dehydrated and stored dry, *f*-sand was prepared as described above. The sand was then removed from the centrifuge tubes using disposable pipettes and placed on small plastic petri dishes. The excess water was removed and care was taken to ensure that sand wasn't removed with the water. The sand was allowed to dry at room temperature overnight. The sand was then stored dry in 15 mL centrifuge tubes for up to 4 months and turbidity removal tests were performed as described.

Protein Isolation and Identification: To confirm that *f*-sand's flocculant and antimicrobial activity was due to MOCP adsorbed to the surface, the protein was removed from the surface of the sand by incubation in salt solutions. 35 grams of *f*-sand was prepared as described and transferred to a glass petri dish where it was incubated with 0.30 M NaCl. The sand was agitated for 10 seconds and then incubated 10 minutes before the 0.30 M NaCl solution was removed with a disposable pipette. This incubation procedure was repeated with 0.60 M NaCl. The absorbance of both solutions was measured at 280 nm. The 0.30 M NaCl showed no significant absorbance but the 0.60 M NaCl solution did show absorbance at 280nm indicating presence of protein in the solution. The 0.60 M NaCl solution was sent to the Proteomics and Mass Spectrometry Core Facility at Penn State for analysis. SDS-PAGE using a 12% Mini-PROTEAN ® TGX TM Precast Gel in a Mini-PROTEAN electrophoresis cell was used to study the solution. The resulting band was digested and analyzed using mass Spectrometry to determine the isoelectric point and the amino acid sequence. A match between the isoelectric point and the amino acid sequence reported in the literature would indicate that MOCP is in fact the protein adhered to the surface of the sand.

RESULTS AND DISCUSSION

The flocculant and antmicrobial activity of MOCP in bulk solution has been studied and explained in the literature[13,14] and it has been shown that *f*-sand retains this functionality (figure 3). This indicates that the active protein MOCP remains adhered to the surface of the sand after rinsing the excess Moringa extract away [1]. In order to confirm this conclusion, it was necessary to conduct studies to confirm the identity of the protein on the surface of the sand. To maximize the potential of this discovery, optimization of the amount of seed needed to functionalize sand was studied along with the storability of the *f*-sand.

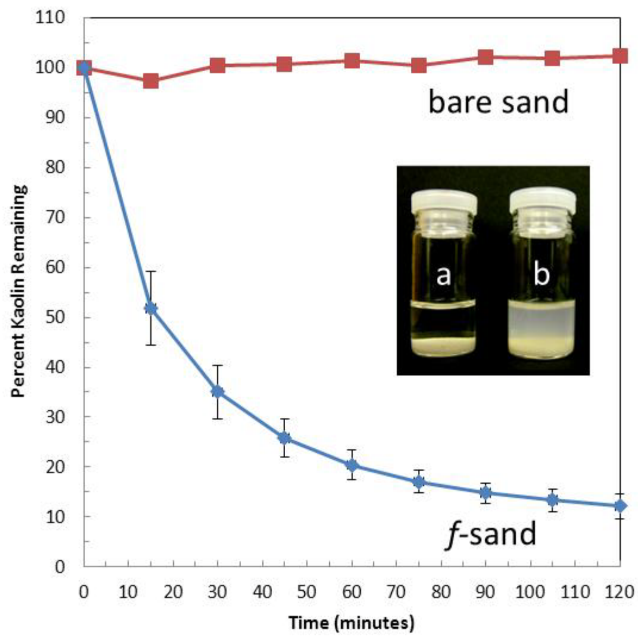

Figure 3. Kaolin is removed from solution using *f*-sand and no removal is measured when kaolin solution is treated with bare sand. The average removal rate was 83% of kaolin in one hour using 2 grams of *f*-sand. Inset: the kaolin removal of *f*-sand is demonstrated. (a) *f*-sand treated kaolin suspension. (b) the same suspension treated with bare sand. The *f*-sand treated sample is visibly less turbid than the sample treated with bare sand[1].

The turbidity removal ability of *f*-sand is comparable to that of the bulk Moringa solution. Lea found that Lea found that bulk Moringa solution could reduce high turbidity solutions by 80-99.5%[15]. It is unknown at this time why the bulk Moringa solution is capable of removing high percentages of turbidity in shorter periods of time. The percentage of turbidity removal that the *f*-sand is capable of achieving within one hour depends on the initial turbidity level. In less turbid solutions, the *f*-sand will be limited by the diffusion of the contaminant particles to the sand surface. It is also possible that the kinetics of the turbidity removal process are altered when the protein becomes adhered to a surface. If this is the case, it may be possible to optimize the kinetics of the system to increase turbidity removal.

