IRON NANOPARTICLES AND BIOPOLYMERS FOR PLANT NUTRIENT FORTIFICATION

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Novel iron (Fe) cross-linked alginate (FCA) beads were used for aqueous phosphate removal. Batch experiments were conducted with the beads using three different concentrations of phosphate (5, 50 and 100 mg PO_4^{3-}-P/L) as well as environmentally relevant (eutrophic lakes) concentration of 100 µg PO_4^{3-}-P/L. About 80-97% phosphate was removed within 3 h. for lower concentrations of phosphate. The maximum phosphate sorption capacity was found to be 78.7 mg PO_4^{3-}-P/g of beads. Phosphate removal was not affected because of the presence of Cl^-, HCO_3^-, SO_4^{2-}, NO_3^- and natural organic matter (NOM). FCA beads were also used with actual lake waters (11-69 µg PO_4^{3-}-P/L) and 81-100% phosphate removal was observed in 24 h. The FCA beads having a point of zero charge (PZC) of 9.2 make it an ideal candidate for phosphate removal in eutrophic lakes.

Phosphate-laden spent iron cross-linked alginate (FCA) beads were used in hydroponics to evaluate the bioavailability of P and Fe using lettuce (*Lactuca sativa*) as a test plant. Phosphate-laden spent FCA beads were found to support the plants throughout the growth period. The bioavailability of P and Fe in the spent beads is promising considering the importance of phosphorus and iron in global nutrient security.

Experiments were also conducted with lettuce and spinach (*Spinacia oleracea*) to evaluate the availability of iron from nanoscale zero-valent iron (NZVI). In both plants, bare NZVI enhanced the uptake of Fe as well as other essential elements. The results indicate that biofortification of spinach and lettuce with Fe is possible. The enhanced uptake of iron and other elements by lettuce and spinach is likely to have implications on global nutrient security.

In another experiment, an iron-regulating gene (*Ls*HA2) in lettuce was investigated to gain insights into the strategy taken by plants for acquisition of Fe from a readily unavailable

source, e.g., NZVI. The gene of interest was found to be regulated by the presence or absence of available iron in the solution. This research is likely to give us insights into the mechanism of plant nutrient fortification with nanoparticles.

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DEDICATION

To my parents and wife

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1. INTRODUCTION

1.1. Background

Phosphorus (P) is important for the growth of plants and microorganisms in most ecosystems (Pierzynski, 2005). However, when excess phosphorus originating from point and non-point sources find its way into waterbodies it results in eutrophication (Almeelbi and Bezbaruah, 2012). Phosphorus affects 42% of the lakes and 66% of the river and streams in the United States (USEPA 2009). Accelerated eutrophication due to high phosphate presence not only impacts the aquatic life but also hampers the economy of communities that rely on aquatic food and other resources (Cleary, et al., 2009). It has been estimated that ~$2.2 billion is lost annually as a result of eutrophication in U.S. freshwaters (Dodds, et al., 2009). The cost of eutrophication to the United Kingdom water industry is estimated at more than £15 M per annum (Babatunde and Zhao, 2010). Therefore, it is imperative to devise effective methods to remove excessive phosphate from waters. There is a significant gap in technology to remove low concentration (eutrophic) phosphate from waters. Currently, there is no universally accepted method for phosphate removal from eutrophic lakes.

On the other hand, phosphorus is heavily intertwined with the global food security (Cordell, et al., 2009). Modern agriculture is heavily dependent on phosphate fertilizer derived from phosphate rock, which is non-renewable in nature. It is estimated that at the present rate of consumption, the global phosphate reserves may be depleted in 50-100 years (Cordell, et al., 2011). It is, therefore, very important to develop effective technologies to reclaim phosphate from other sources (mines) like eutrophic waters.

Iron (Fe) is also essential for plants and humans. Iron deficiency is the most common nutritional deficiency in humans. Iron is vital for oxygen transport in the body and for energy

metabolism as well (WHO/FAO, 1998). Iron deficiency is manifested in anemia and is mainly prevalent in women and children. It was estimated in 2011 that around 43% of children, 38% of pregnant women, and 29% of non-pregnant women have anemia worldwide which correspond to 273 million of children, 32 million of pregnant women, and 496 million of non-pregnant women (WHO, 2015). The African Region has the highest prevalence (62.3%) of anemia in children while the most affected number of children and women resides in the South-East Asia Region, including 96.7 million children and 202.0 million women of reproductive age (15-49 years) (WHO, 2015). Therefore, iron deficiency is a problem of global importance.

Biofortification has been explored as a long-term solution to iron deficiency problem (Zhu, et al., 2007). The fortification of staple crop plants with bioavailable iron is likely to provide a sustainable and economical tool to remedy iron deficiency in target populations worldwide (Jeong and Guerinot, 2008). Biofortification as an agronomic intervention is sometimes not efficient because iron in oxidized ferric form is less soluble in aerobic environments. Innovative iron fortification through soil and foliar application of iron containing compounds has been tried in sorghum (Ortega-Blu and Molina-Roco, 2007), wheat (Aciksoz, et al., 2011), and leafy vegetables (e.g., spinach, (Almeelbi and Bezbaruah, 2014)).

The current work described in this dissertation is aimed at removal of phosphate using innovative iron cross-linked biopolymer. The work also includes examining whether phosphate removed from different water sources can be used as a plant fertilizer. The cross-linked iron in the biopolymer was found to be bioavailable to plants and the work was expanded to include other sources or iron as a possible plant biofortifier. Iron nanoparticles have been investigated as a possible biofortificant.

Over the last decade, nanoparticles have been used in agriculture in different ways. Nanomaterials have been used as smart delivery systems of fertilizers, herbicides, pesticides and plant growth regulators (Perez-de-Luque and Hermosin, 2013). Scientists have also explored the possibility of using nanoparticles to biofortify plants. In a hydroponic study, Almeelbi and Bezbaruah (2014) used nanoscale zero-valent iron (NZVI) for phosphate removal and subsequently used the spent (phosphate-sorbed) NZVI as a source of phosphorus and iron for spinach and algae. They found increased growths of spinach and algae when spent NZVI was used as a sole source of iron and phosphorus. The iron content increased significantly in all plant parts (roots, stems, and leaves) when spent NZVI was used as a source of iron. Iron content increased by 7 and 11 times in the stem and leaves in spinach as compared to the control (conventional $FeSO_4$ as the source of iron). However, in their study, Almeelbi and Bezbaruah did not have a microscale zero-valent iron (MZVI) particles or bulk iron particles as a control. Therefore, it is difficult to say that the enhanced uptake was solely due to the nano size of the iron particles. This dissertation research included microscale zero-valent iron particles along with NZVI in a study with spinach (*Spinacia oleracea*) to elucidate the role of nanosized iron in the enhanced uptake of iron and in the prolific growth of plant.

This dissertation research also included additional plant species and looked at the possible mechanism involved in nanoparticle-triggered iron uptake by plants. Molecular level (genetic) studies were conducted to understand the possible mechanism of iron uptake by plants.

1.2. Need Statement

Phosphorus through accelerated eutrophication impacts aquatic lives and hampers the economy of countries relying on their aquatic food and other resources. Innovative new technologies are needed which will turn this problematic nutrient contaminant into a mineable

resource for the humankind who is anticipating a looming phosphate crisis. Technologies are also needed to solve the iron deficiency problem which is posing a serious risk to the health of a considerable number of people in the world. The new technology or technologies aimed at addressing these needs should be cost-effective.

1.3. Research Objectives

The main objectives of this study are: (1) the development of cost-effective technologies for removal of phosphate and subsequently use the reclaimed phosphate in agriculture, (2) to biofortify plants with iron.

The specific objectives of this study are:

- Investigate phosphate removal by the use of iron crosslinked alginate (FCA) beads.
- Investigate the bioavailability of phosphate and iron from FCA beads.
- Investigate the effect of nano and non-nano iron particles on spinach.
- Investigate the possibility of iron fortification of lettuce plants with nanoscale zero-valent iron NZVI.
- Investigate the iron uptake mechanism in lettuce by looking at the expression level of a particular gene.

1.4. Hypotheses

- Iron present as FCA beads will be able to adsorb phosphate from water.
- Phosphate adsorbed onto alginate beads and iron within the beads will be available for plant uptake.
- Iron nanoparticles will enhance the uptake of iron in plants compared to bulk particles.

- Iron nanoparticles will trigger iron-regulating genes in plants for enhanced uptake of iron.

1.5. Dissertation Organization

There are seven chapters in this dissertation. **Chapter 1** is an overview of the research problem, need statement, and objectives of this research. The other chapters (**Chapter 2-6**) in the dissertation are presented in journal paper formats, and each of these chapters has already been submitted or will be submitted for publication in peer reviewed journals. Each of these chapters has its own introduction, materials and methods, results, discussions and conclusions. **Chapter 2** discusses the use of iron cross-linked alginate (FCA) beads for phosphate removal from water. **Chapter 3** includes the bioavailability of phosphate and iron from phosphate-containing iron cross-linked alginate beads (spent FCA beads) using lettuce (*Lactuca sativa*) as a test plant. **Chapter 4** presents the bioavailability and the effects of NZVI to spinach (*Spinacia oleracea*) compared to microparticles. **Chapter 5** describes the biofortification of lettuce with bare nanoscale zero-valent iron (NZVI) particles and phosphate-containing spent NZVI. **Chapter 6** presents the research with the homologous LsHA2 gene in lettuce (*Lactuca sativa*) to investigate its role in Fe acquisition from NZVI in hydroponics. **Chapter 7** is the conclusions and the possible future directions of this and related research.

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2. BIOPOLYMER BEADS FOR AQUEOUS PHOSPHATE REMOVAL: POSSIBLE APPLICATION IN EUTROPHIC LAKES

2.1. Abstract

Novel iron (Fe) cross-linked alginate (FCA) beads were used for aqueous phosphate removal. Batch experiments were conducted with the beads (0.118 g dry weight) using three different concentrations of phosphate (5, 50 and 100 mg PO_4^{3-}-P/L) as well as environmentally relevant (eutrophic lakes) concentration of 100 µg PO_4^{3-}-P/L. About 97% phosphate (initial phosphate concentration = 5 mg PO_4^{3-}-P/L) was removed by the beads in 360 min from an aqueous solution. With 50 and 100 mg PO_4^{3-}-P/L, the beads were found to remove ~76% and 24%, respectively in 360 min. With 100 µg PO_4^{3-}-P/L, 80% removal was achieved within 20 min. The first order reaction model fitted well for 5 mg PO_4^{3-}-P/L and reaction rate constant (k) was 0.0091 per min. First-order reaction was also observed with 100 µg PO_4^{3-}-P/L with a k = 0.0828 per min. The maximum phosphate sorption capacity was found to be 78.7 mg PO_4^{3-}-P/g of beads. No change in phosphate removal was observed in the presence of Cl^-, HCO_3^-, SO_4^{2-}, NO_3^- and natural organic matter (NOM). To investigate the feasibility of using these FCA beads in real life situation (e.g., in eutrophic lakes), actual lake waters (11-69 µg PO_4^{3-}-P/L) were used and 81-100% phosphate removal was observed in 24 h. Results presented here demonstrate the potential use of the FCA beads for the reclamation of eutrophic lakes (removal of excess of phosphate).

2.2. Introduction

Phosphorus (P) is important for the growth of plants and microorganisms in most ecosystems. However, when excess phosphorus stemming from point sources and non-point sources finds its way into waterbodies it leads to eutrophication. Phosphorus affects 42% of the

lakes and 66% of the river and streams in the United States (USEPA 2009). Municipal and industrial wastewaters are the major point sources that contribute to P build-up in the aquatic environment. About 260,000 metric tons of PO_4^{3-}-P are discharged to US waters every year from wastewater treatment facilities (Litke, 1999). Agricultural run-offs constitute the major non-point sources for phosphorus. In the US, agriculture contributes ~3,629,000 metric tons of PO_4^{3-}-P per year to water bodies. Other non-point sources, especially animal agriculture, contribute ~1,089,000 metric tons of phosphorus to US waters (Litke, 1999). Aquaculture (fish farming) is another growing source of nutrient pollution. For every metric ton of fish, aquaculture operations produce between 7.9 and 11.6 kilograms of phosphorus waste (Strain and Hargrave, 2005). The amount of phosphorus dumped into the European coastal waters is 200,000 to 300,000 metric tons phosphorus per year (measured between 1985 and 2005) with major contributions from more intensively farmed agricultural regions (Grizzetti, et al., 2012).

Accelerated eutrophication due to high phosphate presence not only impacts the aquatic life but also hampers the economy of communities that rely on aquatic food and other resources (Cleary, et al., 2009). It has been estimated that $2.2 billion is lost annually as a result of eutrophication in U.S. freshwaters (Dodds, et al., 2009). The greatest economic losses were attributed to lakefront property values ($0.3-2.8 billion/year) and recreational use of waters ($0.37-1.16 billion/year). The cost of eutrophication to the United Kingdom water industry is estimated at more than £15 M per annum (Babatunde and Zhao, 2010).

Algal growth is the manifestation of excess phosphate in waters. To avoid algae overgrowth problems, total phosphorus should not be >5 µg/L in streams discharging into lakes or reservoirs, 25 µg/L in lakes or reservoirs, and 100 µg/L in streams or flowing waters not discharging into a waterbody (USEPA, 1986). Generally, surface waters with 10-30 µg/L of

total phosphorus remain free from algal blooms (Peleka and Deliyanni, 2009). It is imperative to devise effective methods to remove excessive phosphate from waters. There is a significant gap in technology to remove low concentration (eutrophic) phosphate from waters.

Currently, there is no universally accepted method for phosphate removal from eutrophic lakes. The techniques used in lakes are aimed at the reduction of internal P-loading from sediments which include sediment dredging (Jing, et al., 2015), oxygenation (Yin and Kong, 2015), use of chemical flocculants (Al, Fe or Ca-based chemical products) (Lin, et al., 2015), and in-situ sediment capping. In-situ sediment capping includes P sorbents based on Al, Ca or lanthanum-modified clay minerals. Products based on La-modified bentonite (e.g., Phoslock®) have been extensively used in fresh water lakes in many European countries to effectively lock P in sediments (Lurling, et al., 2016). Other developments in sediment capping include Fe-modified clays (e.g., Bephos™ (Zamparas, et al., 2013) and Sinobent®(Gołdyn, et al., 2014)). Thermally modified calcium-rich attapulgite have been developed for capping sediments (Yin and Kong, 2015).

The high cost involved and concomitant problems of treatment and disposal of the dredged sediment preclude the sediment dredging as an effective method (Lin, et al., 2015). Chemical flocculants result in disadvantages such as toxicity toward aquatic organisms (Pessot, et al., 2014) and the efficacy of iron salt is heavily dependent on environmental factors (e.g., redox and pH) (Immers, et al., 2013). Sediment capping has promising potential as it is very effective and the raw materials are globally available (Reitzel, et al., 2013, Yin, et al., 2011). However, capping materials may also pose problems to the ecosystems (e.g., toxicity to Atlantic salmon (Pessot, et al., 2014) and free lanthanum affects aquatic organisms at low alkalinity (Copetti, et al., 2016)) and are currently cost-prohibitive (Spears, et al., 2013).

Present methods (**Table 2.1**) for phosphate removal from wastewater include biological treatments (de-Bashan and Bashan, 2004, Ekama, 2015), chemical precipitation with aluminum, iron and calcium salts (Tchobanoglous, et al., 1991), adsorption (Almeelbi and Bezbaruah, 2012, Lai, et al., 2016, Liu and Hesterberg, 2011), and reverse osmosis (Dolar, et al., 2011, Luo, et al., 2016). Chemical treatment methods for aqueous phosphate removal use chemicals like lime (Ahn and Speece, 2006, Dunets and Zheng, 2014), alum (Babatunde and Zhao, 2010), and ferric chloride (Caravelli, et al., 2010). However, chemical precipitation is generally not suitable for low concentration phosphate removal, and reverse osmosis is capital intensive. Biological treatment methods do not use chemicals nor produce excess sludge. However, they require more complex plant configurations and operating regimes (Morse, et al., 1998). Biological assimilation and enhanced biological phosphorus removal (EBPR) are employed for phosphate removal from wastewater (Oehmen, et al., 2007). Biological assimilation using photosynthetic organisms (plants, algae, and some bacteria such as cyanobacteria) is also practiced.

Sorption is one of the most attractive options for aqueous phosphate removal because of its simplicity in design, operational ease, range of sorbents available, and their cost effectiveness (Bhatnagar and Sillanpaa, 2011, Mishra, et al., 2010). Sorption has an advantage over other technologies due to its effectiveness at low phosphate concentrations (Loganathan, et al., 2014). An array of adsorbents has been investigated for phosphate removal. Peat-based biosorbent (Robalds, et al., 2016), modified chitosan beads (Liu and Zhang, 2015), dolomite mineral (Yuan, et al., 2015), lanthanum oxide and hydroxide material (Xie, et al., 2015, Xie, et al., 2014), nanoscale zero-valent iron (Almeelbi and Bezbaruah, 2012, Eljamal, et al., 2016, Soliemanzadeh, et al., 2016, Wen, et al., 2014), fly ash (Cheung and Venkitachalam, 2000), red mud (Huang, et al., 2008), iron oxide tailing (Zeng, et al., 2004), ferric sludge (Song, et al., 2011), phosphate

mine wastes (PMW) particles (composed of calcite, fluorapatite, and quartz) (Jaradat, et al., 2016), blast furnace slag (Lee, et al., 2012, Oguz, 2004), half-burned dolomite (Roques, et al., 1991), layered double hydroxides (Cheng, et al., 2009, Das, et al., 2006, Khitous, et al., 2016, Kuwahara, et al., 2016, Novillo, et al., 2014, Sun, et al., 2014, Yan, et al., 2015), magnesium oxide-based biochar (Jung and Ahn, 2016, Li, et al., 2016), and water treatment residuals (Wang, et al., 2011, Wang, et al., 2014) have been used.

Table 2.1. Phosphorus removal and recovery technologies [adapted from (Morse, et al., 1998)].

Phosphorus Removal Technology	Recovery	Technology Advantages	Technology Disadvantages
Chemical Precipitation	Low; binding is too strong	Established Technology	Low recyclability, sludge production, price extensive
Enhanced Phosphorus Biological Removal (EPBR)	Moderate	Established Technology, no chemical used	Complex technology, sludge handling difficult
Crystallization	High	Retrofitting, recyclability	Requires chemicals, process difficult
Ion exchange	High (struvite)	High potential, use of struvite for agriculture	Requires chemicals, process difficult
Magnetic	Low	High Potential	Requires chemicals, process difficult
Tertiary filtration	None	Easy to use, proven technology	No useful product
Sludge treatments	High	Sludge value increased	Requires chemicals, process difficult
Adsorption	Low	High Potential	Regeneration process difficult

In the past, bio-based materials including polysaccharides (biopolymers) have been used as adsorbents for removing pollutants. Biopolymers are unique materials as they are abundant, inexpensive, renewable, and modifiable. They also have chirality, chelation, and adsorption

capacities (Crini, 2005). They are typically non-toxic for the ecosystem components, biocompatible, biodegradable, and polyfunctional (Crini, 2005). Sodium (Na) alginate, the salt of alginic acid, has been investigated as a sorbent for the removal of organic and inorganic pollutants from wastewaters (Li, et al., 2013). It is attractive because of its biodegradability, hydrophilicity, presence of carboxyl and hydroxyl groups, low cost, natural origin, and renewable nature (Li, et al., 2013). Alginates are anionic linear copolymers composed of two monomeric units, β-1-4-linked D-mannuronic acid (M) and α-1,4-linked L-guluronic (G) acid and are produced by brown algae and bacteria (Paques, et al., 2014). The gelling properties of its guluronic residues with divalent ions such as calcium enables the formation of alginate matrices as gels, films, beads, pellets, microparticles and nanoparticles (Sarmento, et al., 2007). Divalent metal ions cross-link to carboxyl groups on adjacent alginate molecules (Hassan, et al., 2014) and form alginate gels. Calcium (Ca) alginate biopolymer is an efficient sorbent for the removal of metal ions, but it is not capable of removing anionic species. Min and Hering (1998) used Iron (Fe^{3+}) to partially displace loosely bound Ca^{2+} ion in Ca-alginate to produce Fe-doped calcium-alginate beads; the iron-calcium alginate beads were found to be effective at removing oxyanionic contaminants, specifically Se (IV), Cr (VI) and As (V) (Min and Hering, 1998, Min and Hering, 1999)

Iron (Fe) cross-linked alginate (FCA) beads were used in this study for phosphate removal. The objective of this research work was to determine if FCA beads can be used for effective removal of aqueous phosphate. A series of experiments were conducted with the FCA beads to investigate the mechanisms of phosphate sorption onto FCA beads. The specific objectives of this work were to: (1) investigate the phosphate sorption characteristics of FCA beads, and (2) determine the feasibility of using FCA beads in eutrophic lakes and wastewater.

2.3. Materials and Methods

2.3.1. Material

Iron (II) chloride tetrahydrate (FeCl2·4H2O, reagent grade, Alfa Aesar), calcium chloride (CaCl2, ACS grade, BDH), monopotassium phosphate (KH2PO4, 99% pure, EMD), sodium alginate (production grade, Spectrum), potassium nitrate (KNO3, 99%, Alfa Aesar), sodium hydroxide (NaOH, ACS Grade, BDH), potassium sulfate (K2SO4, ACS grade, HACH), potassium chloride (KCl, ACS Grade, BDH), potassium bicarbonate (KHCO3, ACS Grade, Alfa Aesar), natural organic matter (Suwannee River NOM, RO isolation, IHSS), and humic acid (H1452, Spectrum) were used as received unless and otherwise specified.

2.3.2. Synthesis of Fe cross-linked alginate (FCA) beads

Sodium alginate (20 g) was dissolved in 1 L of deionized (DI) water by stirring the solution overnight at 60 ^{o}C to form a 2% alginate solution. Fe cross-linked alginate (FCA) beads were synthesized by adding the alginate solution to ferrous chloride ($FeCl_2$) solution (2% w/v) at room temperature (22±2 ^{o}C). The alginate solution (5 mL in each batch) was added drop wise into the $FeCl_2$ solution using a peristaltic pump. FCA beads were formed immediately as the alginate came into contact with the iron solution. The beads from each batch were kept separately in a polypropylene tube fitted with a plastic cap. Enough $FeCl_2$ solution was added to each tube to completely submerge the beads, and the beads in the tubes were allowed to harden in $FeCl_2$ solution for an additional 24 h (Bezbaruah, et al.). The hardened beads were then washed with DI water. The wet FCA beads were stored in batches in DI H_2O. Each batch of wet FCA beads was blotted with tissue papers to remove excess water prior to their use in experiments. The weight of the FCA beads are reported on dry weight basis (60 ^{o}C/12 h).

2.3.3. Kinetics studies

FCA beads produced in a single batch (wet and dry weights 1.516±0.080 g and 0.118±0.008 g, respectively) were added into 50 mL phosphate solution in a polypropylene plastic tube (reactor) fitted with a plastic cap. Four PO_4^{3-}-P concentrations (100 µg/L, 5, 50 and 100 mg/L) were chosen for kinetic studies. The 100 µg PO_4^{3-}-P/L concentration was selected to get insights into the kinetics of removal at environmentally relevant PO_4^{3-}-P concentrations found in eutrophic lakes. Controls (no FCA beads but only PO_4^{3-} solution) were also run. The reactors and controls were then rotated at 28 rpm in a custom-made end-over-end shaker to reduce mass transfer resistance. For 5, 50 and 100 mg/L PO_4^{3-}-P concentrations, a set of sacrificial reactors was withdrawn at specific time intervals (0, 10, 30, 60, 90, 120, 180, 240, 360, 720, 1080, 1440 min), and the phosphate concentrations in the bulk solution were measured and reported as the average (with standard deviations) from three replicates. For 100 µg/L, the sacrificial reactors were withdrawn at more frequent time intervals up to 30 minutes (0, 4, 6, 8, 10, 15, 20, 30 min) and then at longer intervals (60, 90, 120, 180, 240, 360, 720, 1080, 1440 min). Ascorbic acid method was used for phosphate analysis (Eaton, et al., 2005). The phosphate removal efficiency (η) was calculated using the equation: $\eta = (C_0 - C_e)*100\%/C_0$ where C_0 and C_e are the initial and equilibrium concentrations of PO_4^{3-}-P in mg/L. A Hach DR 5000 spectrophotometer (880 nm, detection limit = 9 µg PO_4^{3-}-P/L) was used for phosphate measurement.

2.3.4. Isotherm studies

Experiments were conducted to understand the isotherm behavior of the FCA beads during PO_4^{3-} removal. One batch of FCA beads was used in each batch reactor. The reactors and controls were then rotated at 28 rpm. The phosphate concentration in the bulk solution was

measured after 24 h to calculate the sorption capacity of the beads. Initial concentration of phosphate was varied from 200 to 1300 mg PO_4^{3-}-P/L. The adsorption capacity of the beads were calculated as $q = (C_0 – C_e)*V/m$, where q is the unit mass (mg) of PO_4^{3-}-P per g of FCA bead (dry weight), V is the volume of PO_4^{3-} solution in L and m is dry mass of FCA beads in g.

2.3.5. Interference studies

Effects of possible competing ions and compounds on phosphate sorption by the FCA beads were investigated by adding common coexisting anions and natural organic matter (NOM) to the test solution. Interference studies were carried out with chloride (Cl^-, 50-500 mg/L), bicarbonate (HCO_3^-, 10-100 mg/L), sulfate (SO_4^{2-}, 50-1000 mg/L), nitrate (NO_3^-, 10-100 mg/L as NO_3^--N), humic acid (2 mg/L), and Suwanee River NOM (10-50 mg/L) mixed with the test solution (C_0 = 5 mg PO_4^{3-}-P /L). The specific ion or NOM was first mixed with the PO_4^{3-} solution in a 50 mL plastic tube and one batch of FCA beads was added to it. The reactors were then capped and placed in the end-over-end shaker (28 rpm) for 24 h. The batch studies were carried out at room temperature. Triplicate reactors were run for each study and the average values with standard deviations are reported.

2.3.6. Studies with lake waters

Eutrophic lake water samples were collected in August 2014 from Sarah (45.065771°N, -93.691412°W), Katrina (45.012913°N, -93.623659°W), Minnetonka (44.912489°N, -93.580376°W), Half Moon (45.028521°N, -93.628103°W), and Gleason (44.984119°N, -93.493294°W) lakes located in Minnesota. The lake water samples were filtered using a 0.45 µm pore size cellulose nitrate membrane filter (Whatman, 47-mm-diameter type) and stored in plastic bottles at 4 °C for further analyses. Initial PO_4^{3-}-P concentrations were measured from the lake water samples within 24 h of collection, and batch experiments were then conducted in

triplicate using 50 mL polypropylene plastic tubes as reactors containing 50 mL of lake water and a batch of FCA beads. The reactors were rotated at 28 rpm in the end-over-end shaker and then withdrawn after 24 h. The supernatant from each reactor was filtered through a 0.45 µm filter, and the phosphate concentration was measured.

2.3.7. Studies with wastewater

Wastewater samples were collected from three different locations in Fargo (North Dakota) Wastewater Treatment Plant in August 2015, and treated the same way as in the case of lake waters. Sample collection locations are shown in **Figure 2.1**. The Fargo plant consists of an influent pumping station, screening, grit removal, two pre-aeration channels, seven primary clarifiers, three BOD trickling filters, two intermediate clarifiers, two nitrification trickling filters, one final clarifier, chlorination and dechlorination units (**Figure 2.1**). The BOD trickling filters treat the wastewater biologically. The wastewater is sprayed onto synthetic media stacked to a depth of 15 feet in the tank, where the large surface area of the media offers a place for the aerobic bacteria to grow. After all the carbonaceous material is removed in the BOD filters, the wastewater is directed to the nitrification filters where ammonia and organic nitrogen are removed by a different species of bacteria. Three samples were collected from three points being located after primary clarifiers, after BOD trickling filters and after final clarifiers before chlorination tank. FCA beads were tested for phosphate removal from wastewater. Batch experiments were conducted in triplicate using 50 mL polypropylene plastic tubes as reactors containing 50 mL of wastewater and a batch of FCA beads. The reactors were rotated at 28 rpm in the end-over-end shaker and then withdrawn after 24 h. The supernatant from each reactor was filtered through a 0.45 µm filter, and the phosphate concentration was measured.

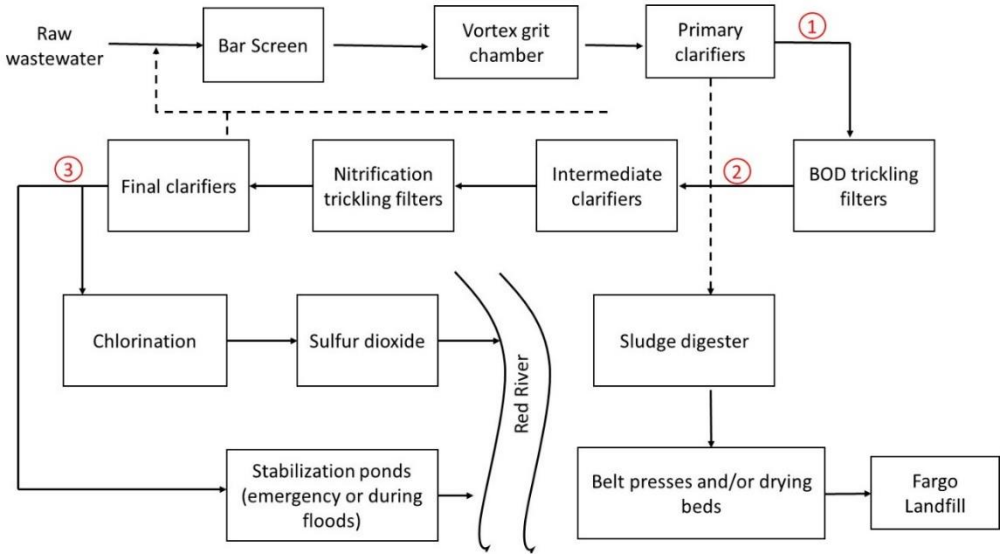

Figure 2.1. A schematic diagram of the Fargo Wastewater Treatment Plant (⟶ : Liquid stream; ⋯▸ : Solid stream; 1 (primary clarifier effluent), 2 (BOD trickling filter effluent), 3 (final clarifier effluent) are the sampling points [adapted from Halis and Eakalak (2012)].

2.3.8. Characterization of alginate beads

Scanning electron microscopy along with energy dispersive spectroscopy (SEM/EDS) was used to observe morphology and characterize the elemental composition of the beads. Fresh (before PO_4^{3-} removal) and spent (after PO_4^{3-} removal) FCA beads were dried overnight in a vacuum oven under a nitrogen environment, and cross sectional samples of the beads were used for imaging and EDS analyses. Dried beads (some intact and some cut in half with a razor blade to reveal the internal structure) were attached to aluminum mounts with carbon tape. To increase conductivity, the beads were coated with a thin layer of carbon using a Cressington 208C carbon coater (TED Pella, Inc., Redding, California). SEM images were obtained with a JEOL JSM-7600F Scanning Electron Microscope (JEOL USA, Inc., Peabody, Massachusetts). Energy dispersive spectroscopy information was acquired using an UltraDry silicon drift X-ray detector and NSS-212e NORAN System 7 X-ray Microanalysis System (Thermo Fisher Scientific, Madison, Wisconsin).

Fourier transform infrared spectroscopic (FTIR) spectra were obtained ex-situ on fresh and spent FCA beads using a Nicolet 8700 FTIR Spectrometer operated with OMNIC software. FTIR was also done on fresh Ca-alginate beads (alginate crossed linked with calcium) for comparison purposes. All spectra were obtained in the range of 4000–400 cm^{-1} using potassium bromide (KBr) as a background. The samples were dried in a vacuum oven under nitrogen environment for 2 days. Pellets were formed by crushing the dried samples with KBr with a mass ratio of 1:10 (sample:KBr). Spectra were recorded at a resolution of 4 cm-1 with each spectrum corresponding to the coaddition of 64 scans. The background collected from KBr was automatically subtracted from the sample spectra. The spectral information was collected and plotted in the same scale on absorbance axis.

2.3.9. Statistical analysis

The results are presented as the mean ± SD (standard deviation, n = 3). The data was checked for homogeneity of variance. One-way analysis of variance (ANOVA) was performed using General Linear Model, followed by a Tukey's pairwise comparison where appropriate. All statistical analysis was performed on Minitab version 17. Significance was determined based on whether p-values were <0.05 or not.

2.4. Results and Discussion

2.4.1. Synthesis and characterization of alginate beads

The synthesized FCA beads (Figure 2.2) were approximately spherical in shape with average diameters of 3.98±0.03 mm (n = 22 batches). Average number of beads produced per batch was 86±6 (n = 26). The average weight of each fresh batch of beads was 1.516±0.080 g (wet) and 0.118±0.008 g (dry, 60 ^{o}C/12h). The dry weight of FCA beads was used for calculating maximum adsorption capacity of the FCA beads.

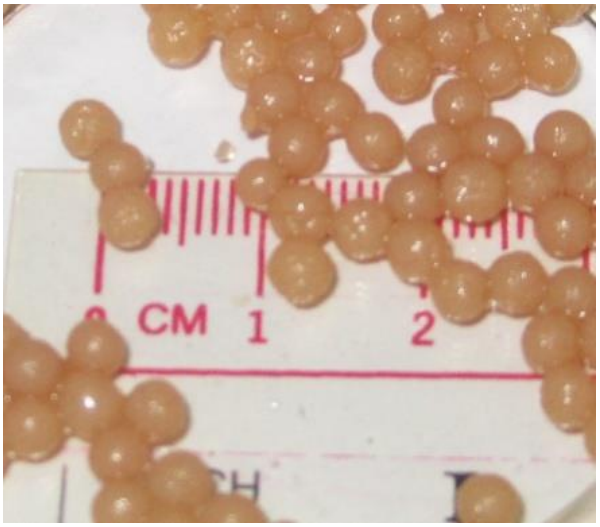

Figure 2.2. Image of the synthesized fresh Fe-cross-linked alginate (FCA) beads. The beads were approximately spherical in shape with average diameters of 3.98±0.03 mm.

Cross-section images were taken at ×80 and ×90,000 magnifications to observe the surface morphologies of the fresh and spent FCA beads. SEM micrographs of fresh (**Figure 2. 3a**) and spent (after phosphate sorption, **Figure 2.3b**) FCA beads indicate that the surface morphology of the beads changed when phosphate was sorbed. An apparent fragile outer layer was formed around the hard core in the spent beads (**Figure 2.3b**). Higher magnification showed nano-sized spheres (average size of 74±35 nm, n = 97) inside the FCA beads (**Figure 2.3c**). The size of nano spheres inside the beads increased marginally to 83± 42 nm (n = 67) after phosphate sorption (**Figure 2.3d**). EDS analysis of fresh beads (**Figure 2.4a**) showed iron, carbon, chloride, calcium, and oxygen while spent beads (**Figure 2.4b**) had phosphorus along with the peaks found in fresh beads.

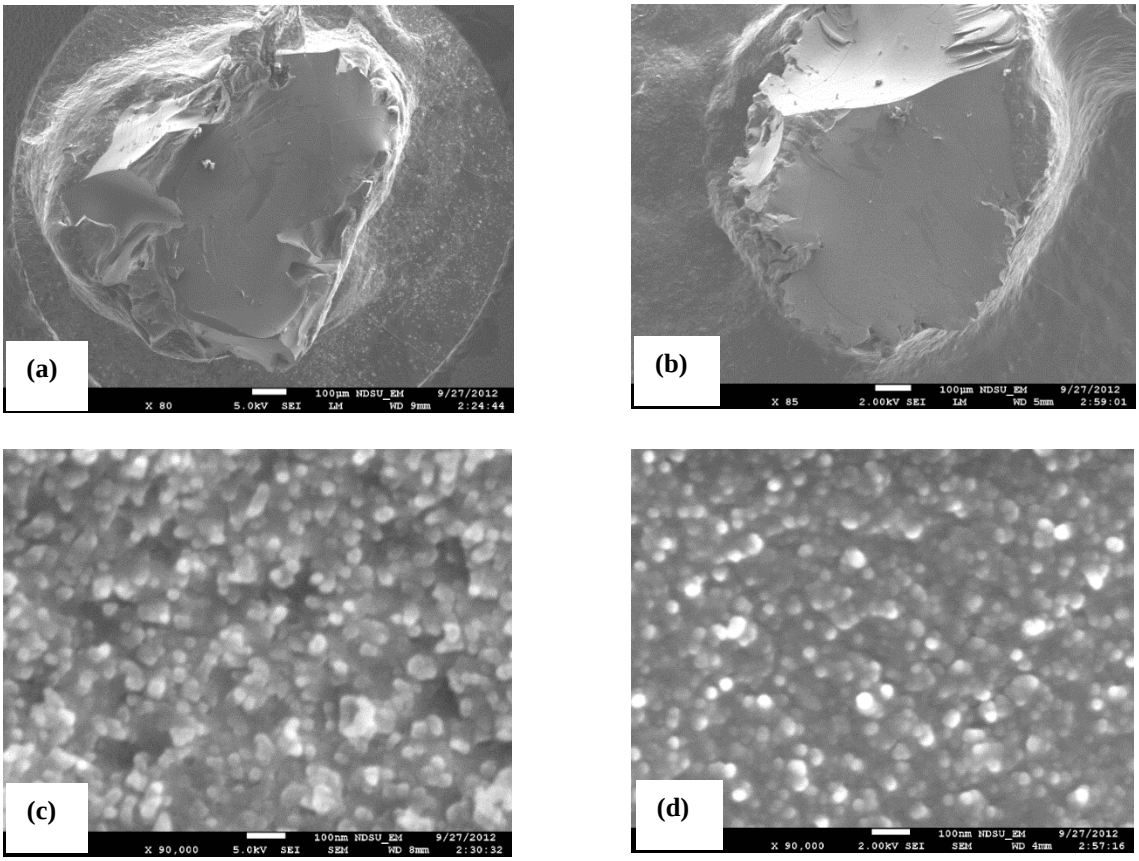

Figure 2.3. (a) SEM image of the surface of a fresh dry FCA bead, (b) SEM image of a used dry FCA bead, (c) SEM image of the cross-section of the center of a fresh dry FCA bead, (d) SEM image of the cross-section of the center of a used dry FCA bead.

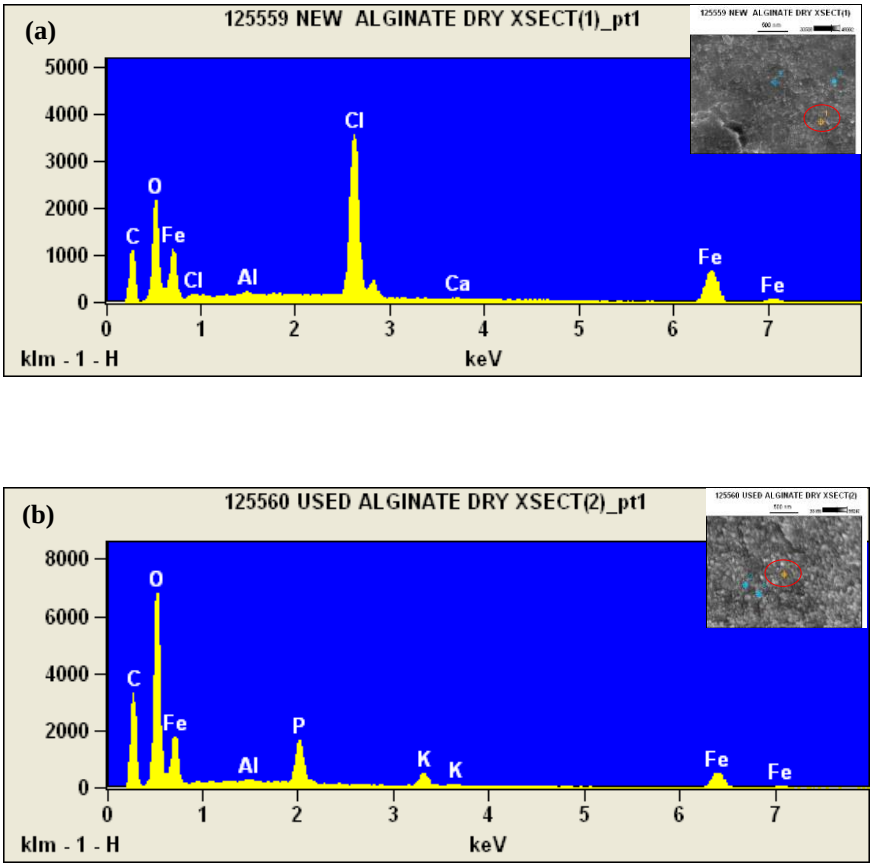

Figure 2.4. (a) EDS spectrum of one point of a fresh FCA bead, and (b) EDS spectrum of one point of a used FCA bead. The iron detected in the bead was probably from both cross-linked as well $FeCl_2$ sorbed onto the beads. Chloride present in the fresh beads might have come from $FeCl_2$ used for bead synthesis. Potassium peak in the spent beads was from KH_2PO_4 used to prepare phosphate solution.

The FTIR data were collected on dried samples to gain information on the molecular structure of fresh FCA beads, spent FCA beads and calcium alginate beads (spectra in **Figure 2.5**). Stretching vibrations of O-H bonds of alginate appeared at 3412 (Ca-alginate bead), 3424 (fresh FCA bead), and 3425 cm^{-1} (spent FCA bead). Singh et al. (2014) reported stretching vibrations of O-H bonds of alginate nanoparticles in the range of 3000-3600 cm^{-1} (Singh, et al., 2014). Stretching vibrations of aliphatic C-H were observed at 2847 and 2921 cm^{-1} in Ca alginate beads, and 2852 and 2923 cm^{-1} in fresh FCA beads. Daemi and Barikani (2012) observed vibrations of aliphatic C-H between 2920 and 2850 cm^{-1} (Daemi and Barikani, 2012).

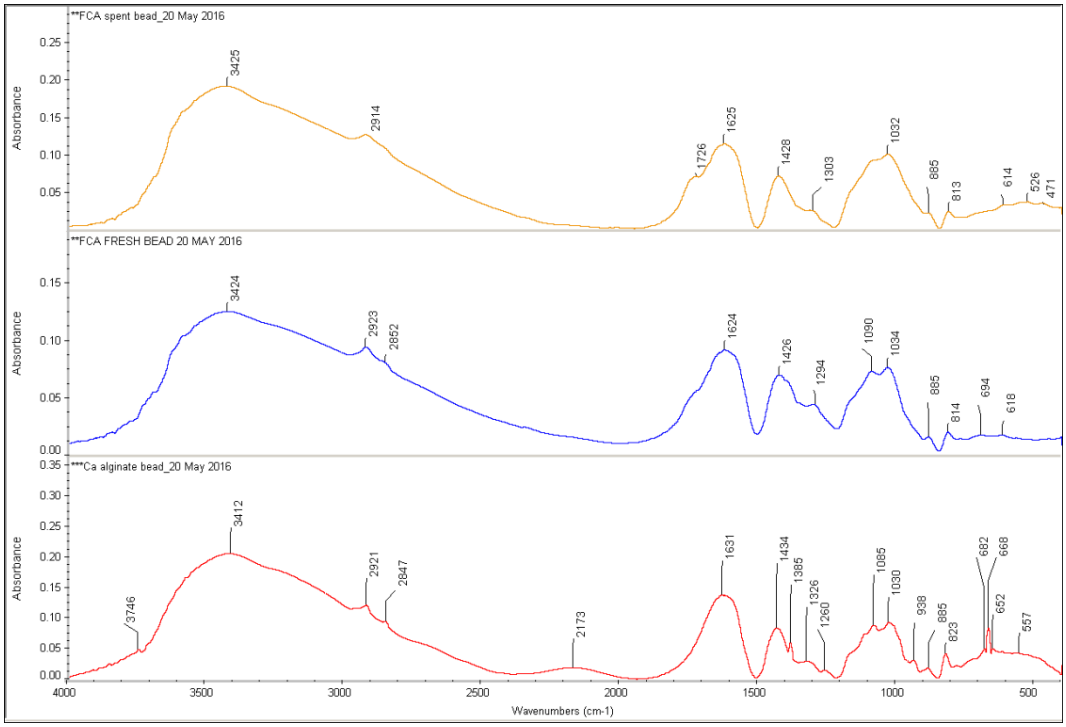

Figure 2.5. FTIR vibrational spectra of fresh FCA beads, spent FCA beads and Ca-alginate beads.

However, in spent FCA beads, there was only one band of C-H stretching vibration at 2914 cm^{-1} indicating there was a slight shift of peak. The peaks at 1631 and 1434 cm^{-1} (Ca alginate), 1624 and 1426 cm^{-1} (fresh FCA) and 1625 and 1428 cm^{-1} (spent FCA) are assigned to the asymmetric and symmetric carboxylate (COO^{-}) vibrations (Wang and He, 2002). Daemi and Barikani (2012) reported asymmetric and symmetric stretching vibrations of carboxylate salt ion in sodium alginate at 1649 and 1460 cm^{-1} (Daemi and Barikani, 2012). Van Hoogmoed et al. (2003) obtained asymmetric and symmetric stretching bands of the COO^{-} group in Ca alginate beads near 1590 and 1410 cm^{-1}, respectively (van Hoogmoed, et al., 2003). The asymmetric stretching vibration of carboxylate ion shifting to lower wave numbers in Ca alginate compared to fresh FCA beads and spent FCA beads is attributed to the difference in charge density, the radius and the atomic weight of the Ca and Fe cations (Daemi and Barikani, 2012). When PO_4^{3-}

is added, the carboxylate band is protonated (COOH) and a new band appears at 1726 cm^{-1} in spent FCA beads. In spent FCA beads two new peaks at 526 and 471 cm^{-1} were observed. The peaks can be assigned to the P–O asymmetric bending of the PO_4 group indicating that there was a formation of iron phosphate compound (Pasparakis and Bouropoulos, 2006). The main peak associated with the P-O is at around 1030 cm^{-1} which is overlapping with other bands of alginates at the same region.

2.4.2. Removal kinetics

Rapid phosphate removal by FCA beads was observed for C_0 = 5 mg PO_4^{3-}-P/L, and about 97% of phosphate was removed within 6 h (**Figure 2.6a**). At 50 mg PO_4^{3-}-P/L, ~76% of phosphate was removed in 6 h, and no additional removal was achieved until the end of 24 h. Removal of phosphate was ~24% after 6 h for C_0 = 100 mg PO_4^{3-}-P/L, and the removal increased to ~46% at 24 h. The data from the batch experiments conducted with initial PO_4^{3-} concentrations (C_0) of 5, 50 and 100 mg PO_4^{3-}-P/L and FCA beads were fitted into zero-, first-, and second-order reaction equations (**Figures A1-A3**). The second order reaction model fitted better for 50 (R^2 = 0.954) and 100 mg PO_4^{3-}-P/L (R^2 = 0.985), and the observed reaction rate constants (k) were found to be 0.0078 and 0.0006 L/mg/min, respectively (**Table A1**). The first order reaction model fitted better for 5 mg PO_4^{3-}-P/L (R^2 = 0.954, k = 0.0091/min).

To investigate efficacy of the FCA beads to remediate phosphate present in low concentration (as in lakes), a 100 µg PO_4^{3-}-P/L test solution was used. About 80% of phosphate was removed within 20 min (**Figure 2.6b**), and then the phosphate concentration remained almost unchanged up to 24 h. The data for this lower concentration also fitted the first order reaction (R^2 = 0.987, k = 0.0828/min, **Figure A4**).

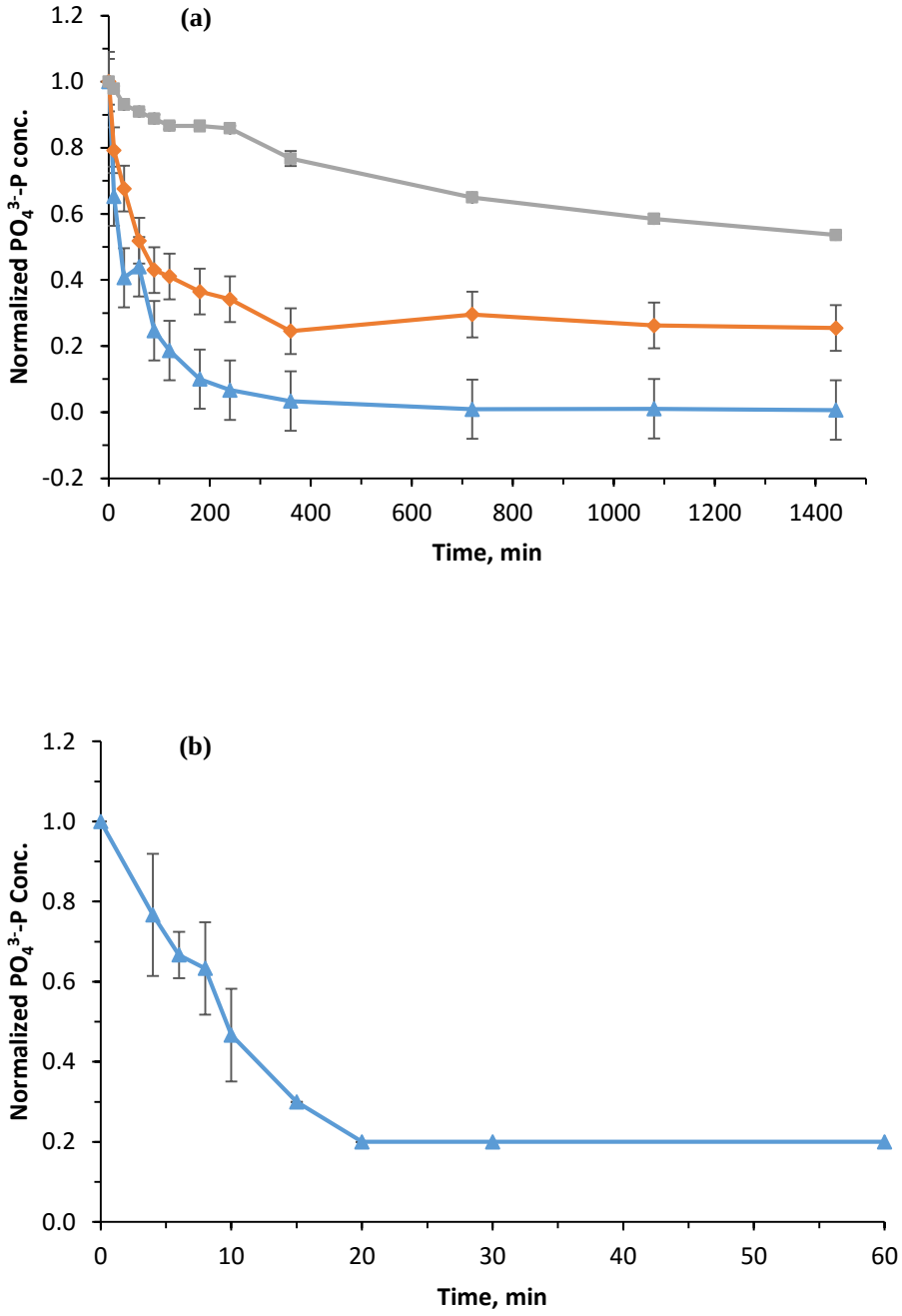

Figure 2.6. (a) Phosphate removal by FCA beads from solutions with different high initial PO_4^{3-} concentration (C_0: ▲: 5 mg PO_4^{3-}P/L; ◆: 50 mg PO_4^{3-}P/L; and ■: 100 mg PO_4^{3-}P/L); (b) low initial PO_4^{3-} concentration (100 µg PO_4^{3-} -P/L). No phosphate removal was observed for any C_0 for the control (blank i.e., phosphate solution with no FCA beads).

2.4.3. Adsorption isotherms

Langmuir, Freundlich, Temkin, and Dubinin-Radushkevich (D-R) isotherm models were tried for the adsorption data obtained using one batch of FCA beads (0.118 g dry weight) with 200-1300 mg PO_4^{3-}-P/L solutions. Langmuir adsorption isotherm model (Langmuir, 1918) (Eq. S1) assumes that all the adsorption sites have equal adsorbate affinity and adsorption at one site is independent of the adsorption at an adjacent site. The bonding to the adsorption sites can be either chemical or physical but strong enough to prevent displacement of the adsorbed molecules.

$$q_e = \frac{bQ_0C_e}{1 + bC_e} \quad \text{(S1)}$$

where C_e is the equilibrium concentration of remaining ions in the solution (mg/L); q_e is the amount of ions adsorbed per mass unit of adsorbent at equilibrium (mg/g); Q_0 is the amount of ions at complete monolayer or the maximum adsorption capacity (mg/g), and b is the Langmuir constant related to the affinity of binding sites (mL/mg) which is a measure of the energy of adsorption.