In order to maximize the potential of this technology, it is important to maintain a high level of turbidity removal while using the least resources possible. Optimization was accomplished through dilution of the original Moringa serum and testing using turbidity removal tests. The results of these tests were compared to the original recipe *f*-sand as well as bare sand and an untreated kaolin solution over time. The result indicated that the original formulation could be diluted to 25% but not as far as 12.5% (figure 4). This indicates that water could be clarified using approximately 40 seeds per liter of water. The current method of using Moringa requires just 1 seed per liter of water. Our result is still far from this efficient. The preparation of *f*-sand should be further studied and optimized to approach the 1 seed per liter that is currently used[1].

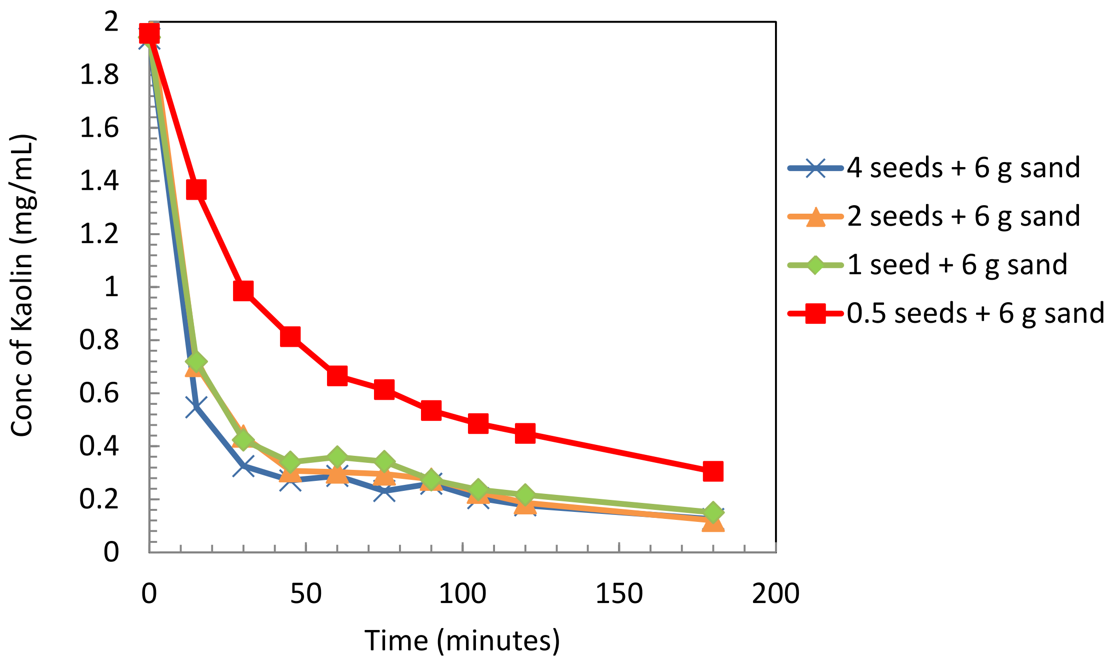

Figure 4. Preparation of *f*-sand was optimized to reduce Moringa seed usage by 75%. 1 seed can be used to functionalize 6 grams of sand and achieve the same kaolin clearance as the initial formulation (4 seeds to functionalize 6 grams of sand).

To determine the storability of *f*-sand, *f*-sand was prepared as described and was stored wet for up to 22 days, or dehydrated, and stored dry for up to 4 months. No decrease in activity was observed in stored *f*-sand, wet or dry. The *f*-sand was prepared periodically throughout the test period and all samples were tested on the same day to eliminate any operational error or differences in the system behavior due to environmental factors such as temperature and humidity. Differences in the kaolin removal curve were observed for dry versus wet stored sand (figure 5). Although both reach the same final removal, the dry stored sand follows a shallower curve. It is important that *f*-sand retains its kaolin removal ability after dehydration and storage because making *f*-sand in large batches for storage and later use eliminates the need for a community to make a fresh batch of *f*-sand each time they want to drink, cook, or bathe. Potable water can be available on a much shorter time scale, which is much more convenient.