Freundlich model (Freundlich, 1906) (Eq. S2) considers monomolecular layer coverage of solute by the adsorbent. It also assumes that the adsorbent has energetically heterogeneous surface and has different affinity for adsorption.

$$q_e = K_F {C_e}^{1/n} \quad \text{(S2)}$$

where C_e is the equilibrium concentration of remaining ions in the solution (mg/L); q_e is the amount of ions adsorbed per mass unit of adsorbent at equilibrium (mg/g); K_F and n are constants related to the adsorption capacity and affinity, respectively.

Adsorption data did not fit very well with any of the isotherm equations. The experimental data fitted comparatively better for Freundlich (R^2 = 0.83) and Langmuir (R^2 =

0.77) isotherms (**Figure 2.7**, **Table 2.2**) compared to Temkin and D-R isotherms; the fits for Temkin, and D-R isotherm (R^2 = 0.73 and 0.50, respectively) were poor. An apparent lag phase was observed in the phosphate adsorption when phosphate concentration was increased from 400 mg/L to 600 mg/L. The adsorption capacity of the FCA beads then spiked again. That Freundlich isotherm most closely represented the experimental data suggests that phosphate experienced multilayer adsorption in FCA beads; multiple sorbent sites played a role in the removal of phosphate from aqueous solution. Freundlich isotherm describes sorption behavior better when heterogeneity is present in the adsorbents (Chitrakar, et al., 2006, Ogata, et al., 2011). The value of n in Freundlich model for phosphate removal were >1 (**Table 2.2**) indicating that this isotherm is nonlinear, and that is indicative of adsorption site heterogeneity. Different species of iron (e.g., cross-linked and ionic) might have worked during the sorption process.

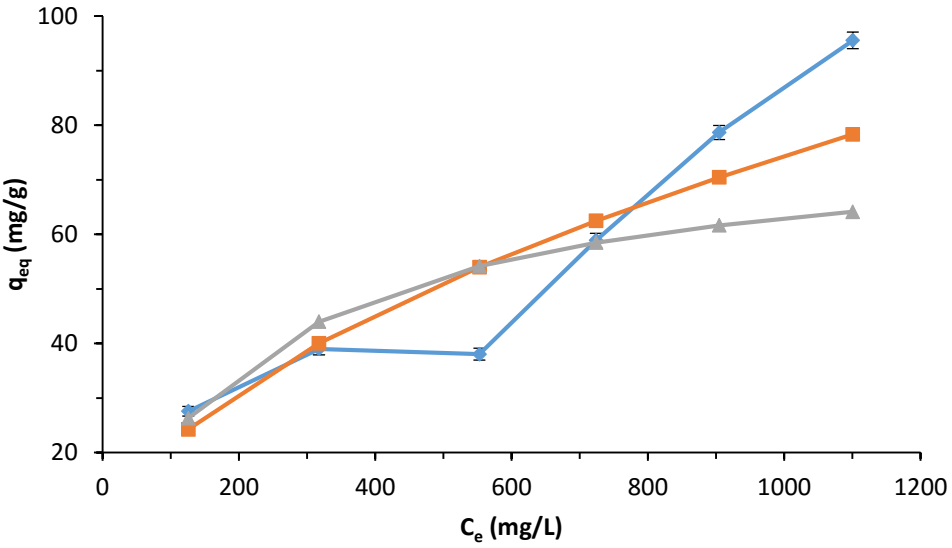

Figure 2.7. Freundlich and Langmuir isotherms models for the PO_4^{3-} removal by wet FCA beads (—◆—, Experimental Data; —■—, Freundlich Equation; and —▲—, Langmuir Equation).

Table 2.2. Isotherm constants and correlation coefficients for the adsorption of phosphate onto iron cross-linked alginate beads.

Model	Parameters	Values
Langmuir isotherm	b (mL/mg)	0.00398
	q_{max} (mg/g)	78.74
	R^2	0.77
Freundlich isotherm	n	1.85
	K_F	1.78
	R^2	0.83
Temkin isotherm	A	118.5
	B	28.11
	R^2	0.73
Dubinin-Radushkevich (D-R) isotherm	q_m	4.12
	B	0.0022
	E (kJ/mol)	0.015
	R^2	0.50

Adsorption heterogeneity was confirmed by another experiment where batches of fresh beads were put in 50 mL deionized water in 50 mL polypropylene vials. Multiple vials (units) were prepared and they were put in the end-over-end shaker. Units were withdrawn after 24 h and the beads were filtered out. The bulk solution was found to be reddish in color indicating leaching out of iron from the beads. The beads from three units were kept separately for phosphate removal studies. The beads from other units were put back to the vials (after cleaning them thoroughly) and fresh deionized water (50 mL) was added to each unit. They were again rotated in the shaker for another 24 h and filtration of the beads were repeated. Beads from three vials were again kept aside for phosphate removal studies and others were put back to the shaker after replacing the bulk water with DI water. The rest of the beads (3 vials) were withdrawn at 72 h. The beads collected at 24, 48 and 72 h were used for phosphate removal studies. Each batch of beads (3 each collected at 24, 48, and 72 h) were transferred to a 50 mL vial. Another 3 vials

were prepared with fresh bead (1 batch in each vial). The phosphate removal studies were conducted in the 50 mL vial with 50 mL solution with an initial phosphate concentration of 100 mg PO_4^{3-}-P/L. The vials (in triplicate) were rotated in the end-over-end shaker for 24 h. The removal percentage was 50%, 47% and 44% for beads shaken for 24 h, 48 h and 72 h, respectively compared to 64% for fresh beads (**Figure 2.8**). Tested by one-way ANOVA, phosphate removal percentages were found to differ significantly (p = 0.000) among the beads. The FCA beads which were not subjected to shaking removed significantly higher amount of phosphate compared to the other FCA beads which were shaken for different period of time. Different phosphate removal capacity of the FCA beads confirms that the phosphate removal mechanism involved two different types of iron sorbents, the iron involved in crosslinking and the physically-adsorbed iron which was leaching out of the beads during the shaking process. However, bulk of the phosphate was removed by the cross-linked iron. There might also have been some strongly physically-adsorbed iron in the beads even after shaking. The results from this experiment and the isotherm studies (where data fitted into the Freundlich equation well) conclusively prove that the removal of phosphate by FCA beads is achieved by cross-linked iron and physically-adsorbed iron.

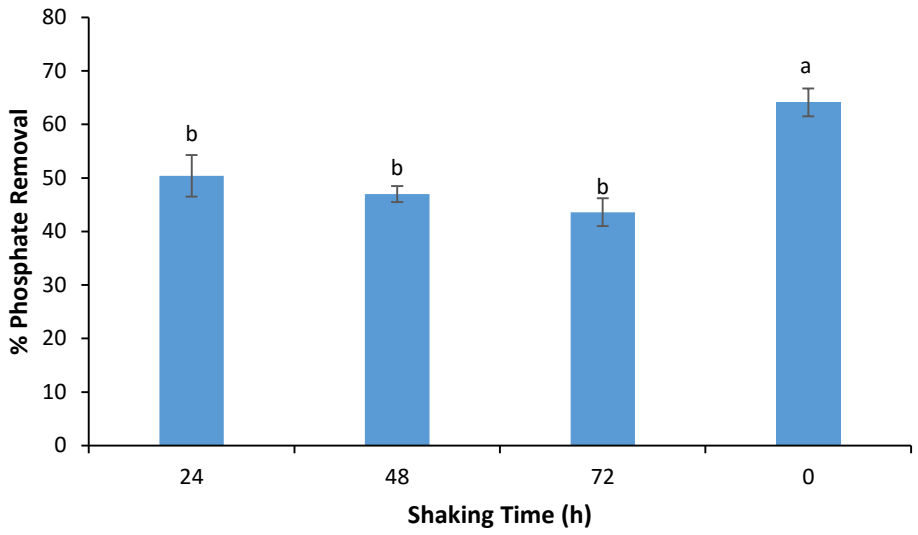

Figure 2.8. Phosphate is removed by cross-linked and physically-adsorbed iron in FCA beads. Beads lose physically-adsorbed iron when they are placed under shaking condition. As a result, phosphate removal is reduced. Initial PO_4^{3-}-P conc. was 100 mg/L. Different letters above bars indicate significant differences between different treatments.

From Langmuir isotherm model, maximum sorption capacity was found to be 78.7 mg/g (dry weight) of FCA beads. Choi et al. (2012) used Freundlich isotherm model to describe PO_4^{3-} adsorption behavior onto sulfate-coated adsorbents (zeolite, hydrotalcite, and activated alumina) (Choi, et al., 2012), whereas adsorption behavior of the same material without coatings were described better by Langmuir isotherm model.

2.4.4. Interference studies

Effect of the presence of Cl^-, HCO_3^-, SO_4^{2-}, NO_3^-, NOM and humic acid on PO_4^{3-} (C_0 = 5 mg PO_4^{3-}-P/L) removal by FCA beads was examined. The phosphate removal percentages varied from 96.3-98.3% in the presence of competing anions and compounds. Tested by one-way ANOVA, phosphate removal percentages were found to differ significantly (p = 0.000) in the presence of competing anions and compounds. Even though the differences are statistically significant compared to the control, the interferences are minimal considering the removal

percentages in the presence and absence of these ions and compounds (**Figure 2.9**). The ions used in this interference study are usually present in surface waters as well as wastewater and groundwater. Other researchers reported a 78% reduction in PO_4^{3-} removal by slag microspheres in the presence of HCO_3^- (Lee, et al., 2012). The addition of SO_4^{2-} also decreased the PO_4^{3-} removal efficiency by ~60% in a polymer-based nanosized hydrated ferric oxides system (Pan, et al., 2009), and the efficiency reduction was 24.5 % in a layered double hydroxides system (Das, et al., 2006). The presence of SO_4^{2-} and Cl^- negatively impacted the PO_4^{3-}reomval from lake water when high gradient layered magnetic separation was used (de Vicente, et al., 2011). In the presence of NO_3^-, PO_4^{3-} removal decreased by 29.2% with double hydroxides (Das, et al., 2006) as the adsorbent and by 6.27% while NZVI (Almeelbi and Bezbaruah, 2012) was used. NOMs are present in surface waters, and known to interfere with PO_4^{3-} removal in adsorption processes (de Vicente, et al., 2011, Guan, et al., 2006). However, no effect of NOM on PO_4^{3-} removal was observed in this study. Similar findings were reported earlier with NZVI (Almeelbi and Bezbaruah, 2012). The lack of interference by the dominant ions and NOM makes an FCA bead system a potential candidate for field application for PO_4^{3-} removal.

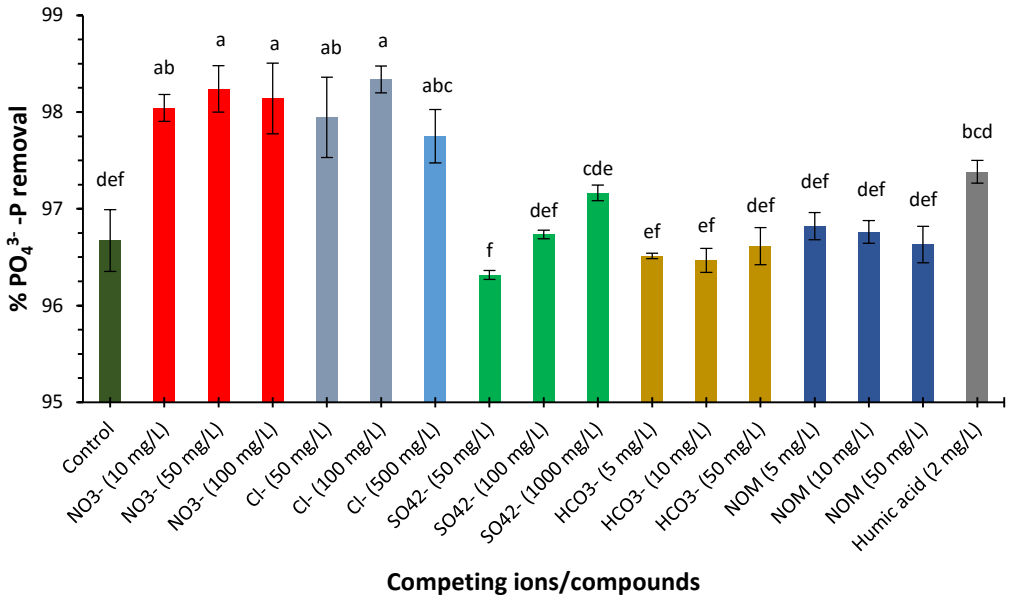

Figure 2.9. Effects of competing compounds on phosphate removal by FCA beads (C_0 = 5 mg PO_4^{3-}-P/L, contact time = 24 h). The control is only PO_4^{3-} solution prepared in DI water and treated with FCA beads. Different letters above bars indicate significant differences between different treatments.

2.4.5. Effect of pH and point of zero charge (PZC)

The effect of pH on phosphate removal (C_0 = 5 mg PO_4^{3-}-P /L) by FCA was investigated at pH of 4, 5, 7, 8 and 9 (**Figure 2.10**). The phosphate removal percentages varied from 95.1-96.2% at different pHs. Tested by one-way ANOVA, phosphate removal percentages were found to differ significantly (p = 0.002) at pH 8 compared to other pHs. However, the removal of phosphate achieved at different pHs was satisfactory and the efficacy of these FCA beads is likely to be unaffected under the range of pH tested. pH not affecting the PO_4^{3-} removal efficiency of FCA beads has important practical implications. The pH in eutrophic lakes ranges from 7.5 to 8.5 (Michaud, 1991). The point of zero charge (PZC) of the beads was found to be 9.2 (**Figure 2.11**) and that explains why the beads were effective even at high pH. When the solution pH is less than 9.2, the FCA beads remain positively charged allowing the sorption of

negatively charged phosphate (PO_4^{3-}). Given their effectiveness at high pH, FCA beads will work well for phosphate removal in eutrophic lakes.

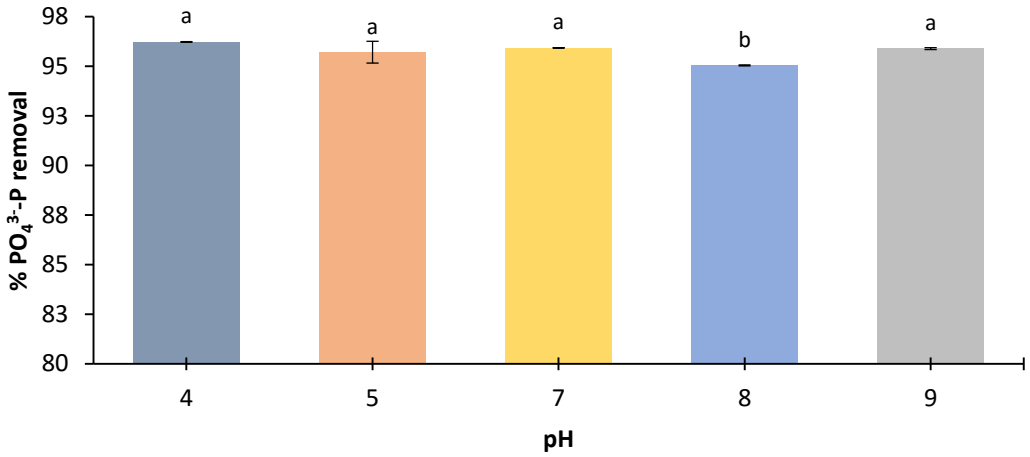

Figure 2.10. PO_4^{3-} removal using FCA beads at pH 4, 5, 7, 8 and 9 (C_0 = 5 mg PO_4^{3-}-P/L, contact time= 24 h). Different letters above bars indicate significant differences between different treatments.

PZC is defined as the pH value at which the charge of the solid surface is zero. Point of zero charge (PZC) was determined for the FCA beads following the potentiometric mass titrations technique (Vakros, et al., 2002). In brief, three solutions were prepared with 3 mL of 0.1 M KNO_3 and 6 mL of DI water in 50 mL polypropylene tubes and their pH values were measured immediately. Three different amounts of dry FCA beads (0.0254 g, 0.0384 g and 0.0488 g) were added into those three polypropylene tubes, followed by the addition of 1 mL of 0.01 M KOH. Then the samples were titrated with 0.01 M HNO_3 and the results were plotted. The PZC of FCA beads was determined as 9.2 based on the point where the three titration curves intersected (**Figure 2.11**). A PZC of 9.2 explains the mechanism of phosphate removal by FCA beads.

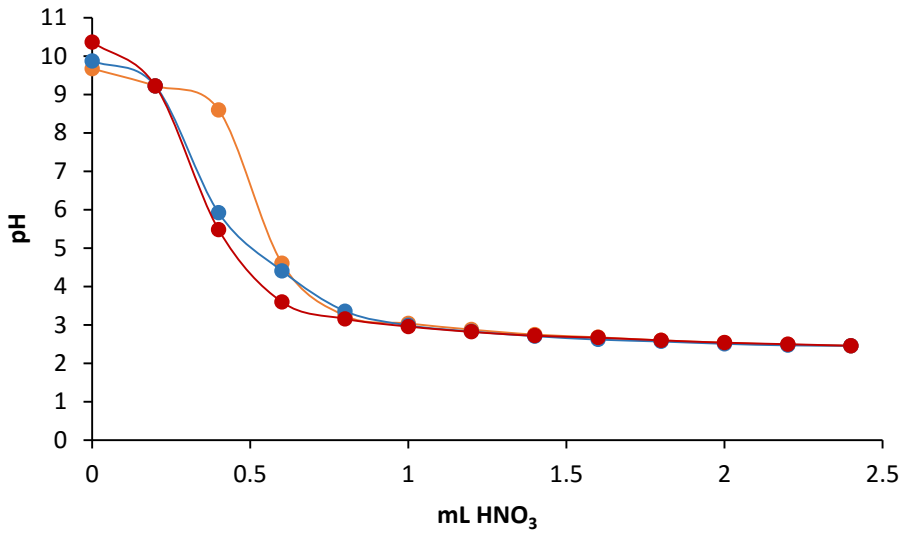

Figure 2.11. Potentiometric mass titrations technique for the determination of the point of zero charge (PZC) of FCA beads. Three curves have a common intersection point at pH 9.2; at this point FCA beads do not have any charges (—●—, 0.0254 g; —●—, 0.0384 g; and —●—, 0.0488 g).

2.4.6. Studies with lake waters

To test the efficacy of FCA beads in eutrophic lake waters, batch studies were conducted with waters from five eutrophic lakes located in Minnesota. The characteristics of lake water are given in Table 2.3. The initial average PO43--P concentrations were 20.1 µg/L at Sarah, 69.2 µg/L at Katrina, 20.1 µg/L at Minnetonka, 11.2 µg/L at Half Moon, and 26.8 µg/L at Gleason lakes. The concentration of PO43--P in the treated Katrina water was 13.4 µg/L after 24 h (~81% removal). For the other four lakes, PO43--P in treated water was below instrument detection limit (Figure 2.12).

Table 2.3. Characteristics of lake water tested for phosphate removal (adapted from Minnesota Pollution Control Agency).

Name	DO (mg/L)	Total P (mg/L)	Turbidity (Secchi depth) (m)
Sarah	12.03	0.154	0.4
Katrina	7.03	0.221	1.50
Minnetonka	9.06	0.047	2.5
Half Moon	7.12	0.265	1.60
Gleason	8.89	0.035	1.0

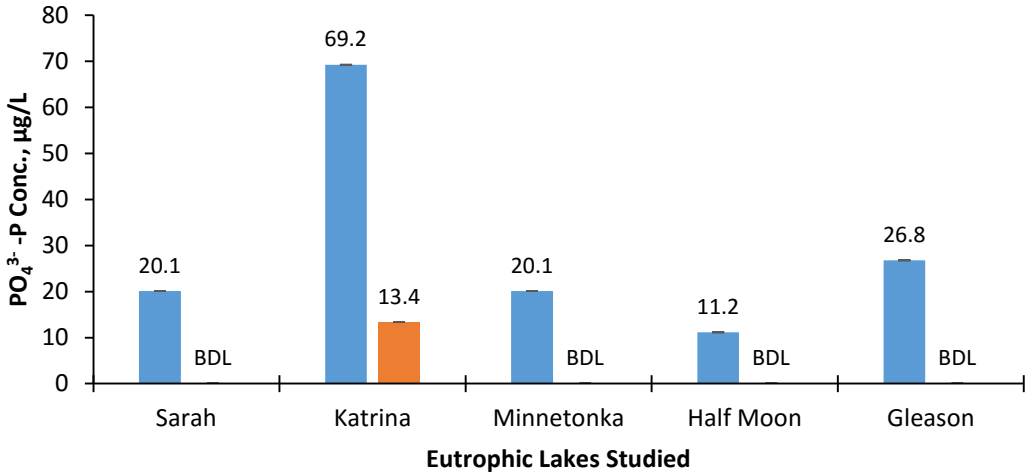

Figure 2.12. PO_4^{3-} removal from eutrophic lakes using FCA beads (■: initial PO_4^{3-}-P conc; ■: final PO_4^{3-}-P conc; Contact time = 24 h); BDL denotes below detection limit.

2.4.7. Studies with wastewater

FCA beads were also tested with wastewater to see their efficacy. The characteristics of wastewater are given in **Table 2.4**. Initial PO_4^{3-}-P concentrations were 4.7 for primary clarifier effluent, 5.3 for BOD trickling filter effluent, and 4.8 for final clarifier effluent. Batch studies were conducted with 50 mL of wastewater samples from each point and 1.516 g of wet FCA beads in 50 mL vials. After 24 h reaction, the concentrations were 1.6 (65% removal), 1.9 (65%), and 2.7 (45%) PO_4^{3-}-P mg/L for primary clarifier effluent, BOD trickling filter effluent, and final

clarifier effluent, respectively (**Figure 2.13**). The phosphate removal was less in the final clarifier effluent compared to the BOD trickling filter effluent. It could be due to the higher concentration of NO_3-N in the final clarifier effluent. The removal capacity of FCA beads was less for wastewater samples compared to artificial samples. This phenomenon is in line with other researchers' findings (Jellali, et al., 2010, Karthikeyan, et al., 2004). Jellali et al. (2010) attributed this phenomenon to anionic competition, possibly from chloride or sulfate ions.

Table 2.4. Characteristics of wastewater tested for phosphate removal (adapted from Fargo Wastewater Treatment Facility Report).

Description	pH	TBOD (mg/L)	COD (mg/L)	NH_3-N (mg/L)	NO_3-N (mg/L)	Alk (mg/L)
Primary clarifier effluent	7.46	204	442.9	25.7	-	-
BOD trickling filter effluent	7.08	35	110.3	11.3	9.6	224
Final clarifier effluent	7.57	9.3	48.1	0.7	20	156.4

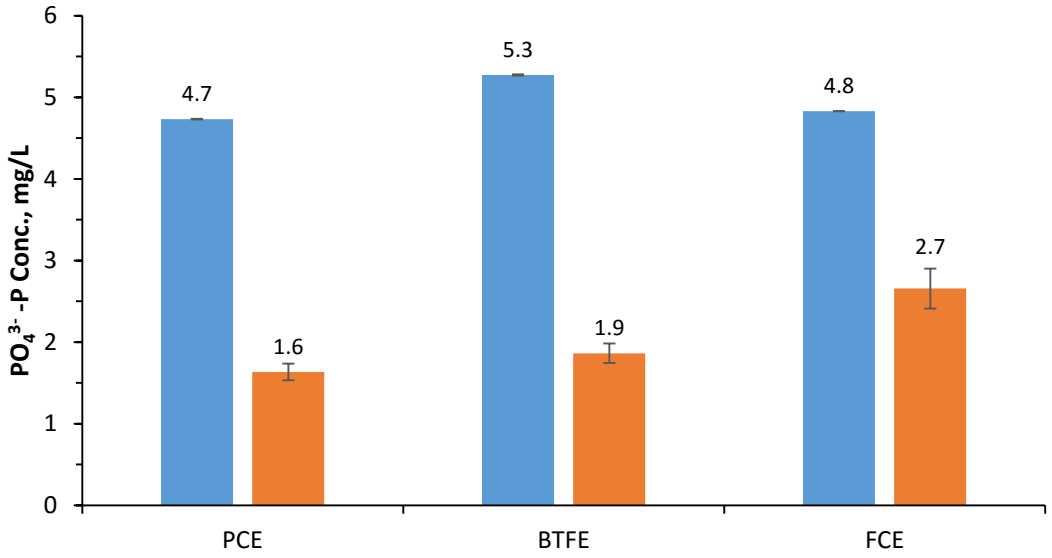

Figure 2.13. PO_4^{3-} removal from wastewater using FCA beads (■: initial PO_4^{3-}-P conc.; ■: final PO_4^{3-}-P conc.; Contact time = 24 h). PCE stands for primary clarifier effluent, BTFE for BOD trickling filter effluent, and FCE for final clarifier effluent.

2.5. Environmental Significance

2.5.1. Eutrophic lake remediation

The PZC of the FCA beads is 9.2, making them ideal for lake applications. The beads are very effective as there was no interference in phosphate removal in the presence of Cl^-, HCO_3^-, SO_4^{2-}, NO_3^-, NOM and humic acid (**Figure 2.9**). About 80% removal of aqueous phosphate was achieved within 20 min at the environmentally relevant concentration of 100 µg/L (**Figure 2.6b**). Further, PO_4^{3-} in actual lake waters were removed up to 100% (**Figure 2.12**). Based on the sorption capacity of 78.7 mg PO_4^{3-}-P/g (dry weight) of beads, the total amount of beads needed to remediate a 10-acre lake (4.05 hectares, 10 m deep, 100 µg PO_4^{3-}-P/L) is 360 kg (dry weight) of beads to reduce phosphate concentration to non-eutrophic level (≤ 30 µg PO_4^{3-}-P/L) (detailed calculation in **Table 2.5**).

Assuming a factor of safety of 4 (i.e., beads are only 25% efficient in lakes), total amount of beads (= 1,440) ≅ 1,500 kg. Total cost of beads = 1,500 kg × \$7/kg (cost analysis details in **Table 2.6**) = \$10,800. With O&M and labor costs (100% of bead cost) of \$10,800, the total cost for phosphate remediation of the 4.05-hectare eutrophic lake is (\$10,800+\$10,800 =) \$21,600. Cost per hectare-m of lake is \$533 (or per acre-ft is \$72). The beads can be placed in porous pouches within the lake while the naturally occurring underwater current and waves are expected to keep the loosely packed beads in direct contact with water. This naturally stirred system should also reduce mass transfer resistance. The beads (containing sorbed phosphate) can be collected back as spent beads.

Table 2.5. Detailed calculations* for amount of beads needed to remediate a eutrophic lake.

Design Data:
Lake area = 10 acres (4.05 hectare)
Depth =30 ft (10 m)
Current Lake PO_4^{3-}-P concentration = 100 µg/L (eutrophic)
Target Lake PO_4^{3-}-P concentration of 30 µg/L (non-eutrophic)
Sorption capacity of the beads = 78.7 mg PO_4^{3-}-P/g (dry weight) of beads
Assumed factor of safety (FOS) = 4 (i.e., beads are only 25% efficient in lakes)
FCA Bead weight 1.000 kg dry weight = 12.847 kg wet weight (based on each batch of FCA beads weighs 1.516±0.080 g (wet weight) or 0.118±0.008 g (dry weight)
Calculations:
Volume of lake/Volume of water to be treated = 405,500 m^3 = 405,000,000 L
The amount of PO_4^{3-}-P to be treated in the lake = 405,000,000 L × (100 µg/L−30 µg/L) = 28,350,000,000 µg = 28,350,000 mg
The amount of beads needed for = (28,350,000 mg × g of beads/78.7 mg) = 360,229 g of beads ≅ 360 kg of beads (on dry weight basis)
Applying the FOS of 4, the total amount of beads needed = (360 × 4) kg (= 1,440) ≅ 1,500 kg (on dry weight basis)
Amount of beads needed = 1500 x 12.847 = 19, 270 kg (on wet weight basis)

*The calculations were done not taking into account the sediment phosphorus which might replenish the depleting phosphate content of lake water during the removal process.

Table 2.6. Cost calculation for eutrophic lake remediation.

Available Data:
The cost of sodium alginate = $5512 per metric ton (Qingdao Yijia Huayi Import and Export Co., Ltd.)
Cost of $FeCl_2$ = $496 per metric ton (Wuhan Golden Wing Industry & Trade Co. Ltd.).
Each batch of alginate was produced using 5 mL of 2% alginate solution and 30 mL of 2% $FeCl_2$ solution (maintaining a molar ratio of 6.7:1 between Fe and sodium alginate)
The dry weight of each batch is 0.118±0.008 g
O&M and Labor costs =100% of bead cost
Lake size = 10 acres (4.05 hectares), 30 ft (10 m) deep
Current Lake PO_4^{3-}-P concentration = 100 µg/L (eutrophic)
Target Lake PO_4^{3-}-P concentration of 30 µg/L (non-eutrophic)
Calculations:
Cost involved in producing 0.118 g of dry beads = ($0.00992/1000 mL of $FeCl_2$) × 30 mL + ($0.1102/1000 mL of sodium alginate)× 5 mL = $0.0002976 + $0.000551 = $0.000849.
Cost of 1 g of dry beads will cost = $0.000849/0.118 = $0.0072
Cost of 1 kg of beads will cost = $0.0072 × 1000 = $7.20
Cost of beads needed to remediate a 4.05-hactare (10 m deep) lake = 1500 kg × $7.20/kg = $10,800.
O&M and Labor costs (= would be 100% of bead cost) = $10,800
Total cost to remediate a 4.05-hectare area lake = $10,800+$10,800 = $21,600
Cost per hectare-meter (lake total = 40.5 hectare-m) = $21,600/40.5 = $533.33 ≅ $533.00
Cost per acre-foot (lake total = 300 acre-ft) = $21,600/300 = $72.00

2.5.2. Sorption capacity and cost of phosphate removal

The adsorption capacity of the adsorbents reported by others was 2-58 mg/g of sorbent (Choi, et al., 2012). The cost of phosphate removal using techniques reported by others varied from $2 to $1775 per g P removed (Choi, et al., 2012). The current FCA beads have a very high sorption capacity of 78.7 mg/g of beads which is 1.36-39 times higher than those reported. The cost of production of the beads is only $0.0072 per g of beads as compared to $0.06-28.77 per g (Choi, et al., 2012) of other sorbent. Higher sorption capacity and lower cost of production make the FCA very competitive with a cost of only $0.09 per g P removed. Assuming a factor of safety

of 4 (i.e., adsorption capacity is only 25% of the experimental value) the cost comes to $0.36 per g P removed (**Table 2.7**) and, thus, remain very affordable.

Table 2.7. Comparison of different sorbents for sorption capacity and cost-effectiveness.

Sorbents	Cost of production per g of sorbent ($)	Max adsorption capacity (mg g^{-1})	Unit price for removal per 1 g P ($)
Layered double hydroxides	28.77	47	612*
Sulphate-coated zeolite	0.06	30	2*
Ion-exchange resin	3.55	2	1775*
Titanium mesostructure	4.25	51	83*
Hydrotalcite	1.82	58	31*
FCA beads (this study)	**0.0072****	**79**	**0.36*****

***Table adapted from Choi et al. (2012)
**Refer to Table 2.6
****A factor of safety of 4 is used meaning the beads are assumed to remove phosphate at only 25% of the current value (from batch studies). Assuming 25% efficiency of phosphate removal means that the maximum adsorption capacity of the FCA beads will be 19.75 mg/g (dry weight) of FCA beads.

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3. PHOSPHATE-CONTAINING BIOPPOLYMER BEADS AS SLOW-RELEASE PHOSPHORUS AND IRON FERTILIZERS

3.1. Abstract

An experiment was conducted to evaluate the potential use of phosphate-laden spent iron cross-linked alginate (FCA) beads biopolymer as slow-release phosphorus and iron fertilizers using lettuce (*Lactuca sativa*) as a model plant. Parris Island variety of lettuce was hydroponically grown in a custom-made growth chamber with three treatments, namely spent FCA beads + modified Hoagland solution containing no Fe and P, spent FCA beads + modified Hoagland solution containing no P, spent FCA beads + modified Hoagland solution containing no Fe, and one control (only Hoagland solution containing all the nutrients). Lettuce was grown until maturity and iron and phosphorus contents were measured. Spent FCA beads were found to support biomass production across the treatments indicating that the phosphorus and iron were available for plant uptake. The uptake of iron was significantly higher in the plants treated with spent FCA beads + modified Hoagland solution containing no Fe and P, and spent FCA beads + modified Hoagland solution containing no P compared to the control. Phosphorus uptake in the plants treated with spent FCA beads + modified Hoagland solution (containing no Fe) was comparable to the control plants. The bioavailability of P and Fe in the spent beads is promising and it suggests that spent FCA beads can be recycled back directly to soils as a slow-release P and Fe fertilizer which could act as supplementary sources of these elements in agriculture.

3.2. Introduction

Phosphorus is an essential element for the growth of organisms and plants and is also an indicator for the quality of surface water (Pierzynski, 2005). Phosphate may also impair water quality in waterbodies (Barlow, et al., 2005). Excessive phosphorus stemming from point and

non-point sources find its way into natural waters and triggers eutrophication (Smith, 2003). The USA suffer an economic loss of $2.2 billion annually as a result of eutrophication in its freshwaters (Dodds, et al., 2009).

On the other hand, modern agriculture is heavily dependent on phosphate fertilizer derived from phosphate rock, which is non-renewable in nature. It is estimated that at the present rate of consumption, the global phosphate reserves may be depleted in 50-100 years (Cordell, et al., 2011). As mineable phosphorus is declining, it will need to be replaced with new sources of phosphorus (Rittmann, et al., 2011). Recovering lost phosphate (the phosphate that has been lost from the agricultural system) from the eutrophic waterbodies and wastewater treatment plants can be a viable solution to the phosphorus scarcity problem. Currently, chemical precipitation involving divalent or trivalent metal ions is a common practice adopted to reclaim phosphate from wastewater (Morse, et al., 1998). Magnesium- and calcium-based precipitation products are commonly used as fertilizer because recovered phosphate is less tightly bound to these metal ions (Morse, et al., 1998). Phosphate recovery from wastewater as struvite (also called magnesium ammonium phosphate hexahydrate, $MgNH_4PO_4 \cdot 6H_2O$) is a fairly common practice (Rittmann, et al., 2011); struvite precipitation readily occurs when phosphate concentration reaches 100-200 mg/L and ammonium is present in the solution (Kataki, et al., 2016). However, the technologies which are employed in wastewater treatment plant are not applicable for eutrophic waterbodies because of the very low concentration of phosphate in these waters. Sorption is regarded to be an effective technology for reclaiming phosphate at low concentrations (Loganathan, et al., 2014). It is, therefore, very important to find out an effective sorbent to reclaim phosphate from eutrophic waterbodies which can be safely disposed of in soil for agricultural purposes.

FCA beads were used as a sorbent for aqueous phosphate removal (**Chapter 2**). Findings from batch studies suggested that FCA beads can adsorb phosphate effectively. The maximum phosphate sorption capacity was found to be 78.7 mg PO_4^{3-}-P/g of dry beads. The present study was undertaken to explore the potential use of spent FCA beads (the beads used for phosphate removal) as a supplementary source of phosphorus and iron (iron is present as a cross-linker and also as adsorbed ions) using lettuce (*Lactuca sativa*) as a model plant. PO_4^{3-}-P concentration at 0.2 mg/L in soil solution is required for plants for optimum growth (Pierzynski, 2005). However, PO_4^{3-}-P concentrations as low as 0.03 mg/L are adequate to produce high yields of some agronomic crops (Pierzynski, 2005). The hypothesis of the present work is that the spent FCA beads would provide plants with the required amount of phosphate and it is also hypothesized that iron present in the beads will also be released for plant uptake. The main objective of this study was to determine whether spent FCA beads used to reclaim phosphate from eutrophic waters can be applied to soils as a P and Fe fertilizer.

3.3. Materials and Methods

3.3.1. Chemicals

Iron(II) chloride tetrahydrate (FeCl2·4H2O, reagent grade, Alfa Aesar), sodium alginate ((C6H7O6Na)n, reagent Grade, Spectrum), calcium nitrate tetrahydrate (CaNO3·H2O, Alfa Aesar), potassium nitrate (KNO3, Mallinckrodt Chemicals), magnesium sulfate heptahydrate (MgSO4·7H2O, Mallinckrodt Chemicals), magnesium nitrate hexahydrate (Mg(NO3)2·6H2O, Alfa Aesar), ammonium dihydrogen phosphate (NH4H2PO4, Alfa Aesar), sodium tetraborate decahydrate (Na2B4O7·10H2O, amresco), copper(II) sulfate pentahydrate (CuSO4·5H2O, BDH), manganese sulfate monohydrate (MnSO4·H2O, Mallinckrodt Chemicals), sodium molybdate dihydrate (Na2MoO4·2H2O, BTC), zinc sulfate monohydrate (ZnSO4·H2O, J.T.

Baker) and ethylenediaminetetraacetic acid, ammonium nitrate (NH4O3, ACS grade, Alfa Aesar), and iron(III) monosodium salt (FeNa(O2CCH2)2NCH2CH2N(CH2CO2)2, ACS Grade, Alfa Aesar) were used as received unless otherwise specified.

3.3.2. Synthesis and preparation of spent FCA beads

FCA beads were synthesized using sodium alginate and ferrous chloride (**Chapter 2**). Sodium alginate (20g) was dissolved in 1 L of water at 60 °C overnight under continuous stirring. FCA beads were synthesized by adding the sodium alginate solution drop by drop to ferrous chloride solution (2%). Then the beads were kept submerged in sufficient amount of ferrous chloride solution for 24 h. This was done to harden the beads and to continue the ion exchange process. The ion exchange process enables the divalent ions crosslink the copolymers of alginate (Min and Hering, 1998). After separating the hardened beads using a strainer, the beads were washed three times with copious amount of water and stored in vials in DI water for further use. Phosphate was then loaded onto and into the beads by running a phosphate sorption experiment. For phosphate loading, 100 mg/L PO_4^{3-}-P solution was used. A measured amount (10 g) of fresh FCA beads (corresponding to 0.78 g of dry beads) were put in a 500 mL of plastic bottle and 250 mL of 100 mg/L of PO_4^{3-}-P solution was added to the beads. Then the bottles were put in an end-over-end shaker for 24 h. After 24 h, the beads were separated from the bulk solution and kept for use in the plant study. The phosphate (as PO_4^{3-}-P) concentration in bulk solution was measured at 0 h and 24 h and a mass balance was done to find the amount of phosphate adsorbed onto the beads.

3.3.3. Desorption study

A desorption study was conducted to see the release pattern of adsorbed phosphate from the spent FCA beads. First a single batch of FCA beads (wet and dry weights are 1.516±0.080 g

and 0.118±0.008 g, respectively) was added to 50 mL of 50 mg/L PO_4^{3-} -P solution. Polypropylene tubes were used as reactors for the sorption study. The reactors were rotated in a custom-made end-over-end shaker for 24 h. After 24 h, the reactors were taken out and the beads were separated from the solution. Then 50 mL of DI water was added to the spent or used FCA beads. The reactors were allowed to stand for 4 d (this constituted one cycle). After 4 days, solution was separated from the beads and tested for phosphate content. The beads were immediately added to 50 mL of DI water in 50 mL vials and were allowed to sit for another 4 days. This process was continued for 28 days and every 4 days the solution was replaced with new DI water. The bulk solution was tested for phosphate content.

3.3.4. Plant growth conditions

3.3.4.1. Germination and plant preparation for hydroponic experiment

Lettuce seeds of Parris Island variety (*Lactuca sativa*, Burpee, Warminster, PA) were purchased from a local outlet. The lettuce seeds were germinated in plug trays with nonabsorbent cotton as media (**Figure 3.1**). The seeds were kept moist using automated misting nozzles in a greenhouse. The germinated seeds were then moved to another room and allowed to grow for ten days. The seedlings were routinely fed with half strength Hoagland solution (Hoagland and Arnon, 1950). The seedlings were provided with cool-white fluorescent light (14 h light/10 h dark cycle). The light intensity was ~100 µmol/m^2/s.

(a)

(b)

Figure 3.1. (a) Lettuce seeds were germinated in a plug tray (b) Schematic of the plug tray.

3.3.4.2. Hydroponic studies

After plants developed a root system and were at four-to-five-leaf stage, healthy plants of similar size were carefully removed from the plug trays and the roots of the plants were rinsed with copious amount of deionized water, keeping the roots unharmed. Plants were then anchored in nutrient solutions contained in 1 L plastic tumblers. One lettuce seedling was placed into a Styrofoam disc float (a hole was made in the disc float using a hole punch) with their roots below the disc and the shoots supported above with a wrap of non-absorbent cotton (Jacob, et al., 2013). The seedlings were held erect by plugging the gaps with non-absorbent cotton. The Styrofoam disc was cut in a way so that it goes inside the plastic tumbler and move up and down with water. The disc also reduces the light entering the nutrient solution beneath the Styrofoam. The growth reactors were wrapped with aluminum foil to prevent light penetration. Modified Hoagland nutrient solutions (Hoagland and Arnon, 1950) (750 ml) was poured into tumblers and the disc with seedling was placed on the solution ensuring continuous root contact with the nutrient solution (Jacob, et al., 2013). The Hoagland solutions were modified according to the need of the experiment (**Tables 3.1, 3.2, 3.3 and 3.4**). Iron, phosphorus and iron and phosphorus were eliminated from the standard Hoagland solution to make three modified solutions to be used with spent FCA beads as treatments. FCA beads were added to the modified nutrient solutions as needed before the seedlings were transplanted. The experiment was a completely

randomized design with three treatments and four replications. The treatments are (**a**) Spent FCA beads and modified Hoagland solution containing no Fe and P, (**b**) Spent FCA beads and modified Hoagland solution containing no P, (**c**) Spent FCA beads and modified Hoagland solution containing no Fe and (**d**) Control (only Hoagland solution containing all the nutrients). The control contained all the nutrients, including FeNaEDTA and $NH_4H_2PO_4$ as sources of iron and phosphorus. There was a total of 16 tumblers with 4 replicates per treatment. The nutrient solution was aerated with bubblers (at a rate of ~2 cc/minute) to provide oxygen to the roots and also to keep the beads in suspension (Trujillo-Reyes, et al., 2014). The solution and spent beads were replaced every four days. The plants were grown 30 d in hydroponics. Light was provided in 14 h light/10 h dark cycles with cool-white fluorescent plant bulbs with a light intensity of ~100 µmol/m2/s.

The amount of FCA beads to be used for plant study in each reactor was decided based on another experiment previously conducted. The earlier experiment was carried out with lettuce to see if there is any toxicity due to ionic iron (which is itself a micronutrient for plants) stemming from FCA beads. Three different concentrations were used for the beads. Alginate beads at 13 g/L of hydroponic solution was found to be nontoxic, and support growth of lettuce. So in the plant growth reactors, 750 mL of hydroponic solution was used and 10 g of spent FCA beads were added to each reactor.

Table 3.1. Modified Hoagland solution (Hoagland and Arnon, 1950).

Chemicals	Final concentration		Important ions
	mM or µM	mg/L	
$Ca(NO_3)_2•4H_2O$	2 mM	472	Ca^{2+}, NO_3^-
KNO_3	6 mM	606	K^+, NO_3^-
$MgSO_4•7H_2O$	0.5 mM	123	Mg^{2+}, SO_4^{2-}
$Mg(NO_3)_2•6H_2O$	0.5 mM	128	Mg^{2+}, NO_3^-
$NH_4H_2PO_4$	2 mM	230	NH_4^+, $H_2PO_4^-$
$Na_2B_4O_7•10H_2O$	20 µM	3.81	$B_4O_7^{2-}$
$CuSO_4•H_2O$	0.5 µM	0.089	Cu^{2+}
$MnSO_4•3H_2O$	10 µM	2.05	Mn^{2+}
$Na_2MoO_4•2H_2O$	0.5 µM	0.12	MoO_4^{2-}
$ZnSO_4•H_2O$	4 µM	0.716	Zn^{2+}
FeNaEDTA	10 µM	1.69	Fe^{2+}

Table 3.2. Modified Hoagland solution containing no iron (modified for this research).

Chemicals	Final concentration		Important ions
	mM or µM	mg/L	
$Ca(NO_3)_2•4H_2O$	2 mM	472	Ca^{2+}, NO_3^-
KNO_3	6 mM	606	K^+, NO_3^-
$MgSO_4•7H_2O$	0.5 mM	123	Mg^{2+}, SO_4^{2-}
$Mg(NO_3)_2•6H_2O$	0.5 mM	128	Mg^{2+}, NO_3^-
$NH_4H_2PO_4$	2 mM	230	NH_4^+, $H_2PO_4^-$
$Na_2B_4O_7•10H_2O$	20 µM	3.81	$B_4O_7^{2-}$
$CuSO_4•H_2O$	0.5 µM	0.089	Cu^{2+}
$MnSO_4•3H_2O$	10 µM	2.05	Mn^{2+}
$Na_2MoO_4•2H_2O$	0.5 µM	0.12	MoO_4^{2-}
$ZnSO_4•H_2O$	4 µM	0.716	Zn^{2+}

Table 3.3. Modified Hoagland solution containing no phosphorus (modified for this research).

Chemicals	Final concentration		Important ions
	mM or µM	mg/L	
NH_4NO_3	2 mM	160	NH_4^+, NO_3^-
$Ca(NO_3)_2•4H_2O$	2 mM	472	Ca^{2+}, NO_3^-
KNO_3	6 mM	606	K^+, NO_3^-
$MgSO_4•7H_2O$	1 mM	246	Mg^{2+}, SO_4^{2-}
$Na_2B_4O_7•10H_2O$	20 µM	3.81	$B_4O_7^{2-}$
$CuSO_4•H_2O$	0.5 µM	0.089	Cu^{2+}
$MnSO_4•3H_2O$	10 µM	2.05	Mn^{2+}
$Na_2MoO_4•2H_2O$	0.5 µM	0.12	MoO_4^{2-}
$ZnSO_4•H_2O$	4 µM	0.716	Zn^{2+}
FeNaEDTA	10 µM	1.69	Fe^{2+}

Table 3.4. Modified Hoagland solution containing no phosphorus and no iron (modified for this research).

Chemicals	Final concentration		Important ions
	mM or µM	mg/L	
NH_4NO_3	2 mM	160	NH_4^+, NO_3^-
$Ca(NO_3)_2•4H_2O$	2 mM	472	Ca^{2+}, NO_3^-
KNO_3	6 mM	606	K^+, NO_3^-
$MgSO_4•7H_2O$	1 mM	246	Mg^{2+}, SO_4^{2-}
$Na_2B_4O_7•10H_2O$	20 µM	3.81	$B_4O_7^{2-}$
$CuSO_4•H_2O$	0.5 µM	0.089	Cu^{2+}
$MnSO_4•3H_2O$	10 µM	2.05	Mn^{2+}
$Na_2MoO_4•2H_2O$	0.5 µM	0.12	MoO_4^{2-}
$ZnSO_4•H_2O$	4 µM	0.716	Zn^{2+}

3.3.5. Analytical procedures

3.3.5.1. Lettuce studies

Plants were harvested after 30 d of hydroponic growth. The harvested plants were washed with copious amounts of DI water and the plants were separated into roots and leaves. The roots were washed with 10 mM $CaCl_2$ solution to remove any attached FCA. The fresh weight of leaves and roots were measured. The harvested plant material was then dried at 65 ^{0}C until

constant weight and the final weight was recorded. Thereafter, the samples were powdered and homogenized using a mortar and pestle.

3.3.5.2. Phosphorus and iron measurements

The powdered plant tissues collected from hydroponic experiments were digested using the protocol by (Jones Jr., 2001). Samples (~0.25 g) were weighed into a digestion tube and 5.0 mL of conc. HNO_3 was added. The mouth of the digestion tubes was covered with watch glasses and then the tubes were allowed to stand overnight. The tubes were then placed on a hot plate and digested at 125 ^{0}C for 1 h. The tubes were then allowed to cool and 3 mL of 30% H_2O_2 was added to the tubes. The contents were again digested at 125 ^{0}C until the digest was clear. The colorless digest was brought to volume adding 1:10 HNO_3 and the solution was analyzed for P and Fe. Phosphorus content was measured spectrophotometrically using yellow color method at 470 nm (Eaton, et al., 2005). Iron content was measured using PinAAcle 900H Atomic Absorption Spectrophotometry (AAS) (Perkin Elmer).

3.3.6. Statistical analysis

Plant biomass (roots, leaves) are reported in g/plant. Phosphorus and iron contents in plant are reported in mg/plant. The data were checked for homogeneity of variance. One-way analysis of variance (ANOVA) was performed for fresh mass of plant parts and elements among the treatments, followed by a Tukey's pairwise comparison (p <0.05). The results are presented as the mean ± SD (standard deviation, n = 4). All statistical analysis was performed on Minitab version 17.

3.4. Results and Discussion

3.4.1. Synthesis and preparation of spent FCA beads

FCA beads were synthesized using sodium alginate and ferrous chloride. Synthesized beads were used for phosphate removal study. The maximum phosphate sorption capacity calculated from the previous experiment was 78.7 mg PO43--P/g of dry beads. Therefore, theoretically the beads should be able to adsorb all the phosphate (25 mg of PO43--P) supplied in the solution. To confirm the presence of phosphorus, the spent FCA beads were digested and analyzed for phosphate content. The amount of phosphorus adsorbed was 19.91±1.86 mg. In the reactor, 750 mL of hydroponic solution was used and 10 g of spent FCA beads were added to each reactor. Based on the chemical analysis, 10 g of spent FCA beads contained ~12.5 mg of Fe which is equivalent to 16.7 mg/L in the solution.

3.4.2. Desorption study

A desorption study was conducted with the spent FCA beads to see the release pattern of adsorbed phosphate (**Figure 3.2**). To assess the bioavailability of P from spent FCA beads, the desorption study was conducted. The desorption study consisted of seven cycles. The collected solutions from seven cycles were tested for phosphate content. The PO_4^{3-}-P content was measured spectrophotometrically using ascorbic acid blue color method (Murphy and Riley, 1986). After the sorption study with 50 mg/L of PO_4^{3-}-P, the solution had 32.2 mg/L of PO_4^{3-}-P. Therefore, FCA beads had sorbed = (50-17.8)*50 = 1,610 µg or 1.61 mg of PO_4^{3-}-P. The amount of PO_4^{3-}-P desorbed over 28 days was = (0.94*50+0.27*50+0.54*50+0.33*50+0.31*50+0.70*50+0.36*50) µg = 171.5 µg or 0.17 mg. The remaining PO_4^{3-}-P sorbed on the beads was = (1.61-0.17) mg = 1.44 mg. Therefore, from the desorption study it was evident that the FCA beads could desorb phosphate at a rate deemed to

be sufficient to meet up the demand of a growing plant. The phosphate concentrations measured ranged from 0.27-0.94 mg/L, which is deemed to be enough for optimum growth of plants (Pierzynski, 2005).

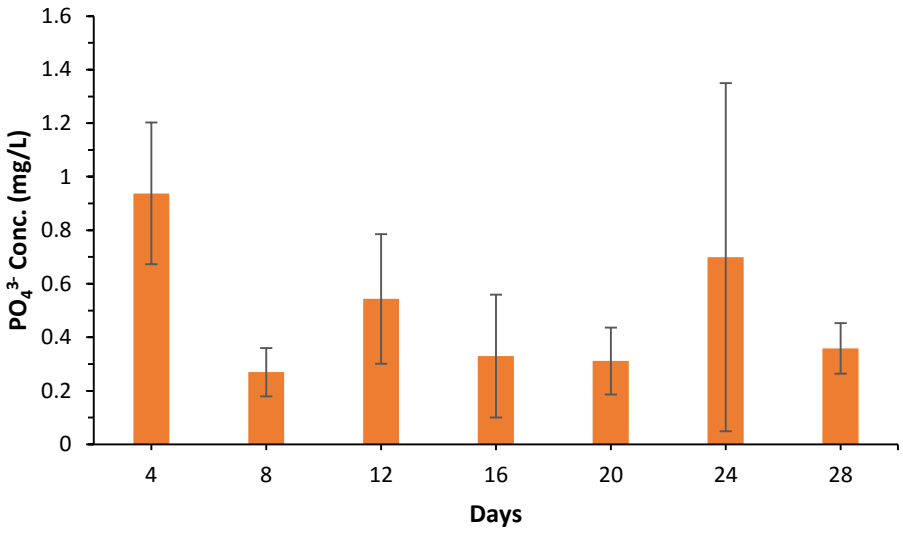

Figure 3.2. Phosphate was released successively from spent FCA beads after the introduction of fresh solution each time to simulate conditions for plant growth. Introduction of fresh solution created a gradient which enabled the desorption of phosphate from spent FCA beads. The desorption study was continued for 28 days.