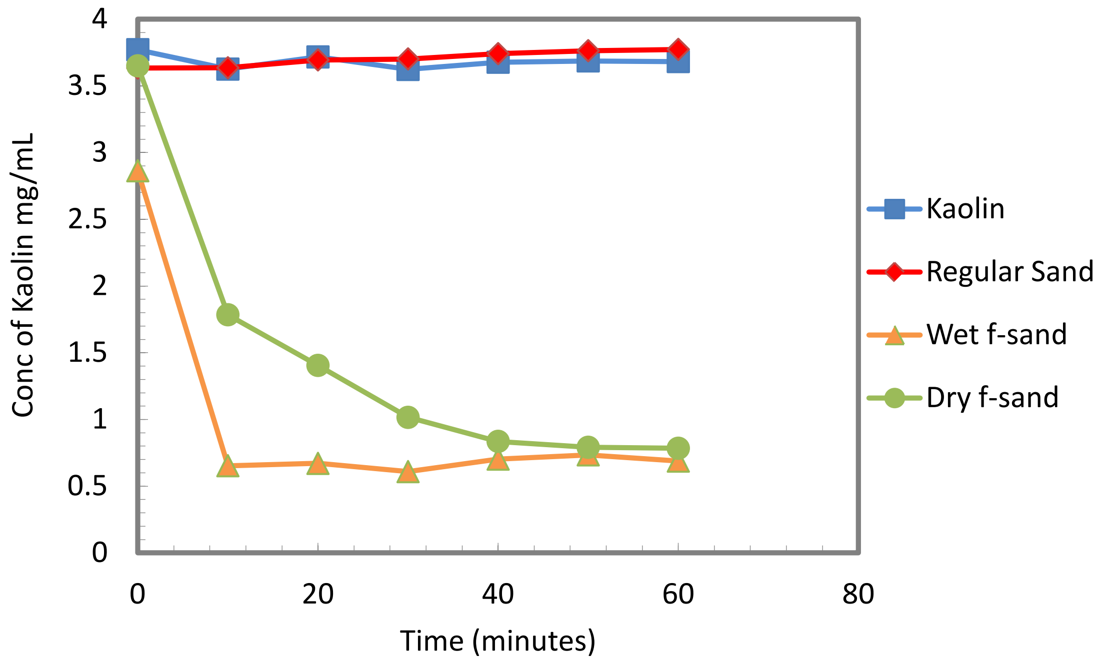

Figure 5. Kaolin removal ability of wet and dry stored sand is compared. Wet and dry stored sand both achieve significant kaolin removal compared to plain sand and untreated kaolin solution. The dry stored sand follows a shallower curve and does not achieve as much kaolin removal in the first 40 minutes. This graph shows average removal of at least 10 samples prepared in triplicate for both the wet and dry sand.

To confirm the presence of MOCP on the sand surface, the protein was eluted from the surface using salt solutions. After each salt rinse, absorbance at 280 nm was used to confirm the presence of protein in the solution. Solutions with protein present were analyzed and compared to literature values. MOCP has been shown to be a cationic dimeric protein. Each monomer has a molecular weight of 6,500 Da. The isoelectric point of the protein is between 10 and 11.[6] SDS-PAGE and mass spectrometry were used to confirm that the molecule adhered to the sand surface had these characteristics. The SDS-PAGE yielded a single band at 6,500 Da. Upon digestion of this band and analysis via mass spectrometry, the amino acid sequence was found to match the amino acid sequence reported by the National Center for Biotechnology. The isolectric point was found to be 10.8, within the reported literature values[6]. This analysis confirms that MOCP is adhered to the surface of the sand. The lack of other proteins adsorbed to the sand is most likely attributed to MOCP's positive charge and to its small size and corresponding high diffusion coefficient.[1] This is important to the further development of this water treatment technology because it confirms that the *f*-sand is functionalized with the suspected protein and that no optimization will be necessary to prevent other proteins from adsorbing to the sand surface.[1] Absorbance at 280 nm and the Beer-Lambert Law are often used to find relative concentrations of protein in solution. Some methods exist for estimating the absorption coefficient necessary for using the Beer-Lambert Law but in order to find the actual concentration of protein in the solution, a calibration curve should be developed for MOCP. Preliminary analysis showed that increasing the amount of Moringa extract used to make the *f*-sand increased the absorbance at 280 nm of the corresponding salt solution.

CONCLUSION

Moringa alone has been shown to provide many extremely important benefits to the communities in which it is grown. Its use to treat drinking water has been promoted and developed by the Peace Corps. Moringa can be used to produce clean and safe drinking water easily, inexpensively and quickly. Finding a way to use Moringa to produce potable water would provide a socially, environmentally and economically sustainable way for a developing community to gain access to potable water. Previous work has shown that MOCP retains its functionality when adhered to the surface of sand and the problematic excess organic matter is rinsed away. In this work, it has been shown that the previously studied antmicrobial MOCP protein is the only protein that is adhered to the surface of the sand.

Having immediate access to potable water is convenient for developing communities and reduces the amount of time that must be spent treating water. Additionally, it is convenient if the *f*-sand can be stored easily and used when needed. In this work, it was shown that *f*-sand can be stored wet for up to 22 days or dehydrated and stored dry for up to 4 months. This is convenient because the *f*-sand can be produced when seeds are available. Storing the *f*-sand instead of the unused seeds reduces the risk of loss to animals or bugs. It also allows community members to obtain clean drinking water within an hour even if the store of potable water is used up.