3.4.3. Plant biomass

The fresh weight of lettuce plants (measured after the harvest) varied depending on the treatment (Figure 3.3). In the plants treated with spent FCA beads in nutrient solution and conventional modified Hoagland solution (containing no Fe and P), the average weight of fresh leaves and root were 12.23±5.52 g/plant and 4.90±2.10 g/plant, respectively. The plants treated with spent FCA beads and modified Hoagland solution (containing no P) yielded 7.92±3.23 g/plant of leaf biomass and 3.76±1.44 g/plant of root biomass. The average weight of fresh leaves and roots were 18.04±11.68 g/plant and 4.30±1.79 g/plant, respectively for plants treated with spent FCA beads and modified Hoagland solution (containing no Fe). In the positive control

where the plants were provided with all the nutrients and no spent beads, the leaf and root biomass were 28.47±9.43 g/plant and 6.12±0.73 g/plant, respectively. There was a significant difference ($p = 0.021$) between the Control and No P hydroponic solution (with spent FCA beads) in terms of leaf biomass. No statistically significant differences were observed between other treatments. There were no significant differences ($p = 0.240$) among the treatments in terms of root biomass.

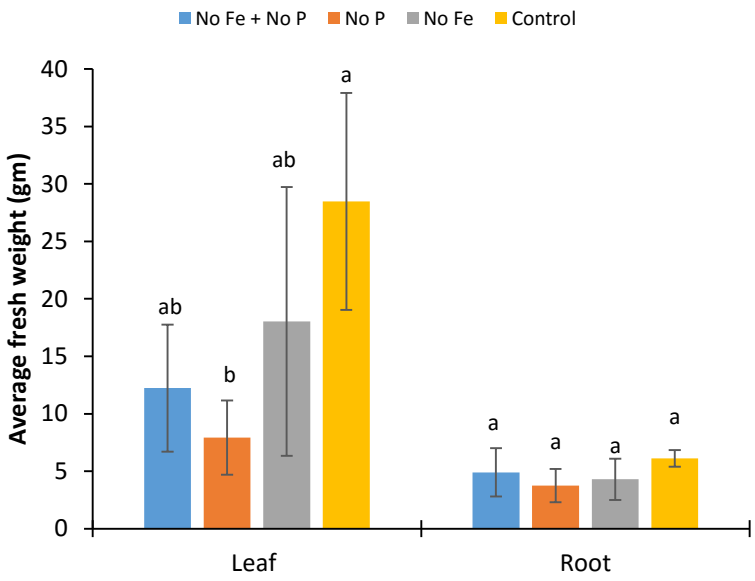

Figure 3.3. Average fresh weights of lettuce leaves and roots treated with spent FCA beads. Treatments are (i) No Fe + No P: Spent FCA beads and Hoagland solution containing no Fe and P, (ii) No P: Spent FCA beads and Hoagland solution containing no P, (iii) No Fe: Spent FCA beads and Hoagland solution containing no Fe, and (iv) Control (only Hoagland solution containing all the nutrients). Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison ($p<0.05$). Different letters above bars indicate significant differences between different treatments.

From the leaf and root biomass data, it is clear that plants treated with FCA beads and modified nutrient solution were able to take up phosphorus and iron for use. Even though the average leaf and root biomass is less in plants treated with spent FCA beads and different modified solutions compared to the control (containing all the elements), the results are

promising. The spent FCA beads used for phosphate removal from eutrophic waters could be used as a supplementary source of phosphorus in agriculture. The biodegradability of the FCA beads is likely to enable the spent FCA beads to release adsorbed phosphorus to soil to be taken up by plants.

3.4.4. Phosphorus and iron analysis

Phosphorus and iron contents in mature lettuce were analyzed and reported here as mg/plant. Uptake of elements was calculated by multiplying the dry weight of plant material by the concentration (mg/kg or µg/kg) of an element of particular interest. Uptake of elements will give insight into the bioavailability of P and Fe in the nutrient solution treated with spent FCA beads.

3.4.4.1. P uptake

Phosphorus uptake in mature lettuce leaves varied among the treatments (Figure 3.4 and Table 3.5). Phosphorus uptake in the leaves was high (5.51±2.03 mg/plant) in the plants treated with Hoagland solution containing all the elements. The uptake was significantly different ($p = 0.008$) than in the plant leaves treated with no P modified Hoagland solution and spent FCA beads (1.42±0.49 mg/plant) and no Fe and P modified Hoagland solution and spent FCA beads (2.13±0.64 mg/plant) and spent FCA beads. As for root, the uptake of P was the highest (1.35±0.39 mg/plant) in plants treated with no Fe and spent FCA beads. The uptake of P was 0.95±0.10 mg/plant in the plants treated with the control (Hoagland solution containing all the elements and no spent FCA beads). The uptake was significantly different ($p = 0.001$) than in the plants treated with no P modified Hoagland solution and spent FCA beads (0.44±0.03 mg/plant) and no Fe and P modified Hoagland solution and spent FCA beads (0.62±0.29). It appears that

additional Fe the in nutrient solution (control) decreased the uptake of P; spent FCA beads had sufficient Fe to replenish the nutrient solution with Fe.

Some interesting results were observed from this study. Phosphorus uptake in the plants treated with spent FCA beads and modified Hoagland solution (containing no Fe and P) was higher (the uptake was 58% more) than in the plants treated with spent FCA beads and modified Hoagland solution (containing no P). It appears that excess Fe from the nutrient solution reduced the uptake of P. It is likely that additional Fe might have reacted with P being desorbed (Pierzynski, 2005). As a result, plants suffered from phosphorus deficiency. However, the spent FCA beads used in this study are likely to be degraded in soil over time and will be available for plant uptake.

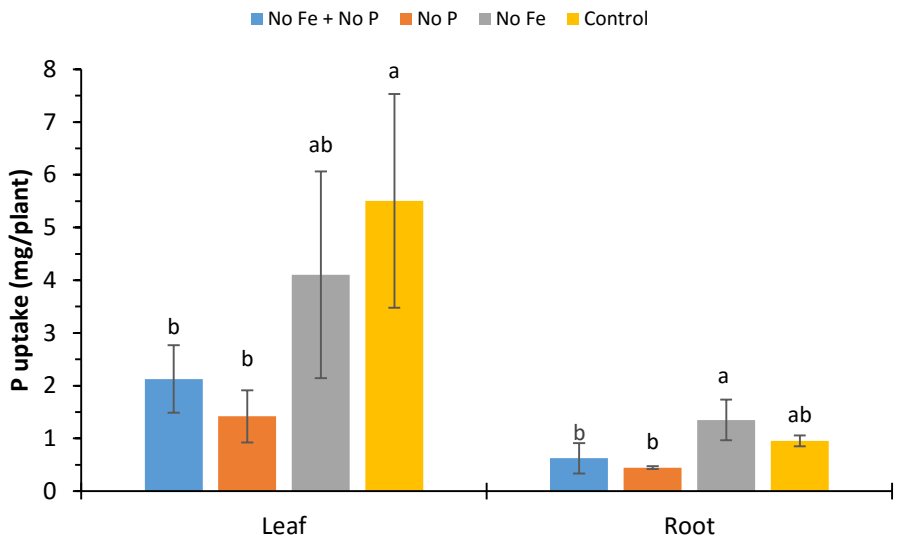

Figure 3.4. Average phosphate uptake in lettuce leaves and roots treated with spent FCA beads. Treatments are (i) No Fe + No P: Spent FCA beads and Hoagland solution containing no Fe and P, (ii) No P: Spent FCA beads and Hoagland solution containing no P, (iii) No Fe: Spent FCA beads and Hoagland solution containing no Fe, and (iv) Control (only Hoagland solution containing all the nutrients). The values represent the average phosphate uptake of four plants. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison ($p<0.05$). Different letters above bars indicate significant differences between different treatments.

Table 3.5. Uptake of P in lettuce leaves and roots. Treatments are (i) No Fe and P: Spent FCA beads and Hoagland solution containing no Fe and P, (ii) No P: Spent FCA beads and Hoagland solution containing no P, (iii) No Fe: Spent FCA beads and Hoagland solution containing no Fe, and (iv) Control (only Hoagland solution containing all the nutrients). The values represent the average phosphate uptake of four plants. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison ($p<0.05$). Different letters in the same column indicate significant differences between different treatments.

Treatment	Uptake of P (mg/plant)	
	Leaf	Root
No Fe and P	2.13±0.64b	0.62±0.29b
No P	1.42±0.49b	0.44±0.03b
No Fe	4.10±1.96ab	1.35±0.39a
Control	5.51±2.03a	0.95±0.10ab

3.4.4.2. Fe uptake

Iron uptake also varied in mature lettuce leaves subjected to different treatments (**Figure 3.5**). Iron uptake was the highest (0.88±0.24 mg/plant) in the plant leaves treated with no Fe and P and spent FCA beads (**Table 3.6**). The uptake was 0.85±0.23 mg/plant in the plant leaves treated with no P in the nutrient solution plus spent FCA beads. The uptake of iron was significantly higher (p = 0.004) in these two treatments compared to plants (0.32±0.10 mg/plant) treated with the regular Hoagland solution (Control). As for roots, the uptake of iron was 0.21±0.03 mg/plant, 0.23±0.01 mg/plant, and 0.19±0.04 mg/plant for no Fe and P plus spent FCA beads, no P plus spent FCA beads and No Fe plus spent FCA beads nutrient solutions, respectively. The uptake was significantly higher (p = 0.000) in these three treatments than in the plants (0.10±0.02 mg/plant) treated with the control (regular Hoagland solution without spent FCA beads).

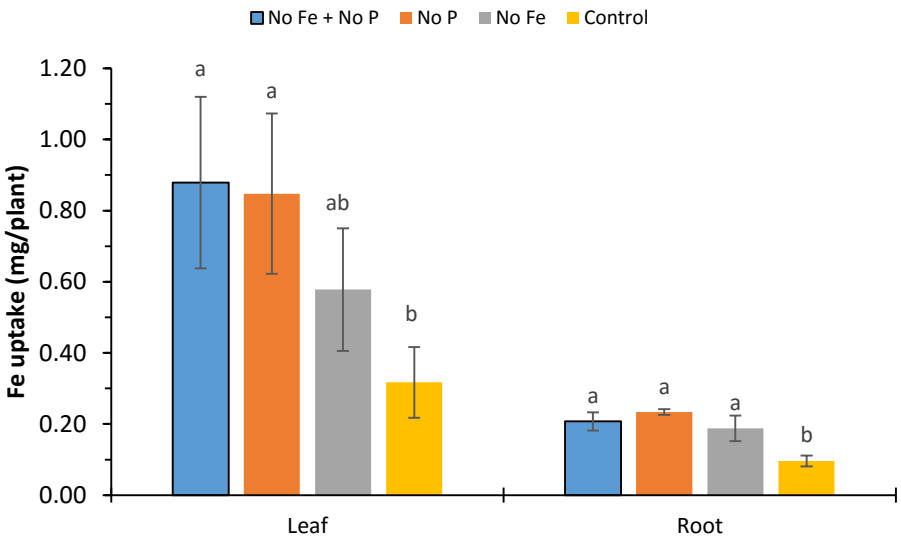

Figure 3.5. Average iron uptake in lettuce leaves and roots treated with spent FCA beads. Treatments are (i) No Fe + No P: Spent FCA beads and Hoagland solution containing no Fe and P, (ii) No P: Spent FCA beads and Hoagland solution containing no P, (iii) No Fe: Spent FCA beads and Hoagland solution containing no Fe, and (iv) Control (only Hoagland solution containing all the nutrients). The values represent the average iron uptake of four plants. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison ($p<0.05$). Different letters above bars indicate significant differences between different treatments.

Table 3.6. Uptake of Fe in lettuce leaves and roots. Treatments are (i) No Fe and P: Spent FCA beads and Hoagland solution containing no Fe and P, (ii) No P: Spent FCA beads and Hoagland solution containing no P, (iii) No Fe: Spent FCA beads and Hoagland solution containing no Fe, and (iv) Control (only Hoagland solution containing all the nutrients). The values represent the average iron uptake of four plants. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison ($p<0.05$). Different letters in the same column indicate significant differences between different treatments.

Treatment	Uptake of Fe (mg/plant)	
	Leaf	Root
No Fe and P	0.88±0.24a	0.21±0.03a
No P	0.85±0.23a	0.23±0.01a
No Fe	0.58±0.17ab	0.19±0.04a
Control	0.32±0.10b	0.10±0.02b

From the uptake pattern of lettuce plants, it is evident that plants were able to take up more Fe from the hydroponic solution supplemented with spent FCA beads. It is likely that Fe was leaching out of spent FCA beads increasing the availability of Fe in the solution. As a result, plants were able take up more iron from the solution.

The spent FCA beads, which is laden with phosphate and iron, behaved as a slow release fertilizer and could release P and Fe into aqueous solution as P and Fe was being depleted from the hydroponic solution. The desorption study, which was done to mimic conditions for plant growth, also proved that the spent FCA beads could be used as a slow-release P and Fe fertilizer. However, the growth of plants under no P hydroponic solution and spent FCA beads was not comparable to the control. It could be attributed to either the reduced availability of P because of the additional Fe from hydroponic solution, or the toxic effects of excess iron in the solution. Additional Fe from the Hoagland solution might have reacted with P (Pierzynski, 2005) which formed iron phosphate compound, thereby rendering phosphate unavailable for plant uptake. This hypothesis is supported by the fact that a relatively better plant growth was observed under no P and no Fe hydroponic solution and spent FCA beads. Even phosphate uptake was higher in these plants compared to the plants treated with no P hydroponic solution and spent FCA beads. The uptake of Fe in plants was almost similar in these two treatments which disproves the hypothesis that the growth and the uptake of P by lettuce was affected by the toxic effects of excess Fe.

3.5. Conclusions

It is obvious that spent FCA beads were able to supply lettuce with phosphorus and iron to a certain extent. Even though the growth was not comparable to the control plants, the spent beads are promising as a slow-release fertilizer. While these spent FCA beads were not meant for

the primary source of phosphate and iron fertilizer, they can certainly be used as supplementary sources of phosphorus and iron. Therefore, FCA beads could be used for phosphate removal from eutrophic lakes and wastewater and then the phosphate-laden FCA beads could be applied directly to soil as a fertilizer. The biodegradability of the FCA beads is likely to allow the spent FCA beads to release adsorbed phosphorus to soils. Soil properties will determine whether released phosphorus will be readily available for plant uptake or not. Soil studies are needed with the spent beads to see the immediate availability of adsorbed phosphate to plants. The concept and findings from this work can be used to develop new technologies to combat eutrophication of waterbodies as well as to meet the rising demand of phosphate.

3.6. References

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4. UPTAKE OF IRON BY SPINACH (*SPINACIA OLERACEA*) EXPOSED TO NANOSCALE IRON

4.1. Abstract

An experiment was conducted to evaluate the availability of iron for plant use from nanoscale zero-valent iron (NZVI) using spinach (*Spinacia oleracea*). The main purpose of this study was to biofortify spinach with iron, which is an essential element for both plants and human. In a hydroponics study, spinach was grown until maturity using three doses of iron (11, 55 and 110 mg/L) using NZVI, microscale zero-valent iron (MZVI) and ferrous sulfate ($FeSO_4$). Plants treated with bare NZVI at 55 mg/L yielded profuse biomass (78.1 mg root/plant and 674.2 mg shoot/plant on dry weight basis) and exhibited an enhanced uptake of iron (19.77−64.9 µg/plant) compared to the control. Because of the higher biomass, the uptake of other elements was high as well. The plants did not respond in the same manner when dosed with higher concentration (110 mg/L) of NZVI. The enhanced uptake of iron by spinach is promising and is likely to have implications in agronomy.

4.2. Introduction

Human needs various nutrients for body metabolism (Graham, 2001). Some of these nutrients are present in low quantities in most staple foods resulting in deficiency of these elements in our diet (Gomez-Galera et al., 2010). Iron deficiency is one of the most common nutritional deficiencies prevalent across the globe. Iron has a number of functions in the human body. Iron constitutes the functional core of the heme complex in hemoglobin (oxygen carrier in blood) and myoglobin (oxygen storage unit in muscles). It is also found in the catalytic center of cytochromes which perform redox reactions. Therefore, iron is vital for oxygen transport in the body and for energy metabolism (WHO/FAO, 1998).

Iron deficiency is one of the leading factors for disabilities and deaths worldwide (Boccio, 2003). Iron deficiency causes anemia. In the year 2011, it was estimated that around 43% of children, 38% of pregnant women, and 29% of non-pregnant women have anemia worldwide, corresponding to 273 million of children, 32 million of pregnant women, and 496 million of non-pregnant women (WHO, 2015). Children in the African Region had the highest proportion (62.3%) of individuals suffering from anemia while the most affected number of children and women resided in the South-East Asia Region, including 96.7 million of children and 202.0 million women of reproductive age (15-49 years) (WHO, 2015).

Several interventions were proposed to combat iron deficiency. The most effective intervention to alleviate iron deficiency is dietary diversification which includes consumption of meat, vegetable, fish and fruits with staple foods (Gomez-Galera et al., 2010). Supplementation through the ingestion of micronutrients in tablet or sachets forms and food fortification through the addition of minerals to processed foods are other interventions proposed earlier (Gomez-Galera et al., 2010). Biofortification is regarded to be a more efficient and cost-effective solution. Biofortification involves "increasing bioavailable concentrations of an element in edible portions of crops before harvesting" (White and Boradley, 2009). Biofortification is achieved by fertilization, conventional breeding and/or genetic engineering. It is also done with microorganisms (for example, biofortification of selenium (Se) in wheat) (Duran et al., 2013). The fortification of staple plants with bioavailable Fe is likely to provide a sustainable and economical tool to remedy Fe deficiency in target populations worldwide (Jeong and Guerinot, 2008).

Agronomic intervention is sometimes not efficient because iron in oxidized ferric form is less soluble in aerobic environments. The oxidized iron Fe (III) has a very low solubility at basic

pH, and high bicarbonate which leads to reduced uptake of iron by plant roots (Lucena et al., 2007). Therefore, even though iron is the fourth abundant element in the earth's crust, it is the third most limiting nutrient for plant growth (Gomez-Galera et al., 2010). Innovative iron fortification through soil and foliar application of iron containing compounds has been tried in maize, wheat, barley, common beans, oats, and leafy vegetables (e.g., spinach) (Almeelbi and Bezbaruah, 2014; Ortega-Blu and Molina-Roco, 2007).

During the last decade, there has been increased interest in the application of nanomaterials for agronomic purposes. Nanomaterials have been used as smart delivery systems of fertilizers, herbicides, pesticides and plant growth regulators (Perez-de-Luque and Hermosin, 2013). Scientists have also explored the possibility of using nanoparticles to biofortify plants. It is an agronomic intervention where nanoparticles are applied to enhance the growth of plants, thereby increasing the uptake of mineral elements. In a hydroponic study, Almeelbi and Bezbaruah (2014) used nanoscale zero-valent iron (NZVI) for phosphate removal and subsequently used the spent (phosphate-sorbed) NZVI as a source of phosphorus and iron for spinach and algae. They found increased growths of algae and spinach when spent NZVI was used as a sole source of iron and phosphorus. The iron content increased significantly in all plant parts (roots, stems and leaves) when spent NZVI was used as a source of iron. Iron content increased by 7 and 11 times in the stem and leaves of the plant of spinach as compared to the control. Superparamagnetic iron oxide nanoparticles (SPIONs) was taken up and translocated by hydroponically-grown soybean plants and increase in chlorophyll content in the plants were reported and no toxicity to plants was observed (Ghafariyan et al., 2013).

Keeping all these in mind, a study was undertaken to examine whether NZVI can be used for iron biofortification and whether the presence of NZVI affects the availability of other

minerals in hydroponically grown spinach (*Spinacia Oleracea*). In this study, micro zero-valent iron (MZVI) particles were also added as a treatment for comparison purposes. This will give insights into the nano effects of zero-valent iron particles on the uptake of iron by plants. Spinach was selected for this experiment because this plant is one of the most consumed vegetables with global cultivation area and production of approximately 890,000 ha and 14,000,000 tons, respectively (Citak and Sonmez, 2010). Spinach plant is fast growing, and readily available worldwide. It is also an important source of minerals making it an important crop to be studied. The hypothesis of the research is that NZVI will outperform MZVI in enhancing the uptake of iron because of the nano size.

4.3. Materials and Methods

4.3.1. Chemicals

Iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$, 99 % pure, Alfa Aesar), micro zero-valent iron powder (<10 micron, 99.9+%, SIGMA-ALDRICH), sodium borohydride ($NaBH_4$, 98 %, SIGMA-ALDRICH)), sodium hydroxide (5 N NaOH, Alfa Aesar), HNO_3 (68 %, J.T. Baker), methanol (production grade, BDH), ethanol (ACS grade, Mallinckrodt Chemicals), calcium nitrate tetrahydrate ($CaNO_3 \cdot H_2O$, Alfa Aesar), potassium nitrate (KNO_3, Mallinckrodt Chemicals), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, Mallinckrodt Chemicals), magnesium nitrate hexahydrate ($Mg(NO_3)_2 \cdot 6H_2O$, Alfa Aesar), ammonium dihydrogen phosphate ($NH_4H_2PO_4$, Alfa Aesar), sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$, amresco), copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, BDH), manganese sulfate monohydrate ($MnSO_4 \cdot H_2O$, Mallinckrodt Chemicals), sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$, BTC) and zinc sulfate monohydrate ($ZnSO_4 \cdot H_2O$, J.T. Baker) were used as received unless otherwise specified.

4.3.2. Synthesis and preparation of NZVI

4.3.2.1. NZVI synthesis

NZVI particles were synthesized using sodium borohydride reduction method (Almeelbi and Bezbaruah, 2012). Iron (II) sulfate heptahydrate (10 g) was dissolved in 100 mL of 30% of methanol (30 mL methanol + 70 mL deoxygenated de-ionized (DI) water) (Solution A). The pH of the solution was then adjusted to 6.1 adding 5 N NaOH drop by drop. In the meantime, 3.94 g of sodium borohydride was dissolved in 100 mL of deoxygenated DI water in a 100 mL volumetric flask (Solution B). Once the pH reached 6.1, Solution A was immediately added dropwise to Solution B using a burette under vigorous stirring conditions (using a magnetic stirrer). The combined solution was then allowed to stand for 20 min. The resultant black precipitates (NZVI) were centrifuged and washed with ethanol. The NZVI in slurry form was then dried in a vacuum oven under nitrogen environment. Finally, the dried NZVI particles were ground using a mortar and pestle and stored in 20 mL vials (headspace flushed with nitrogen) for later use.

4.3.3. Experimental set-up for spinach study in hydroponics

4.3.3.1. Germination and plant preparation for hydroponic experiment

Spinach (Double Choice Hybrid, Spinacia oleracea, Burpee, Warminster, PA) seeds were purchased from a local outlet. Seeds were initially treated with liquid nitrogen for cracking up the shells for faster germination. The spinach seeds were then placed on Perlite media in Petri dishes and kept in the dark at room temperature (22+2 0C) for three days for germination. After three days, the Petri dishes were moved to a custom-made growth chamber (Figure 1) and germinated seedlings were allowed to grow for another three days. The seedlings were provided

with cool-white fluorescent light (14 h light/10 h dark cycle). The light intensity was ~100 µmol/ m^2/s.

4.3.3.2. Growth studies

After 3 d of growth in the growth chamber (**Figure 4.1**), the spinach seedlings were ready for transplantation to the hydroponic growth reactors. The growth reactor (890 mL) was 18.4 cm tall plastic tumblers with 8.9 cm top diameter (**Figure 4.2**). The roots of spinach plants were washed with DI water before transplantation. Three spinach seedlings were then placed into a Styrofoam disc float (three holes in the disc float) with their roots below the disc and the shoots supported above with a wrap of non-absorbent cotton (Jacob et al., 2013).

Figure 4.1. Growth chamber used for spinach study. The temperature and relative humidity was maintained at ~75 °F and ~60%.

Hoagland nutrient solutions (Hoagland and Arnon, 1950) were modified according to the treatments of the experiment. Iron was taken out from one of the nutrient solutions different

doses of NZVI or MZVI were added (**Table 4.1**). $FeSO_4$ was included in the treatments to serve as an active control. The nutrient solution which was used for negative control (Control) contained no iron (**Table 4.1**).

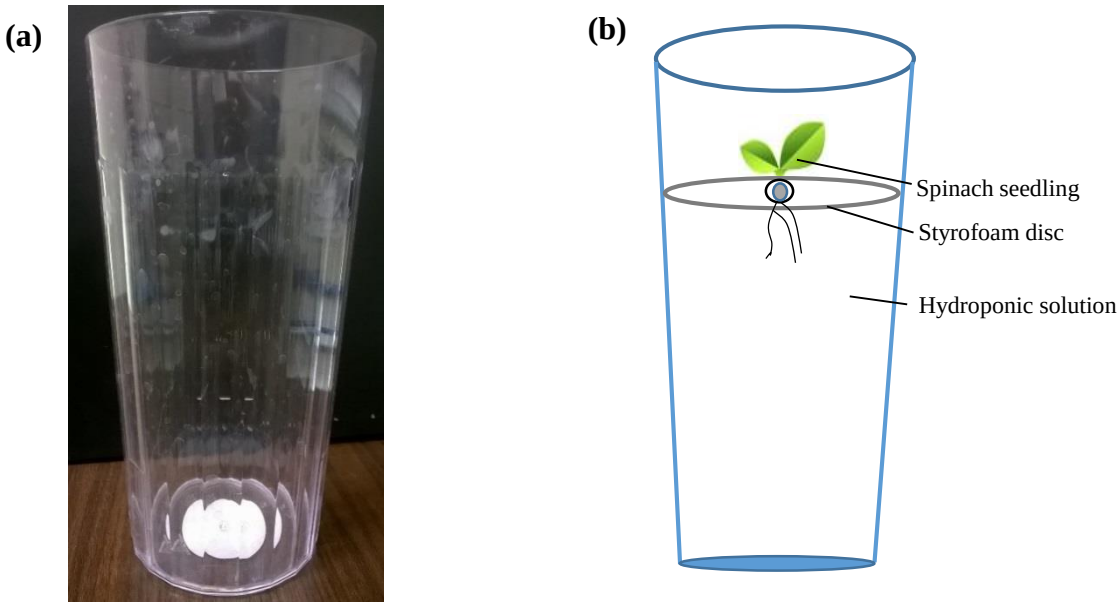

Figure 4.2. (a) Spinach growth reactor (b) Schematic of the growth unit.