In order for this process to be implemented and be sustainable, the process must be optimized. The optimization process was begun in this work with optimization of the amount of seed material needed to maintain turbidity removal functionality. The amount of seed necessary was reduced by 75%. This result should be replicated with bacterial studies before implementation in an actual community. In addition, the rest of the process must be optimized. The amount of *f*-sand necessary to treat a given volume of contaminated water with a given bacterial concentration should be determined. The surface area of the sand and quality of the seeds must also be taken into consideration. Further work is being conducted to determine if seeds harvested at different points in their life cycle have different concentrations of MOCP. This would impact the optimization process.

This process is still far from being implemented in a developing community but previous work and the results shown here indicate that this is a promising possibility for combating the worlds problem with access to drinking water. Use of Moringa to treat water in developing communities would increase the health of community members in a way that is empowering to the community and provides additional opportunities such as the nutritional benefits of incorporating the leaves into their diets or the economic benefits of selling the high quality oil pressed from the seedcake.

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EDUCATION

PhD in Chemical and Biological Engineering 2017
Northwestern University

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RESEARCH EXPERIENCE

Undergraduate Research | May 2010 – Current **|** University Park, PA
Directed by Professor Darrell Velegol
Research Goal: Develop water clarification technology for use in developing nations

- Applied knowledge of mass transfer, colloidal science and bacterial methods to improve and optimize the process through two Undergraduate Summer Research Fellowships and continued work throughout the school year
- Initiated additional research goals leading to an application for a Gate's Foundation Grant and multi-country collaboration
- Appointed Student Lead of an EPA P3 grant team dedicated to translating lab success into a working prototype and presented at the EPA National Sustainability Expo in April 2011 in Washington, D.C.
- Conducted assessment visits to towns in Puerto Rico to find a suitable implementation site

PUBLICATIONS AND CONFERENCE PRESENTATIONS

Jerri, Huda A., Adolfsen, Kristin J., McCullough, Lauren R., Velegol, Darrel, Velegol, Stephanie B."Anti-microbial sand via adsorption of cationic Moringa oleifera protein," Langmuir (*accepted Nov 2011).*

Velegol, Darrell, Jerri, Huda A., Adolfsen, Kristin J., McCullough, Lauren R., Velegol, Stephenie B. "Naturally-Functionalized Antimicrobial Sand (f-sand) Using Moringa Oleifera Seeds," 2011 American Institute for Chemical Engineering Annual Conference, Minneapolis, MN, 16-21 October 2011.

ACTIVITIES

COP 17 – United Nations Negotiations on Climate Change | 28 Nov – 2 Dec 2011 **|** Durban, South Africa
Delegate of and Student Representative of the American Chemical Society

- Chosen as one of five university students from across the country to represent the American Chemical Society at the Conference as a supplement to the UN International Year of Chemistry Initiative
- Supported by travel grants from the Chemical Engineering Department, Global Programs, College of Engineering, Presidential Leadership Academy and the Schreyer Honors College at Penn State
- Responsibilities include developing strategies for use of social media to convey knowledge about climate change negotiations to peers and inspire others to work towards a solution

Resident Assistant | Jan 2010 – Current **|** University Park, PA
Selected for placement in the highly competitive Schreyer Honors College

- Utilized ongoing training in communication, diversity and interpersonal relations to build community and resident satisfaction for 60 diverse undergraduate students
- Employed crisis management applied to personal crises such as suicide intervention and building wide emergencies such as bomb threat evacuations

Presidential Leadership Academy | Jan 2009 – Current **|** University Park, PA
Selected as one of 30 outstanding students for inaugural class of university-wide leadership program

- Learned leadership, team building, and ethical decision making from classroom experiences with President Graham Spanier and Dean Christian Brady including visits to US Steel, FBI, and CIA headquarters to see leadership in action
- Conducted a one semester case study on possible solutions to the high risk drinking problem prevalent in college students and provided recommendations to University administrators that were later implemented

Engineering Leadership Development Minor Program | Aug 2010 – Jun 2011 **|** Budapest, Hungary

- Applied classroom lessons in global engineering leadership to a collaboration with Hungarian business students from Corvinus University in Budapest to plan a water treatment plant for implementation in Nan Plim, Haiti,
- Travelled to Corvinus University to finalize project details and present final results and findings

AWARDS AND HONORS

- Chemical Engineering Undergraduate Research Scholarship - Summer 2010 and Summer 2011
- Paul C. Morrow Endowed Scholarship - Fall 2008 through Spring 2012
- Academic Excellence Scholarship - Fall 2008 through Spring 2012
- Award from the Samuel A. Shuman Endowment in Engineering - Fall 2011
- J. Larry Duda Undergraduate Award - Fall 2011
- Induction into Omega Chi Epsilon Chemical Engineering Honor Society - Fall 2009
- Dean’s List: Fall 2008, Spring 2009, Fall 2009, Spring 2010, Spring 2011