Table 4.1. Modified Hoagland solution containing no iron (modified for this research).

Chemicals	Final concentration		Important ions
	mM or µM	mg/L	
$Ca(NO_3)_2 \cdot 4H_2O$	2 mM	472	Ca^{2+}, NO_3^-
KNO_3	6 mM	606	K^+, NO_3^-
$MgSO_4 \cdot 7H_2O$	0.5 mM	123	Mg^{2+}, SO_4^{2-}
$Mg(NO_3)_2 \cdot 6H_2O$	0.5 mM	128	Mg^{2+}, NO_3^-
$NH_4H_2PO_4$	2 mM	230	NH_4^+, $H_2PO_4^-$
$Na_2B_4O_7 \cdot 10H_2O$	20 µM	3.81	$B_4O_7^{2-}$
$CuSO_4 \cdot H_2O$	0.5 µM	0.089	Cu^{2+}
$MnSO_4 \cdot 3H_2O$	10 µM	2.05	Mn^{2+}
$Na_2MoO_4 \cdot 2H_2O$	0.5 µM	0.12	MoO_4^{2-}
$ZnSO_4 \cdot H_2O$	4 µM	0.716	Zn^{2+}

The experiment was a completely randomized design with three nanoparticles (NZVI) treatments 11, 55 and 110 mg Fe/L), three microparticles (MZVI) treatments 11, 55 and 110 mg Fe/L), and three ferrous sulfate treatments 11, 55 and 110 mg Fe/L), with three replicates per treatment for a total of 30 pots. Once the nutrient solution was transferred, the disc was then placed on the surface of 450 mL nutrient solution in the hydroponic growth reactors ensuring continuous root contact with the nutrient solution. The growth reactors were wrapped with aluminum foil to prevent light penetration. The nutrient solution was aerated with bubblers (at a rate of ~2 cm^3 air/min) to provide oxygen to the roots and also to keep the nanoparticles in suspension (Trujillo-Reyes et al., 2014). The growth solution and NZVI/MZVI were replaced every five days. Light was provided in 14 h light/10 h dark cycles with cool-white fluorescent plant bulbs with a light intensity of ~100 µmol/m^2/s.

4.3.4. Analytical procedures

4.3.4.1. Spinach studies

Plants were harvested after 39 days of hydroponic growth. The harvested plants were washed with copious amounts of DI water and the plants were separated into three parts—the roots, the lower part of the aboveground portion and the upper part of the aboveground portion. The lower part consisted of the stem, first two leaves on the plant and the larger leaves that grew sideways, and the upper part consisted of the stem and the leaves not included in the lower part (**Figure 4.3**). Roots were washed with 10 mM $CaCl_2$ solution to remove any NZVI attached to the root surface (Almeelbi and Bezbaruah, 2014). The harvested plant material was then dried at 65 ^{0}C until constant weight (three plants grown in each tumbler were dried together). After drying, the roots, the lower part of the plants and upper part of the plants were weighed. The

roots yielded very less mass and were not analyzed for macro and micro elements. The samples were then powdered and homogenized using a mortar and pestle.

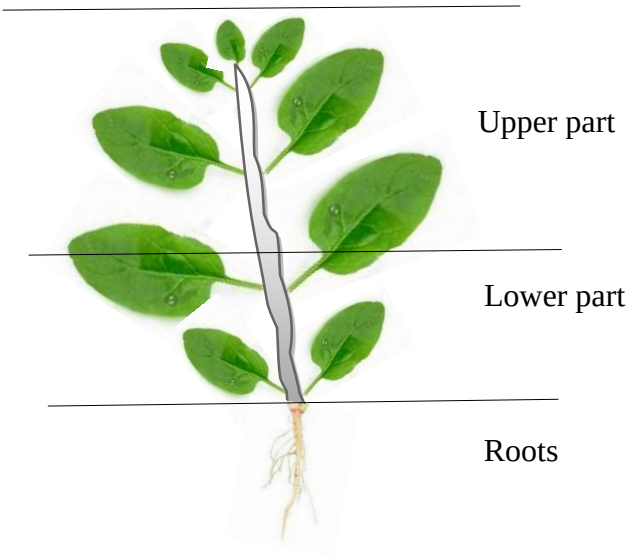

Figure 4.3. Schematic of spinach plant.

4.3.4.2. Macro- and micro-nutrient measurements

The powdered plant tissues collected from hydroponic experiments were digested using the protocol by Jones Jr. (2001). Samples (~0.25 g) were weighed into a digestion tube and 5.0 mL of conc. HNO_3 was added. The mouth of the digestion tubes was covered with watch glasses and then the tubes were allowed to stand overnight. The tubes were then placed on a hot plate and digested at 125 ^{0}C for 1 h. The tubes were then allowed to cool to room temperature (22±2 °C) and 3 mL of 30% H_2O_2 was added to the tubes. The contents were again digested at 125 ^{0}C until the digest was clear. The colorless digest was brought to volume adding 1:10 HNO_3 and the solution was analyzed for P, K, Ca, Mg, Na, Zn, Fe, Mn, Cu, B using a Perkin Elmer ICP-OES (5300 DV Model). A control standard was run after every ten samples to check whether the

values were within acceptable limits (10% of the expected values). Total N in plant tissue was measured using a nitrogen combustion analyzer (Vario MAX cube, Elementar Americas Inc).

4.3.5. Statistical analysis

All elements are reported in mg/plant or µg/plant. However, the data was transformed where needed using Johnson transformation and Box-cox transformation prior to statistical analysis to increase the homogeneity of variance. One-way analysis of variance (ANOVA) was performed for elements among the treatments, followed by a Tukey's pairwise comparisons. The results are presented as the mean ± SD (standard deviation, n = 3). Pearson correlation analysis was also performed between the elements for each treatment. Significant correlations with $r > 0.5$ (therefore explaining 25% of total variation) was only considered for further discussion. All statistical analysis was performed on Minitab version 17. Significance was determined based on whether *p*-values <0.05 or not.

4.4. Results and Discussion

4.4.1. Particles characterization

Average particles size of virgin NZVI was 16.24 ± 4.05 nm (Almeelbi and Bezbaruah, 2012). The percentage of oxygen in the virgin NZVI was found to be 12.10%. Krajangpan et al. (2012) reported it as 15.66%. The presence of a very low amount (0.51%) of Na was observed in the virgin NZVI. Sodium (Na) was possibly left behind as a residual from sodium borohydride ($NaBH_4$) used in the NZVI synthesis process.

4.4.2. Spinach growth study

4.4.2.1. Seed germination

Spinach seed germination in Petri dishes started after 2 d and the percent of seed germination varied from 80% to 100%.

4.4.2.2. Plant biomass

The similar parts (e.g., leaves) from each reactor (three each) were combined together after harvesting at maturity. After drying at 65 ^{0}C, the roots, the lower part of the plants and the upper part of the plants were weighed. The average combined weight (mg) is reported. The dry weight of spinach varied depending on the treatment (**Figure 4.4**).

4.4.2.2.1. Roots

In the plants treated with MZVI 110 mg/L, the weight of roots was 263.2 mg which was significantly higher than the control (solution not containing iron) (17.9 mg). There was a 1370% increase in root mass. Plants treated with NZVI 55 mg/L yielded 234.3 mg of roots; root mass was increased by 1208% compared to the control. Root mass was increased by 176% (biomass was 49.4 mg) compared to the control in the plants treated with NZVI 11 mg/L.

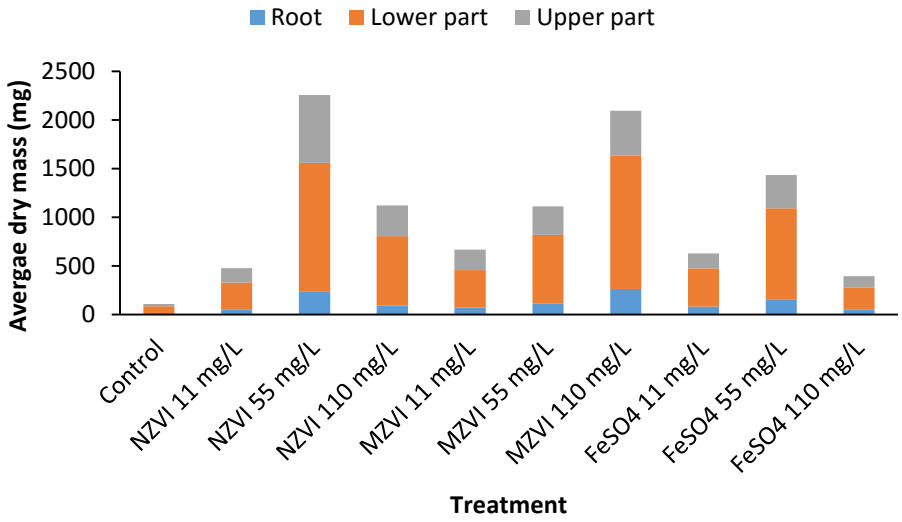

Figure 4.4. Dry biomass of spinach exposed to NZVI, MZVI and $FeSO_4$. Treatments are: (i) Control: all nutrients but Fe, (ii) NZVI 11 mg/L (iii) NZVI 55 mg/L (iv) NZVI 110 mg/L (v) MZVI 11 mg/L (vi) MZVI 55 mg/L (vii) MZVI 110 mg/L (viii) $FeSO_4$ 11 mg/L (ix) $FeSO_4$ 55 mg/L (x) $FeSO_4$ 110 mg/L. Except the control, the rest of the treatments were supplemented with the modified Hoagland solution containing all elements but Fe.

4.4.2.2.2. Lower part

In the case of lower part of the plants, plants treated with NZVI 55 mg/L and MZVI 110 mg/L yielded 1324.8 mg (1834% increase in biomass on the control) and 1372.1 mg (1903% increase in biomass on the control) of biomass, respectively that were significantly higher than the control (68.5 mg). At NZVI 110 and 11 mg/L application, the plants produced 711.7 mg (939% increase in biomass on the control) and 280.9 mg (310% increase in biomass on the control) of biomass, respectively.

4.4.2.2.3. Upper part

For upper part of the plants, plants treated with NZVI 55 mg/L (698.6 mg) (2925% increase in biomass on the control) had significantly more biomass than in plants treated with NZVI 11 mg/L (147.0 mg) (536% increase in biomass on the control), MZVI 11 mg/L (210.1 mg) (810% increase in biomass on the control), $FeSO_4$ 11 mg/L (156.1 mg) (576% increase in biomass on the control), $FeSO_4$ 110 mg/L (114.1 mg) (394% increase in biomass on the control) and the control (23.1 mg).

4.4.3. Macro- and micro-elements analysis

All the macro-elements and micro-elements in spinach plants were analyzed and reported here as mg or µg per plant (Almeelbi and Bezbaruah, 2014). Uptake of elements was calculated by multiplying the dry weight of plant material by the concentration (mg/kg or µg/kg) of an element of particular interest.

4.4.3.1. Fe uptake

The total uptake of iron in the upper part of spinach plant could not be compared with the control (hydroponic solution without iron) because the control did not yield enough dry mass for measurement. Therefore, the comparison was made among the three treatments (NZVI, MZVI,

and $FeSO_4$) at different concentrations (**Table 4.2, Figure 4.5**). The uptake of iron in the upper part of spinach was the highest (19.77 µg/plant) in plants treated with NZVI at 55 mg/L. At the same concentration, iron accumulation was 11.36 and 11.27 µg/plant for MZVI and $FeSO_4$, respectively. So there was 75% increase in iron uptake by plants treated with NZVI compared to MZVI and $FeSO_4$ treatments. For MZVI, iron accumulation was the highest at 110 mg/L (16.46 µg/plant), which is comparable with the highest uptake value with NZVI treatment. Enhanced iron uptake is directly related with the higher biomass of plants. NZVI at 55 mg/L of application produced the maximum biomass in spinach. NZVI at 55 mg/L and MZVI at 110 mg/L apparently elicited the same response from plants in terms of iron accumulation. This phenomenon could be attributed to the surface area of NZVI and MZVI particles and thus possible transformation to Fe^{2+} and Fe^{3+}. The NZVI particles used in this experiment had a surface area of ~25 m^2/g, whereas MZVI had a surface area of ~2 m^2/g (Bezbaruah et al., 2009).

Table 4.2. Uptake* of elements in the lower part of spinach (per plant) treated with NZVI, MZVI and $FeSO_4$. Data from P, K, S, Ca, Mg, Na, Fe, Cu, and B were subjected to Johnson transformation prior to statistical analysis. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison ($p<0.05$). Different letters in the same column indicate significant differences between different treatments.

Treatment	P (mg)	K (mg)	S (mg)	Ca (mg)	Mg (mg)	Na (mg)	Zn (µg)	Fe (µg)	Mn (µg)	Cu (µg)	B (µg)
NZVI 110 mg/L	1.7±0.4abc	28.0±9.0ab	0.97±0.24ab	2.8±0.9a	2.4±1.2ab	0.31±0.11a	50.3±11.0a	38.9±5.8a	125±52a	3.7±0.4ab	58±31ab
NZVI 55 mg/L	2.9±0.9a	48.5±14.6a	1.4±0.4a	3.5±0.8a	3.7±1.2a	0.23±0.05ab	58.2±9.9a	64.9±8.4a	132±23a	3.9±0.5a	74±32a
NZVI 11 mg/L	0.85±0.49abc	8.9±4.6abc	0.32±0.17abc	1.2±0.6ab	0.7±0.3abc	0.04±0.02cd	28.4±14.6	12.9±6.8ab	42±22ab	2.3±1.4abc	9±4cd
MZVI 110 mg/L	3.4±2.7a	45.6±34.1a	1.5±1.1a	4.0±2.7a	3.4±2.4a	0.22±0.07abc	48.6±2.1a	57.3±39.0a	101±17a	4.1±0.5a	28±17abc
MZVI 55 mg/L	1.9±1.2abc	25.9±16.8abc	0.78±0.41abc	1.9±0.9ab	1.8±1.0abc	0.10±0.02abcd	46.4±14.9ab	38.0±21.4a	88±28ab	3.3±1.0abc	16±10abcd
MZVI 11 mg/L	1.1±0.6abc	12.6±8.0abc	0.48±0.28abc	1.2±0.2ab	1.2±0.8abc	0.10±0.06abcd	40.3±30.2abc	13.0±6.4ab	74.0±61.8ab	2.6±1.3abc	10±4
$FeSO_4$ 110 mg/L	0.48±0.44bc	6.0±6.9bc	0.23±0.22bc	0.49±0.39b	0.44±0.46bc	0.04±0.04d	6.0±6.8bc	14.2±14.2ab	7±7b	0.83±0.73c	6±6cd
$FeSO_4$ 55 mg/L	2.2±1.0ab	35.5±19.8ab	0.92±0.42ab	2.0±0.9ab	2.2±1.1ab	0.09±0.03abcd	37.9±19.1abc	38.6±18.9a	62±39ab	3.0±1.1abc	26±13abc
$FeSO_4$ 11 mg/L	1.0±0.3abc	12.4±4.2abc	0.45±0.10abc	1.3±0.4ab	1.0±0.3abc	0.05±0.04bcd	33.1±7.4abc	18.2±16.0ab	58±10ab	2.8±0.3abc	12±4bcd
Control	0.18±0.13c	1.8±1.0c	0.10±0.06c	0.43±0.24b	0.2±0.1c	0.04±0.02cd	5.0±2.6c	1.2±0.7b	6±3b	0.81±0.48bc	2±1d

*****Nutrient uptake per plant was calculated by multiplying dry weight by nutrient concentrations

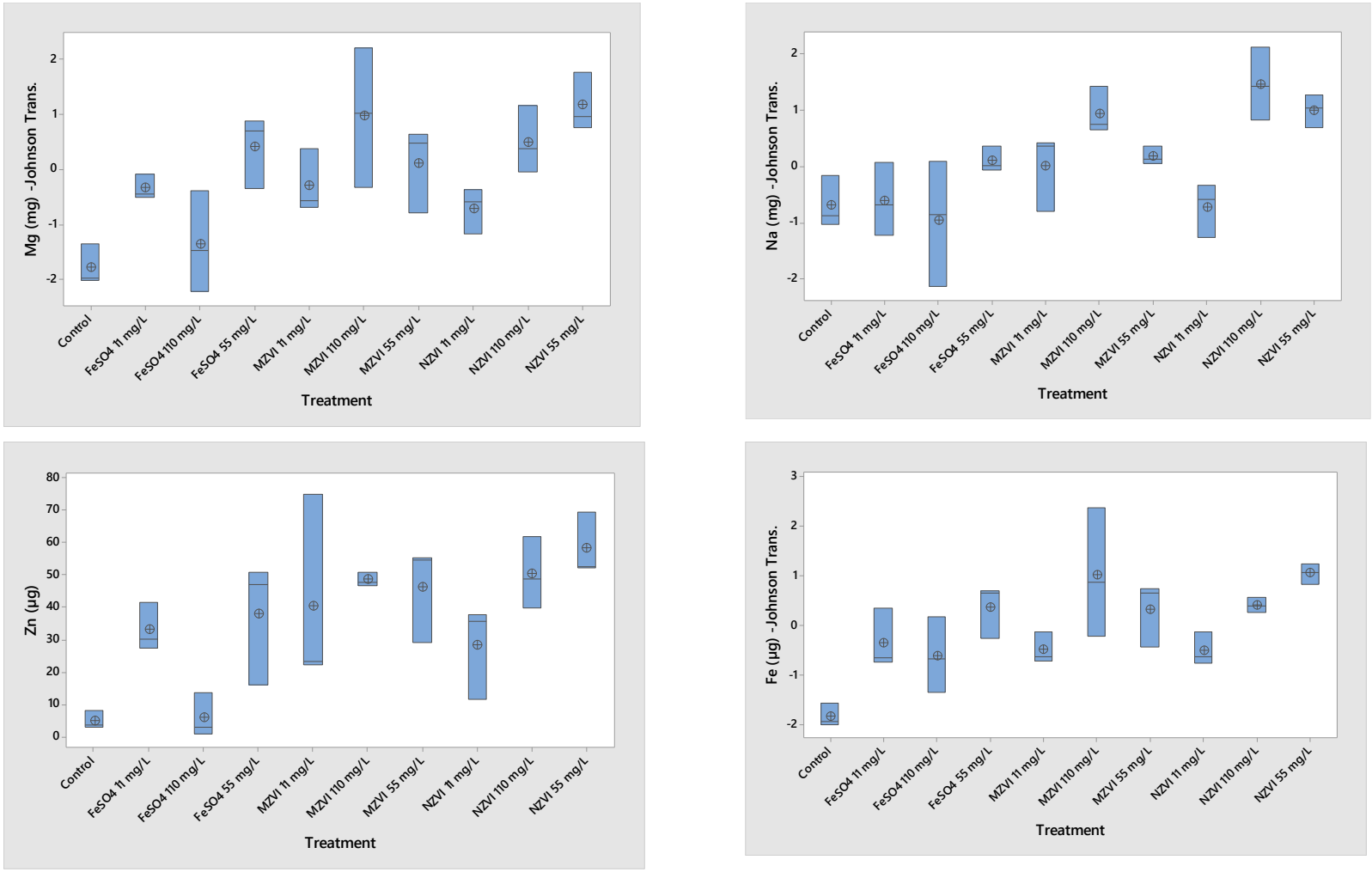

Figure 4.5. Total uptake of Mg, Na, Zn, and Fe in the upper part of plants dosed with different treatments. Treatments are: (i) Control: all nutrients but Fe, (ii) NZVI 11 mg/L (iii) NZVI 55 mg/L (iv) NZVI 110 mg/L (v) MZVI 11 mg/L (vi) MZVI 55 mg/L (vii) MZVI 110 mg/L (viii) $FeSO_4$ 11 mg/L (ix) $FeSO_4$ 55 mg/L (x) $FeSO_4$ 110 mg/L.

The same trend was also observed in the lower part of spinach (**Table 4.3, Figure 4.6**). The plants took up more Fe when dosed with NZVI at 55 mg/L. At that concentration, iron uptake was significantly higher (64.9 µg/plant) than the control (1.2 µg/plant); the uptake was 5308% higher compared to the control. The uptake was 71% higher compared to MZVI (Fe uptake was 38.0 µg/plant) and 68% higher compared to $FeSO_4$ (Fe uptake was 38.6 µg/plant). At 11 mg/L of NZVI application, the uptake was 12.9 µg/plant, respectively. Like the upper part of the plant, Fe uptake was also on the higher side in the lower part of the plants treated with MZVI at 110 mg/L (57.3 µg/plant), which was comparable to the highest uptake with NZVI at 55 mg/L. At the same concentration of NZVI, Fe uptake was less in plants (38.9 µg/plant). Plants treated with $FeSO_4$ at 110 mg/L (14.2 µg/plant) also showed lower uptake of Fe compared to 55 mg/L (38.6 µg/plant) and 11 mg/L (18.2 µg/plant) of application. Therefore, it is evident from this study that NZVI was able to provide spinach with needed active iron.

Table 4.3. Uptake* of elements in the upper part of spinach (per plant) treated with NZVI, MZVI and $FeSO_4$. Data from P, K, S, Ca, Mg, Na, and B were subjected to Johnson transformation and Mn data was subjected to Box-cox transformation prior to statistical analysis. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison ($p<0.05$). Different letters in the same column indicate significant differences between different treatments.

Treatment	P (mg)	K (mg)	S (mg)	Ca (mg)	Mg (mg)	Na (mg)	Zn (µg)	Fe (µg)	Mn (µg)	Cu (µg)	B (µg)
NZVI 110 mg/L	0.8±0.5ab	9.1±5.1ab	0.42±0.23ab	0.64±0.37ab	0.69±0.30ab	0.09±0.05a	11.5±2.6	8.5±4.5	20.4±3.5ab	1.2±0.5	12.4±8.1ab
NZVI 55 mg/L	1.7±0.4a	20.1±5.6a	0.84±0.25a	1.11±0.09a	1.39±0.40a	0.08±0.02a	18.7±2.7	19.8±9.7	21.8±12.3ab	1.8±0.1	21.9±9.6a
NZVI 11 mg/L	0.4±0.1ab	4.3±0.9ab	0.19±0.04ab	0.41±0.14ab	0.40±0.09ab	0.02±0.00a	13.7±6.2	3.2±0.5	26.2±17.3ab	1.2±0.3	4.9±1.0ab
MZVI 110 mg/L	1.2±0.6ab	11.8±5.0ab	0.58±0.27ab	0.87±0.46a	0.92±0.47ab	0.09±0.02a	17.0±4.8	16.5±7.4	24.4±11.0ab	1.3±0.4	8.2±4.5ab
MZVI 55 mg/L	0.8±0.6ab	8.4±6.5ab	0.40±0.31ab	0.47±0.36ab	0.66±0.56ab	0.06±0.03a	13.3±10.5	11.4±8.6	19.7±16.3ab	1.4±1.2	6.0±4.8ab
MZVI 11 mg/L	0.6±0.3ab	6.2±1.7ab	0.30±0.08ab	0.46±0.20ab	0.56±0.14ab	0.05±0.05a	18.1±5.2	5.5±2.0	33.3±10.8a	1.6±0.5	4.7±1.2ab
$FeSO_4$ 110 mg/L	0.3±0.2b	2.8±1.9b	0.13±0.10b	0.13±0.11b	0.23±0.23b	0.02±0.02a	3.4±2.4	7.0±3.6	2.1±1.6b	0.3±0.3	2.3±1.3b
$FeSO_4$ 55 mg/L	0.9±0.5ab	9.3±5.3ab	0.44±0.25ab	0.46±0.28ab	0.72±0.41ab	0.05±0.02a	10.8±5.1	11.3±6.8	11.3±9.5ab	1.2±0.6	6.2±3.5ab
$FeSO_4$ 11 mg/L	0.4±0.1ab	4.0±0.8ab	0.21±0.05ab	0.29±0.01ab	0.35±0.06ab	0.02±0.01a	8.5±0.8	4.7±2.6	14.2±6.8ab	1.1±0.1	3.2±0.2b

*****Nutrient uptake per plant was calculated by multiplying dry weight by nutrient concentrations

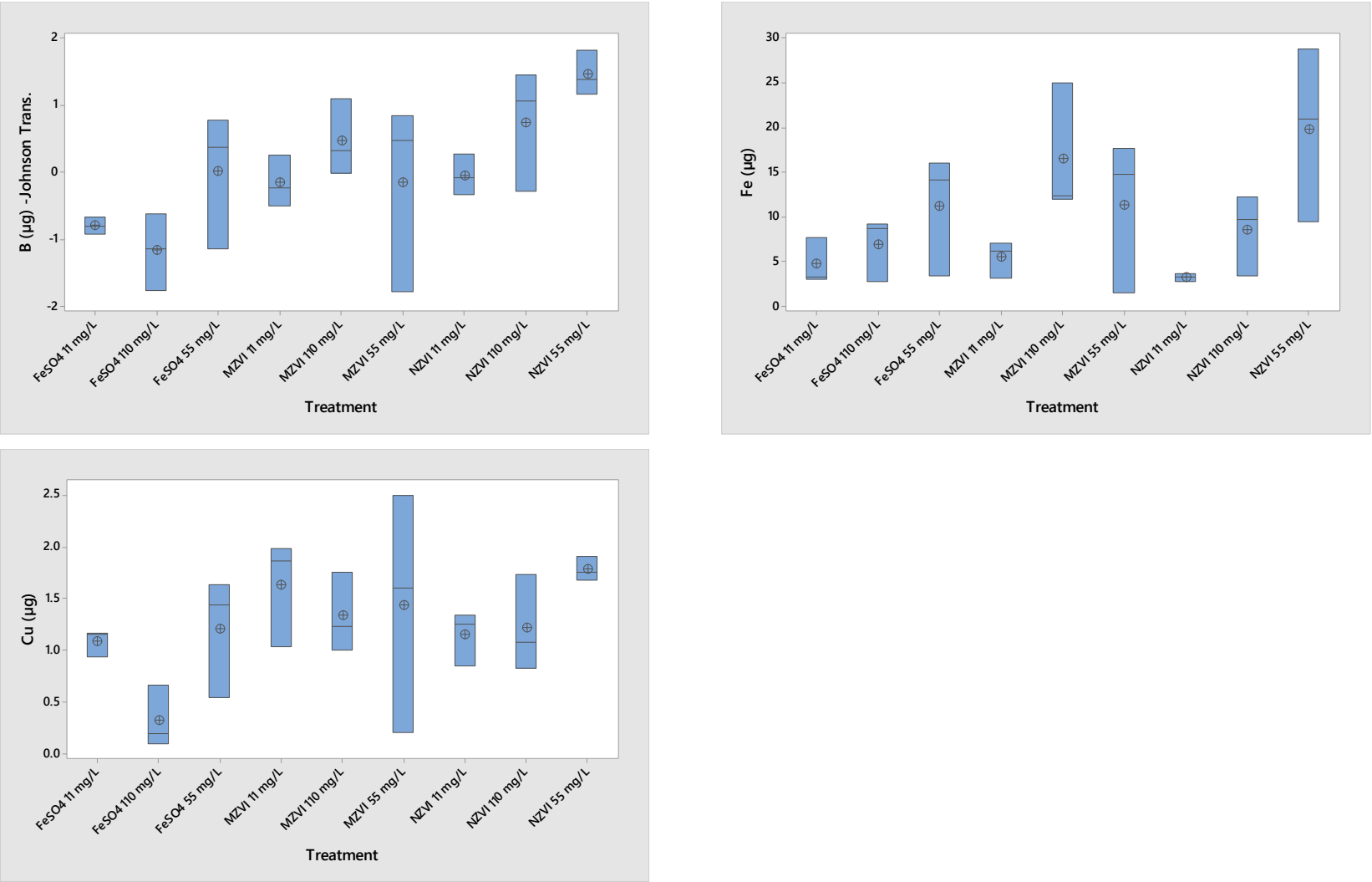

Figure 4.6. Total uptake of B, Fe and Cu in the lower part of plants dosed with different treatments. Treatments are: (i) Control: all nutrients but Fe, (ii) NZVI 11 mg/L (iii) NZVI 55 mg/L (iv) NZVI 110 mg/L (v) MZVI 11 mg/L (vi) MZVI 55 mg/L (vii) MZVI 110 mg/L (viii) $FeSO_4$ 11 mg/L (ix) $FeSO_4$ 55 mg/L (x) $FeSO_4$ 110 mg/L.

4.4.3.2. Element uptake

In this study with spinach, uptake patterns of different elements were analyzed in plants treated with NZVI, MZVI, $FeSO_4$ and the control (**Tables 4.2 and 4.3 and Figures 4.5-4.10).** The total uptake of all the elements varied among the treatments.

4.4.3.2.1. P and K uptake

The uptake of P (1.68 mg/plant) and K (20.09 mg/plant) was the highest at NZVI 55 mg/L of application in the upper part of spinach. In the lower part of spinach, the uptake of P and K was higher at MZVI 110 mg/L (3.37 mg/plant) and NZVI 55 mg/L (48.5 mg/plant) of application compared to the control and other NZVI, MZVI and $FeSO_4$ concentrations.

4.4.3.2.2. Ca, Mg, and S uptake

In the upper part of spinach, the uptake of S (0.84 mg/plant), Ca (1.11 mg/plant), and Mg (1.39 mg/plant) was the highest at NZVI 55 mg/L of application. In the lower part of spinach, the uptake of Mg was higher (3.66 mg/plant) at 55 mg/L of NZVI compared to the control and other NZVI, MZVI and $FeSO_4$ concentrations. The uptake of Ca (4.02 mg/plant) and S (1.48 mg/plant) was higher in plants treated with MZVI at 110 mg/L of application compared to the control and other MZVI, NZVI and $FeSO_4$ concentrations.

4.4.3.2.3. Zn, Mn, Cu, B and Na uptake

The uptake of Cu (1.78 µg/plant), Zn (18.74 µg/plant), and B (21.92 µg/plant) in the upper part of spinach was the highest at NZVI 55 mg/L of application. But the uptake of Mn was higher at MZVI 11 mg/L (33.32 µg/plant). In the lower part of spinach, the uptake of Zn, Mn, and B was higher at 55 mg/L of NZVI compared to the control and other NZVI, MZVI and $FeSO_4$ concentrations. The uptake of Zn, Mn and B was 58.2 µg/plant, 132 µg/plant, and 74 µg/plant, respectively. The uptake of Cu was higher (4.1 µg/plant) in plants treated with MZVI at

110 mg/L of application compared to other treatments. Na uptake was higher (0.31 mg/plant) at 110 mg/L of NZVI application.

Uptake of minerals are modulated when plants are exposed to nanoparticles (Rico et al., 2015). The uptake of elements was influenced by the application of NZVI in this study. In the present study, NZVI at 55 mg/L was seen to increase the uptake of most of the elements in the upper and lower parts of the plants. This is directly associated with the higher yield of biomass. As for MZVI, with a few exceptions, the uptake of mineral elements in the upper part of the plants increased with increasing doses of MZVI. However, the uptake of macro- (P, K, S, Ca, and Mg) and micro-elements (Fe, Zn, Cu, B) showed no statistically significant differences. This trend was not observed for NZVI and $FeSO_4$. For these two treatments, the general trend demonstrated an initial increase in total uptake followed by a decrease in the uptake; total uptake increased from 11 to 55 mg/L of application and then the total uptake decreased at 110 mg/L of application. At higher concentrations, $FeSO_4$ and NZVI might have caused toxicity resulting in reduced yield of biomass, thereby decreasing the total uptake of elements.

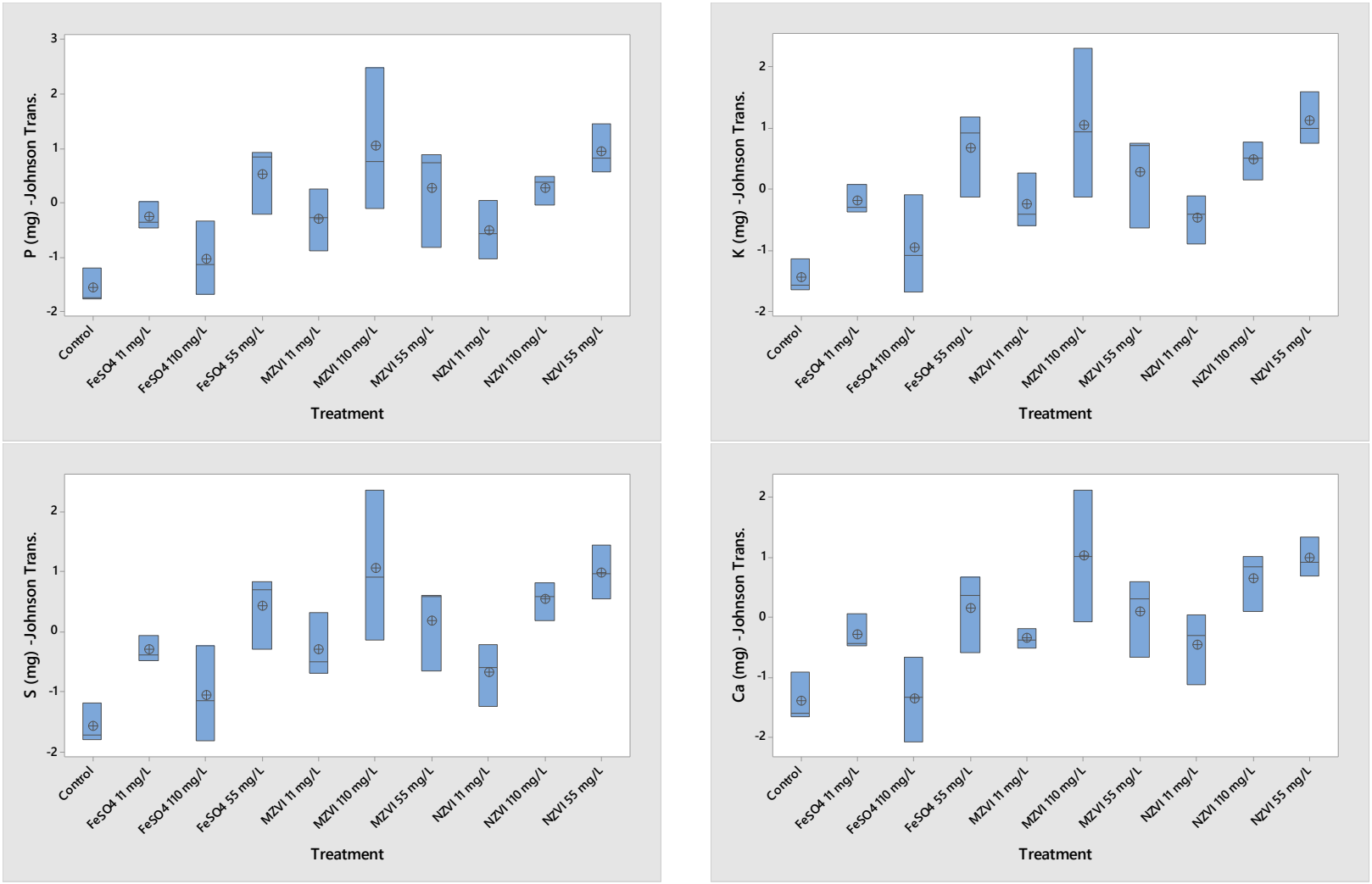

Figure 4.7. Total uptake of P, K, S and Ca in the upper part of plants dosed with different treatments. Treatments are: (i) Control: all nutrients but Fe, (ii) NZVI 11 mg/L (iii) NZVI 55 mg/L (iv) NZVI 110 mg/L (v) MZVI 11 mg/L (vi) MZVI 55 mg/L (vii) MZVI 110 mg/L (viii) $FeSO_4$ 11 mg/L (ix) $FeSO_4$ 55 mg/L (x) $FeSO_4$ 110 mg/L.

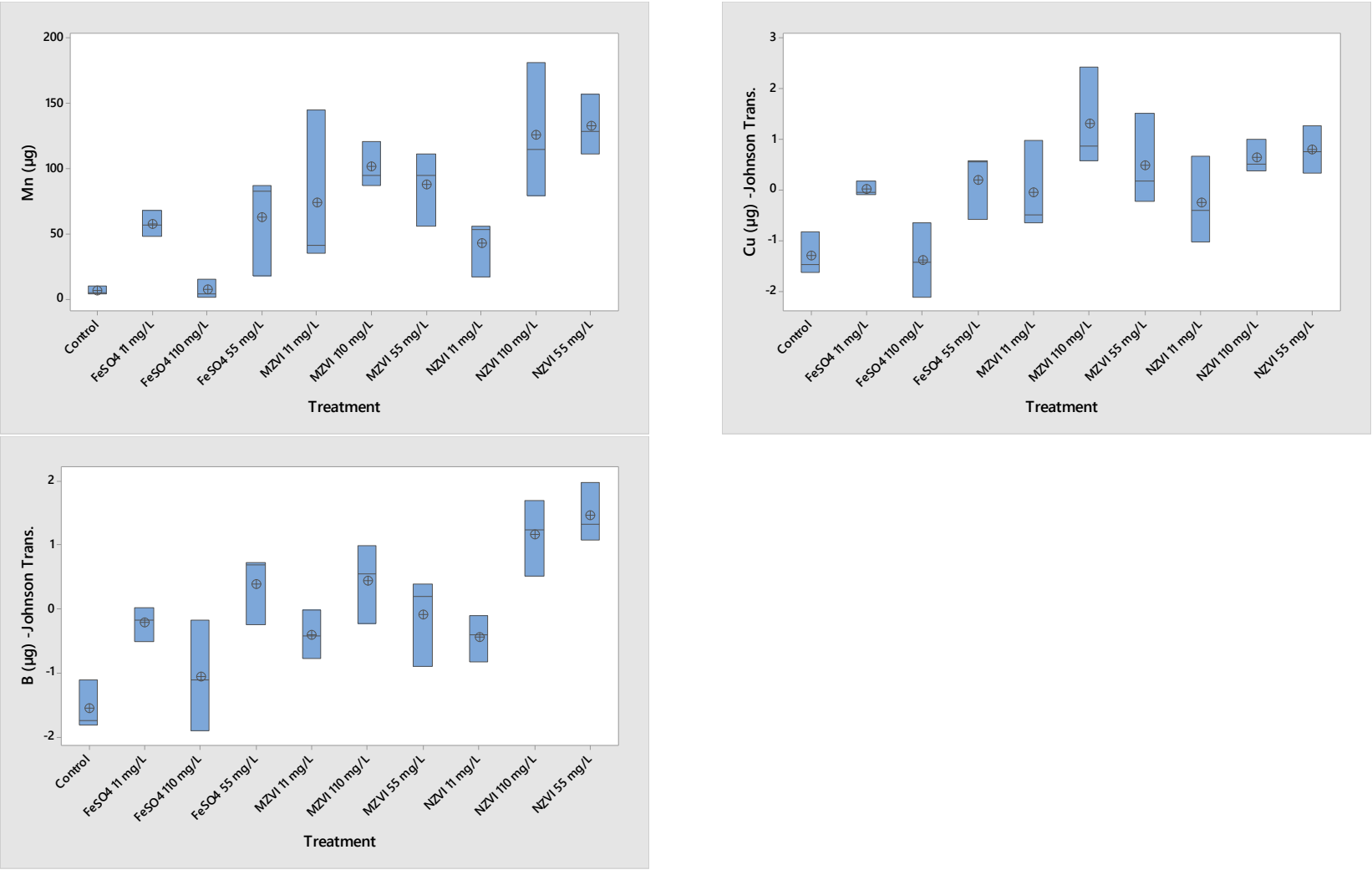

Figure 4.8. Total uptake of Mn, Cu and B in the upper part of plants dosed with different treatments. Treatments are: (i) Control: all nutrients but Fe, (ii) NZVI 11 mg/L (iii) NZVI 55 mg/L (iv) NZVI 110 mg/L (v) MZVI 11 mg/L (vi) MZVI 55 mg/L (vii) MZVI 110 mg/L (viii) $FeSO_4$ 11 mg/L (ix) $FeSO_4$ 55 mg/L (x) $FeSO_4$ 110 mg/L.

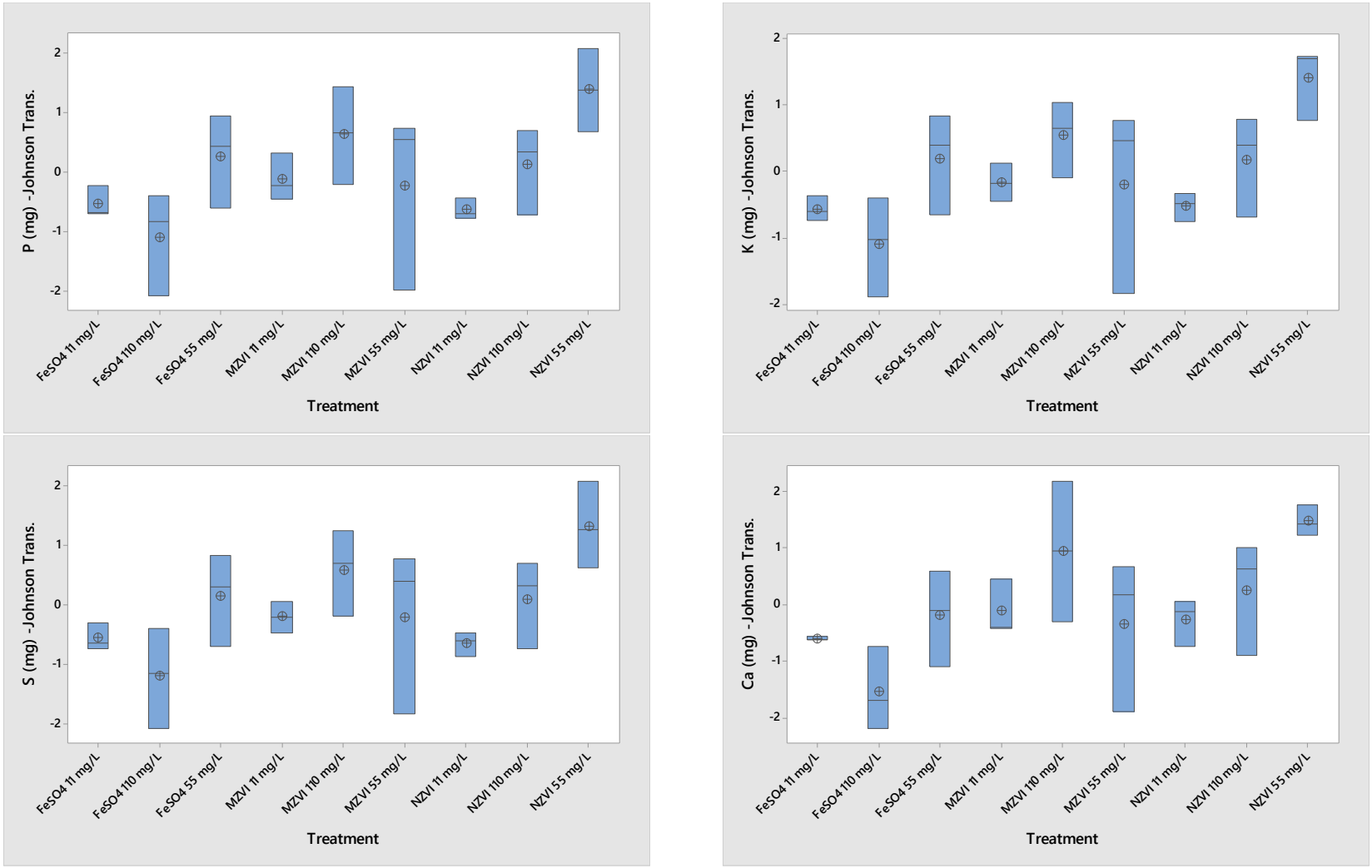

Figure 4.9. Total uptake of P, K, S and Ca in the lower part of plants dosed with different treatments. Treatments are: (i) Control: all nutrients but Fe, (ii) NZVI 11 mg/L (iii) NZVI 55 mg/L (iv) NZVI 110 mg/L (v) MZVI 11 mg/L (vi) MZVI 55 mg/L (vii) MZVI 110 mg/L (viii) $FeSO_4$ 11 mg/L (ix) $FeSO_4$ 55 mg/L (x) $FeSO_4$ 110 mg/L.

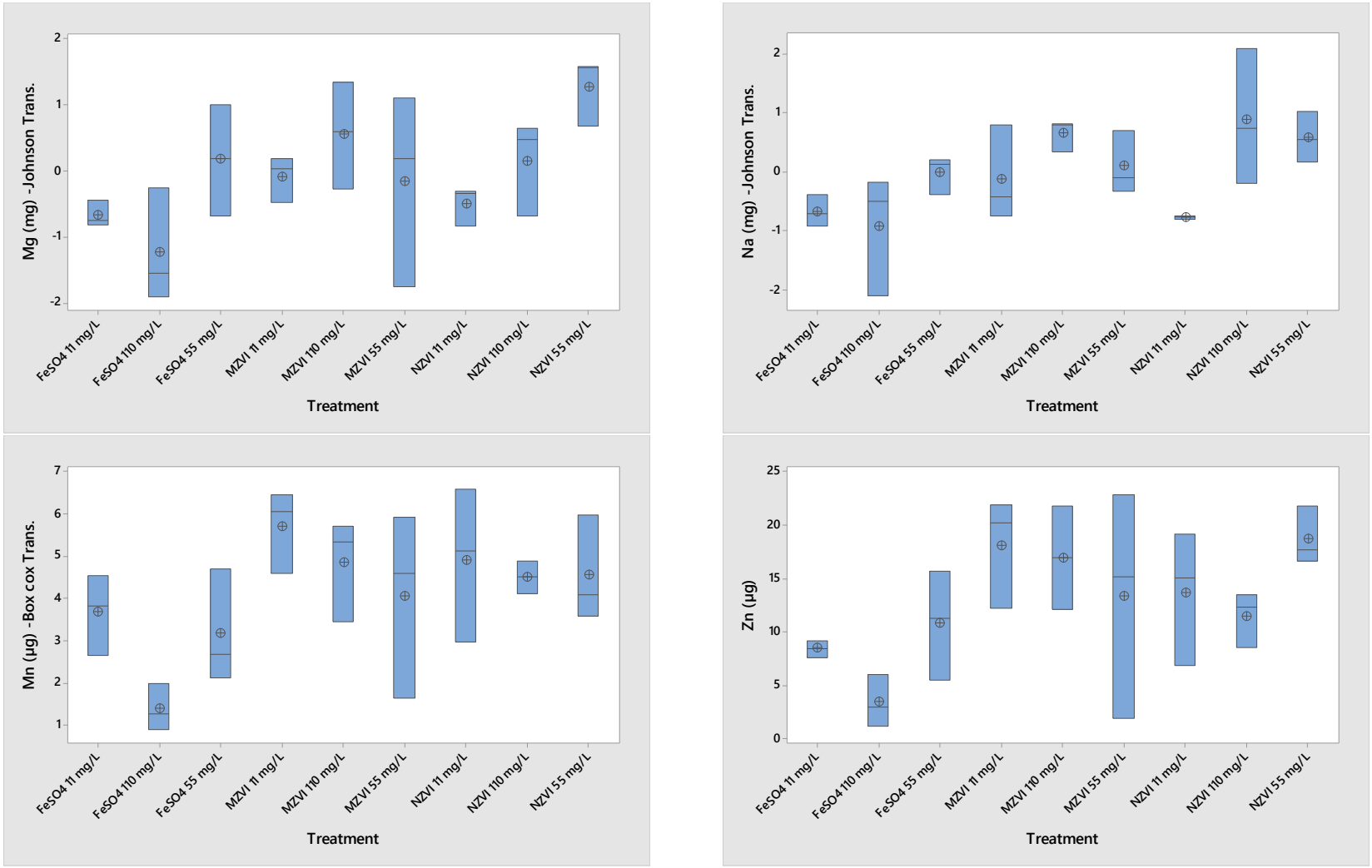

Figure 4.10. Total uptake of Mg, Na, Mn, and Zn in the lower part of plants dosed with different treatments. Treatments are: (i) Control: all nutrients but Fe, (ii) NZVI 11 mg/L (iii) NZVI 55 mg/L (iv) NZVI 110 mg/L (v) MZVI 11 mg/L (vi) MZVI 55 mg/L (vii) MZVI 110 mg/L (viii) $FeSO_4$ 11 mg/L (ix) $FeSO_4$ 55 mg/L (x) $FeSO_4$ 110 mg/L.

Excess B in the plants treated with NZVI might have come from the NZVI used for the experiment. NZVI used in this experiment was synthesized using sodium borohydride method and boron remained in NZVI as a residual. Almeelbi and Bezbaruah (2014) characterized bare NZVI with X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy with energy dispersive spectroscopy (SEM/EDS) and they indicated a significant concentrations of B (**Figure 4.11 and Table 4.4**). At higher dose, NZVI might have hampered the growth of plants because of the higher concentration of Fe^{3+} ions (Ma et al., 2013). Under reduced condition, NZVI is oxidized to Fe^{2+} ions which is further oxidized to its less soluble form (Fe^{3+}) by the oxidizing agents that are exuded from plant roots forming a cover of an insoluble Fe^{3+} compound on the root surface (Ma et al., 2013). This insoluble compound along with NZVI could block the membrane pores and appreciably reduce the efficacy of root uptake of water and nutrients.

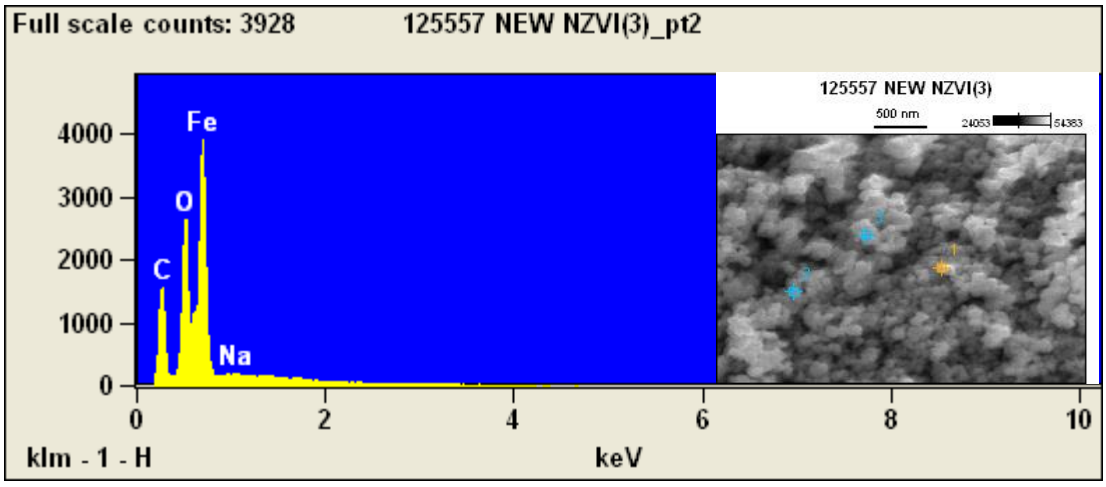

Figure 4.11. EDS spectrum of virgin NZVI (reproduced from Almeelbi and Bezbaruah, 2014).

Table 4.4. Weight percentage of elements present in virgin NZVI (reproduced from Almeelbi and Bezbaruah, 2014).

Particle Type	Part number*	Weight %		
		O	Fe	Na
Virgin NZVI	1	12.10	87.39	0.51
	2	10.37	89.32	0.31
	3	10.90	88.70	0.39

*****The part numbers used for analysis are identified in the SEM images

From the study with spinach, it is very evident that the presence of iron nanoparticles modified the uptake of iron and other elements. The objective of this present study was to see whether NZVI could be used as a biofortificant for plants. It is obvious that not only biofortification of plants with Fe was achieved but also NZVI increased the uptake of some other elements. The presence of iron nanoparticles affected the uptake of iron and other elements (P, K, S, Ca, Mg, Zn, Fe, Mn, and Cu). NZVI also enhanced biomass growth. That NZVI increased the uptake of iron at 55 mg/L concomitantly increased the uptake of most of the elements in both upper and lower part of the plants.

The availability of Fe from NZVI as well as from MZVI indicates that plants were able to use Fe using some Fe acquisition strategies. The oxidation of NZVI and its subsequent dissolution might have provided Fe^{2+}/Fe^{3+} needed for spinach growth (Kadar et al., 2012). It is not clear how bare NZVI caused an increase in nutrient uptake for other elements in spinach. However, in a previous study with *Arabidopsis thaliana*, Kim et al. (2014) revealed that NZVI enhanced root elongation by triggering OH radical-induced cell wall loosening (degradation of polysaccharides occurred as a result), and that in turn increased endocytosis in root cells. This could be an avenue for enhanced uptake of elements in spinach. In another study, Kim et al. (2015) demonstrated that NZVI triggered high plasma membrane H^+-ATPase activity in *Arabidopsis thaliana*, which resulted in a decrease in apoplastic pH, an increase in leaf area, and

also wider stomatal aperture. They attributed these phenomena to a gene called AHA2 (Plasma Membrane H^+-ATPase gene), which exhibited enhanced expression in the roots and leaves of *Arabidopsis thaliana*. This gene is involved in rhizosphere acidification (Kim et al., 2015). In this study, it is likely that a combination of these factors facilitated the enhanced uptake of the nutrient elements.

4.5. Conclusions

In this study, the feasibility of using nanoscale zero-valent iron (NZVI) as a biofortificant was tested using spinach. The experimental results suggest that NZVI can be used as a biofortificant for plants. However, the dose of NZVI appeared to be an important factor for biofortification. It was evident from the results that spinach did not respond in the same manner when the plants were dosed with a higher concentration of NZVI (110 mg/L was the highest concentration used in this experiment). Spinach plants produced more biomass with the application of 55 mg/L of nanoscale zero-valent iron. The presence of bare NZVI at 55 mg/L enhanced the growth of plant and the uptake of iron (19.77 µg/plant and 64.9 µg/plant for upper and lower part, respectively) and the uptake of other elements as well. There was a ~75% and ~70% increase in iron uptake in the part of the plants treated with NZVI compared to MZVI and $FeSO_4$ treatments. NZVI at 55 mg/L of application produced the maximum biomass in spinach. The availability of iron from bare NZVI particles was also evident from the total uptake and concentration of Fe in plants. It is believed that NZVI dissolution might have given the plant enough ionic iron in the solution for iron acquisition. The oxidation of NZVI and subsequent dissolution might have provided Fe^{2+}/Fe^{3+} needed for spinach growth. However, recommendation on the optimum dose of NZVI could not be made based on this research. Optimum dose of NZVI should be determined for each plant species by carrying out extensive

studies with specific species. The combination of plant species, type and dose of nanoparticles is likely to determine how a given plant will respond to a given nanoparticle. Further research is required to consolidate the findings and on how to apply NZVI and other nanomaterials as a biofortificant in agricultural fields.

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5. UPTAKE OF IRON BY LETTUCE (*LACTUCA SATIVA*) EXPOSED TO NANOSCALE IRON

5.1. Abstract

Experiments were conducted to evaluate the availability of iron for plant use from nanoscale zero-valent iron (NZVI) using lettuce (*Lactuca sativa*) as a model plant. The main purpose of this study was to biofortify lettuce with iron. Three lettuce (*Lactuca sativa*) varieties (Iceberg, Black Seeded Simpson, and Parris Island) were hydroponically grown in a greenhouse with bare NZVI (100 mg/L) and spent NZVI (phosphate sorbed) (100 mg/L) as sources of iron. Lettuce was grown until maturity (30 days) and iron and other nutrient elements were measured in the harvested plants. Bare NZVI was found to enhance biomass production across the varieties. The uptake of Fe and some other nutrient elements were positively affected by iron nanoparticles. The enhanced uptake of iron by lettuce is promising and will contribute towards plant nutrient fortification research.

5.2. Introduction

Socioeconomically-developing and -developed countries alike are increasingly facing the difficult question of how to feed their citizen amidst a host of emerging demographic, environmental, and health challenges. In addition to food quantity, increased attention is being paid to food quality attributes, especially nutrient content (Fan and Brzeska, 2014). Nutrition security is a broad and complex issue that encompasses a number of dimensions. Even though there are other aspects of nutrition security, in developing countries nutrition security is more geared towards malnutrition eradication efforts that address “hunger”. Micronutrient malnutrition, commonly known as ‘hidden hunger’, is one of the pressing issues in many parts of the world and affects as many as 3 billion people across the world (Welch and Graham, 2005).

Malnutrition in all its forms is directly linked to 300,000 deaths per year worldwide and is indirectly responsible for about half of all deaths in young children in the world (Black, et al., 2008, Black, et al., 2003, Victora, et al., 2008). The vast majority of world population live in poverty and lack access to a secure supply of safe and nutritious food and they do not get the recommended dietary allowances (RDAs) of mineral nutrients (**Table 5.1**).

Table 5.1. Recommended dietary allowances (RDAs) and tolerable upper limits (ULs) for the five key minerals for US adults.

Element	RDA	UL
Iodine (μg/d)	150	1,100
Iron (mg/d)	8-18	45
Zinc (mg/d)	8-11	40
Calcium (mg/d)	1,000-1,200	2,500
Selenium (μg/d)	55	400

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In human nutrition, essential minerals are defined as inorganic chemical elements or their dissociated ions that are needed for biological or biochemical processes (Gomez-Galera, et al., 2010). Carbon, hydrogen, nitrogen and oxygen are not included due to their abundance in common organic molecules. Apart from these four, there are sixteen elements which are considered vital for human beings (**Table 5.2**). Of these sixteen elements, eleven elements are needed in small quantities and are typically supplied to the body through various routes, and their deficiency is rarely seen in human. The other five elements [iodine (I), iron (Fe), zinc (Zn), calcium (Ca) and selenium (Se)] have limited presence in our typical foods. Therefore, it is highly likely that a monotonous diet will easily lead to the deficiency of these nutrients (Christou and Twyman, 2004). Mineral deficiency is most prevalent in developing countries, where people have limited access to foods in general and fresh food in particular.

Table 5.2. Deficiency of sixteen essential elements for human.

Prevalence of deficiency	Elements
Deficiency is rare	P, Mg, Na, K, Cl, Cu, Cr, Mn, Mo, Ni, F
Deficiency is common	Ca, Fe, Zn, I, Se

Adapted from Gomez-Galera et al. (2010)

Iron (Fe) deficiency is the most common nutritional deficiency. Iron has a number of functions in the human body and it constitutes the functional core of the heme complex in hemoglobin (oxygen carrier in blood) and myoglobin (oxygen storage unit in muscles). It is also found in the catalytic center of cytochromes which perform redox reactions. Therefore, iron is vital for oxygen transport in the body and for energy metabolism (WHO/FAO, 1998). Iron deficiency is mainly prevalent in women and children and causes anemia. It was estimated (2011) that around 43% of children, 38% of pregnant women, and 29% of non-pregnant women have anemia worldwide which correspond to 273 million of children, 32 million of pregnant women, and 496 million of non-pregnant women (WHO, 2015). The African Region had the highest prevalence (62.3%) of anemia in children while the most affected number of children and women resided in the South-East Asia Region, including 96.7 million of children and 202.0 million women of reproductive age (15-49 years) (WHO, 2015).

The most effective intervention to alleviate iron deficiency is the administration of a diversified diet that include fish, meat, fruit and vegetables. However, it is a challenge to provide such a balanced diet to a large number of people in socio-economically challenged countries. Food fortification and supplementation are other conventional strategies that have been adopted. Iron nutrition can be improved using oral supplements (usually in tablet form) or fortification (where iron is added to processed foods). However, these strategies are focused on a small subset of population. Some countries in Latin America have made wheat fortification with iron mandatory (Shrimpton, et al., 2005), but it involves a strong food processing and distribution

infrastructure as well as monitoring. Moreover, it increases the price of the product making fortified products unaffordable for people living in socio-economically challenged countries, especially people living in rural areas (Gomez-Galera, et al., 2010). Iron fortification with fortificants is also technically challenging because iron compounds which are easily absorbable in the human gut are also easily leachable (Frossard, et al., 2000). Fortificants also tend to make food less palatable (Frossard, et al., 2000) . On the other hand, fortificants which have less impact on the palatability aspect of the food are less absorbable (Frossard, et al., 2000).

Biofortification has been explored as an alternative long-term solution (Zhu, et al., 2007). Biofortification is defined as the process of augmenting the concentrations of essential elements in the edible part of the plants through soil application, foliar application, fertilization with irrigation water or genetic improvement (Márquez-Quiroz, et al., 2015). Biofortification is also done with microorganisms [for example, biofortification of selenium (Se) in wheat, (Duran, et al., 2013)]. The fortification of staple crop plants with bioavailable iron is likely to provide a sustainable and economical tool to remedy iron deficiency in target populations worldwide (Jeong and Guerinot, 2008).

Agronomic intervention is sometimes not efficient because iron in oxidized ferric form is less soluble in aerobic environments. The oxidized iron Fe (III) has a very low solubility at basic pH, and high bicarbonate complexation which leads to reduced uptake of iron by plant roots (Lucena, et al., 2007). Therefore, even though iron is the fourth abundant element in the earth's crust, it is the third most limiting nutrient for plant growth (Gomez-Galera, et al., 2010). Innovative iron fortification through soil and foliar application of iron containing compounds has been tried in sorghum (Ortega-Blu and Molina-Roco, 2007), wheat (Aciksoz, et al., 2011), and leafy vegetables (e.g., spinach) (Almeelbi and Bezbaruah, 2014).

During the last decade, there has been increased interest in the application of nanomaterials for agronomic purposes. Nanomaterials have been used as smart delivery systems of fertilizers, herbicides, pesticides and plant growth regulators (Perez-de-Luque and Hermosin, 2013). Scientists have also explored the possibility of using nanoparticles to biofortify plants. It is an agronomic intervention where nanoparticles are applied to enhance the growth of plants, thereby increasing the uptake of mineral elements. In a hydroponic study, Almeelbi and Bezbaruah (2014) used nanoscale zero-valent iron (NZVI) for phosphate removal and subsequently used the spent (phosphate-sorbed) NZVI as a source of phosphorus and iron for spinach and algae. They found increased growths of spinach and algae when spent NZVI was used as a sole source of iron and phosphorus. The iron content increased significantly in all plant parts (roots, stems and leaves) when spent NZVI was used as a source of iron. Iron content increased by 7 and 11 times in the stem and leaves in spinach as compared to the control (conventional $FeSO_4$ as the source of iron). Superparamagnetic iron oxide nanoparticles (SPIONs) was found to be taken up and translocated by hydroponically-grown soybean plants and increase in chlorophyll content in the plants were reported and no toxicity to plants was observed (Ghafariyan, et al., 2013). FeOx NPs and MnOx NPs (<50 pp) were found to stimulate the growth of lettuce seedlings by 12-54% compared to their ionic counterparts (Liu, et al., 2016). Zinc nanoparticles (Zn NP) have also been shown to enhance growth in rice (*Oryza sativa*) when the plants were dosed with 25 and 50 mg/L of Zn NP (Upadhyaya, et al., 2015). Calcium phosphate nanoparticles in association with an arbuscular mycorrhizal fungus (*G. mosseae*) and endosymbiont (*P. indica*) was found to enhance growth, root proliferation and vitality improvement properties in *Zea mays* (Rane, et al., 2015).

Keeping all these in mind, studies were undertaken to examine whether NZVI can be used for iron biofortification and whether the presence of NZVI affects the availability of other minerals in hydroponically grown lettuce (*Lactuca sativa*). Lettuce was selected for this experiment because this plant is one of the most consumed vegetables worldwide with a global production of about 24 million tons (FAOSTAT, 2011). Mean daily consumption of lettuce in Europe is 22.5 g, which corresponds to 6.5% of the total dietary intake of vegetables (WHO, 2003). Lettuce also contains several macro-elements (e.g., K, Na, Ca and Mg) and micro-elements (e.g., Fe, Mn, Cu, Zn and Se) which are vital for human nutrition (Kawashima and Valente Soares, 2003). Fresh lettuce is eaten in salads by a large number of people in the world. This work will provide insights into the effects of NZVI on the uptake of Fe and other essential mineral elements. The premise of the work is that NZVI will trigger an enhanced uptake of Fe when present in the hydroponic solution as a sole source of iron.

5.3. Materials and Methods

5.3.1. Chemicals

Iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$, 99 % pure, Alfa Aesar), micro zero-valent iron powder (<10 micron, 99.9+%, SIGMA-ALDRICH), sodium borohydride ($NaBH_4$, 98 %, SIGMA-ALDRICH)), sodium hydroxide (5 N NaOH, Alfa Aesar), HNO_3 (68 %, J.T. Baker), methanol (production grade, BDH), ethanol (ACS grade, Mallinckrodt Chemicals), calcium nitrate tetrahydrate ($CaNO_3 \cdot H_2O$, Alfa Aesar), potassium nitrate (KNO_3, Mallinckrodt Chemicals), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, Mallinckrodt Chemicals), magnesium nitrate hexahydrate ($Mg(NO_3)_2 \cdot 6H_2O$, Alfa Aesar), ammonium dihydrogen phosphate ($NH_4H_2PO_4$, Alfa Aesar), sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$, amresco), copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, BDH), manganese sulfate monohydrate

($MnSO_4 \cdot H_2O$, Mallinckrodt Chemicals), sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$, BTC) zinc sulfate monohydrate ($ZnSO_4 \cdot H_2O$, J.T. Baker) and ethylenediaminetetraacetic acid, ammonium nitrate (NH_4O_3, ACS grade, Alfa Aesar), and iron(III) monosodium salt ($FeNa(O_2CCH_2)_2NCH_2CH_2N(CH_2CO_2)_2$, ACS Grade, Alfa Aesar) were used as received unless otherwise specified.

5.3.2. Synthesis and preparation of NZVI

5.3.2.1. NZVI synthesis

NZVI particles were synthesized using sodium borohydride reduction method (Almeelbi and Bezbaruah, 2012). Iron (II) sulfate heptahydrate (10 g) was dissolved in 100 mL of 30% of methanol (30 mL methanol + 70 mL deoxygenated de-ionized (DI) water) (Solution A). The pH of the solution was then adjusted to 6.1 adding 5 N NaOH drop by drop. In the meantime, 3.94 g of sodium borohydride was dissolved in 100 mL of deoxygenated DI water in a 100 mL volumetric flask (Solution B). Once the pH reached 6.1, Solution A was immediately added dropwise to Solution B using a burette under vigorous stirring conditions (using a magnetic stirrer). The combined solution was then allowed to stand for 20 min. The resultant black precipitates (NZVI) were centrifuged and washed with ethanol. The NZVI in slurry form was then dried in a vacuum oven under nitrogen environment. Finally, the dried NZVI particles were ground using a mortar and pestle and stored in 20 mL vials (headspace flushed with nitrogen) for later use.

5.3.2.2. Spent NZVI preparation

One gram of NZVI was added to 250 mL of phosphate solution (500 mg PO_4^{3-}-P/L) in a reactor. The reactor was rotated end-over-end at 28 rpm in a custom-made shaker for 24 h, and then the contents were centrifuged at 4000 rpm. The supernatant was separated and analyzed for

phosphate concentration using ascorbic acid method (Eaton, et al., 2005). The spent iron particles (with phosphate sorbed in and onto them) were dried in a vacuum oven under nitrogen environment and ground using a mortar and pestle. A measured amount of the dried spent NZVI particles were used for lettuce studies.

5.3.3. Experimental set-up for lettuce study in hydroponics

5.3.3.1. Germination and plant preparation for hydroponic experiment

Three commercial varieties of lettuce (*Lactuca sativa*, Burpee, Warminster, PA) seeds, namely Iceberg, Black Seeded Simpson and Parris Island, were purchased from a local outlet. The lettuce seeds were germinated in plug trays with nonabsorbent cotton as media (**Figure 5.1**). The seeds were kept moist using automated misting nozzles in a greenhouse. The germinated seeds were then moved to another room and allowed to grow for ten days. The seedlings were fed with half strength Hoagland solution (Hoagland and Arnon, 1950) every day. The seedlings were provided with cool-white fluorescent light (14 h light/10 h dark cycle). The light intensity was ~300 µmol/m^2/s.

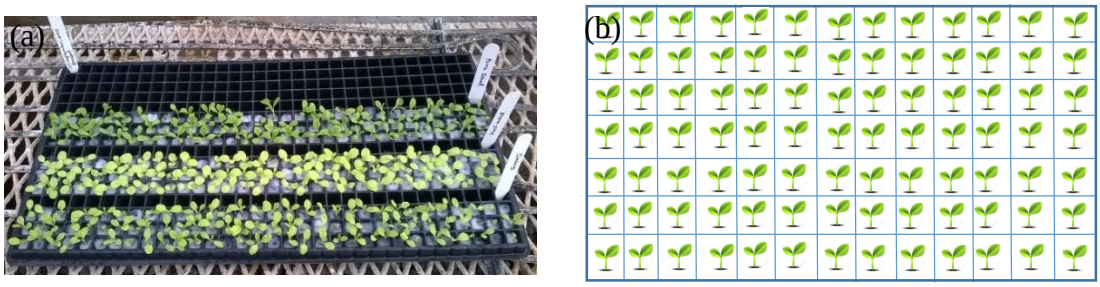


Figure 5.1. (a) Lettuce seeds were germinated in a plug tray (b) Schematic of the plug tray.

5.3.3.2. Growth studies

After plants developed a root system and were at four-to-five-leaf stage, healthy plants of similar size were carefully removed from the plug trays and rinsed with deionized water while keeping the roots intact. Plants were then anchored to a Styrofoam sheet with nine holes made

with a hole punch. The Styrofoam was cut in a way so that it snugly fitted the opening of a plastic container; this was done to reduce the light entering the nutrient solution placed beneath the Styrofoam. Light would otherwise stimulate algal growth in the water and could modify the growth condition for the plants. Nine plants (three plants from each variety) were anchored through the holes and held in place by plugging in nonabsorbent cotton (**Figure 5.2**). Ten liters (10 L) of Hoagland nutrient solution (Hoagland and Arnon, 1950) was modified according to the treatments (**Tables 5.3, 5.4, 5.5, and 5.6**) were used (Hoagland and Arnon, 1950). The treatments were (1) spent NZVI plus all nutrients but Fe and P, (2) No Fe and P: all nutrients but Fe and P, (3) No Fe: all nutrients but Fe, (4) No P: all nutrients but P, (5) bare NZVI plus all nutrients but Fe, and (6) Control 4: all nutrients. The experiment was a completely randomized design and the doses of bare and spent NZVI were 100 mg/L. This concentration was chosen based on earlier works of other scientists. Concentrations higher than 200 mg/L were found to pose toxicity towards cattail and hybrid poplar and concentrations around 20 mg/L did not affect the growth of lettuce (Ma, et al., 2014, Trujillo-Reyes, et al., 2014). It was hypothesized that lettuce will not be affected by this concentration of NZVI (100 mg/L) and will rather promote the uptake of Fe. The experiment was conducted in duplicate in 12 plastic containers and the nutrient solution was aerated with bubblers at a rate of ~2 cm^3 air/minute. The nutrient solutions and nanoparticles were replaced every five days. Light was provided in 14 h light/10 h dark cycles with cool-white fluorescent bulbs with a light intensity of ~300 μmol/m^2/s.

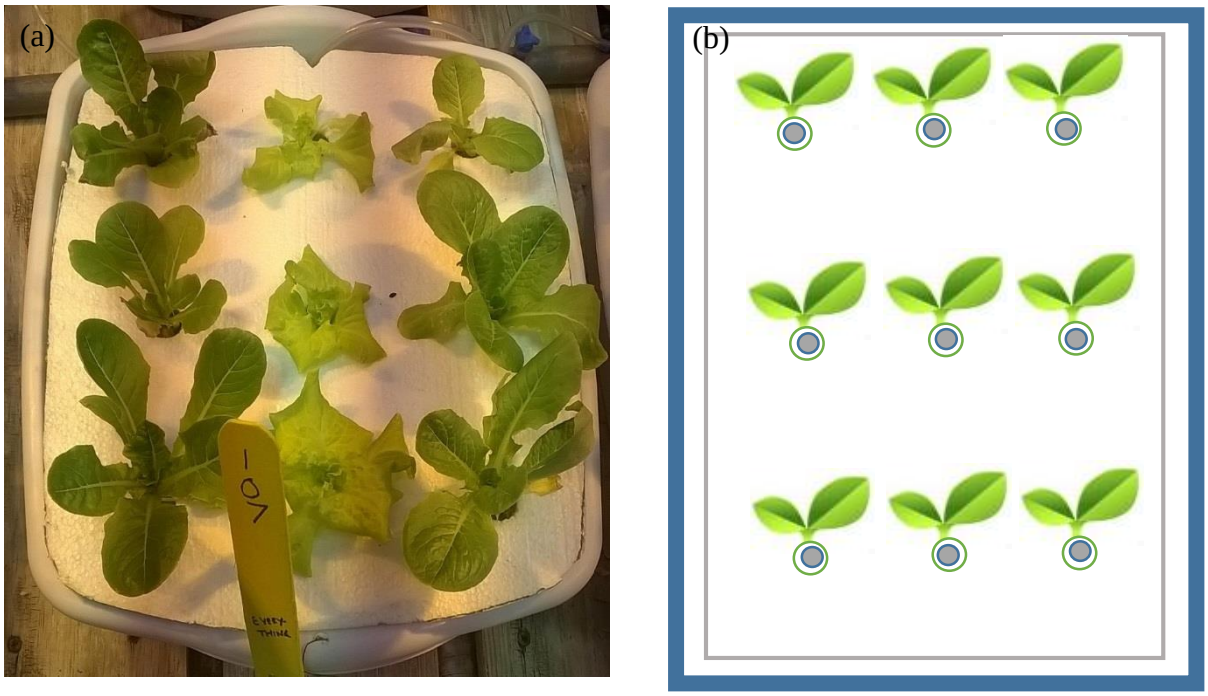


Figure 5.2. (a) Plant growth unit (b) Schematic of the growth unit.

Table 5.3. Modified Hoagland solution (Hoagland and Arnon, 1950).

Chemicals	Final concentration		Important ions
	mM or µM	mg/L	
$Ca(NO_3)_2 \cdot 4H_2O$	2 mM	472	Ca^{2+}, NO_3^-
KNO_3	6 mM	606	K^+, NO_3^-
$MgSO_4 \cdot 7H_2O$	0.5 mM	123	Mg^{2+}, SO_4^{2-}
$Mg(NO_3)_2 \cdot 6H_2O$	0.5 mM	128	Mg^{2+}, NO_3^-
$NH_4H_2PO_4$	2 mM	230	NH_4^+, $H_2PO_4^-$
$Na_2B_4O_7 \cdot 10H_2O$	20 µM	3.81	$B_4O_7^{2-}$
$CuSO_4 \cdot H_2O$	0.5 µM	0.089	Cu^{2+}
$MnSO_4 \cdot 3H_2O$	10 µM	2.05	Mn^{2+}
$Na_2MoO_4 \cdot 2H_2O$	0.5 µM	0.12	MoO_4^{2-}
$ZnSO_4 \cdot H_2O$	4 µM	0.716	Zn^{2+}
FeNaEDTA	10 µM	1.69	Fe^{2+}

Table 5.4. Modified Hoagland solution containing no iron (modified for this research).

Chemicals	Final concentration		Important ions
	mM or µM	mg/L	
$Ca(NO_3)_2•4H_2O$	2 mM	472	Ca^{2+}, NO_3^-
KNO_3	6 mM	606	K^+, NO_3^-
$MgSO_4•7H_2O$	0.5 mM	123	Mg^{2+}, SO_4^{2-}
$Mg\ (NO_3)_2•6H_2O$	0.5 mM	128	Mg^{2+}, NO_3^-
$NH_4H_2PO_4$	2 mM	230	NH_4^+, $H_2PO_4^-$
$Na_2B_4O_7•10H_2O$	20 µM	3.81	$B_4O_7^{2-}$
$CuSO_4•H_2O$	0.5 µM	0.089	Cu^{2+}
$MnSO_4•3H_2O$	10 µM	2.05	Mn^{2+}
$Na_2MoO_4•2H_2O$	0.5 µM	0.12	MoO_4^{2-}
$ZnSO_4•H_2O$	4 µM	0.716	Zn^{2+}

Table 5.5. Modified Hoagland solution containing no phosphorus (modified for this research).

Chemicals	Final concentration		Important ions
	mM or µM	mg/L	
NH_4NO_3	2 mM	160	NH_4^+, NO_3^-
$Ca(NO_3)_2•4H_2O$	2 mM	472	Ca^{2+}, NO_3^-
KNO_3	6 mM	606	K^+, NO_3^-
$MgSO_4•7H_2O$	1 mM	246	Mg^{2+}, SO_4^{2-}
$Na_2B_4O_7•10H_2O$	20 µM	3.81	$B_4O_7^{2-}$
$CuSO_4•H_2O$	0.5 µM	0.089	Cu^{2+}
$MnSO_4•3H_2O$	10 µM	2.05	Mn^{2+}
$Na_2MoO_4•2H_2O$	0.5 µM	0.12	MoO_4^{2-}
$ZnSO_4•H_2O$	4 µM	0.716	Zn^{2+}
FeNaEDTA	10 µM	1.69	Fe^{2+}

Table 5.6. Modified Hoagland solution containing no phosphorus and no iron (modified for this research).

Chemicals	Final concentration		Important ions
	mM or µM	mg/L	
NH_4NO_3	2 mM	160	NH_4^+, NO_3^-
$Ca(NO_3)_2•4H_2O$	2 mM	472	Ca^{2+}, NO_3^-
KNO_3	6 mM	606	K^+, NO_3^-
$MgSO_4•7H_2O$	1 mM	246	Mg^{2+}, SO_4^{2-}
$Na_2B_4O_7•10H_2O$	20 µM	3.81	$B_4O_7^{2-}$
$CuSO_4•H_2O$	0.5 µM	0.089	Cu^{2+}
$MnSO_4•3H_2O$	10 µM	2.05	Mn^{2+}
$Na_2MoO_4•2H_2O$	0.5 µM	0.12	MoO_4^{2-}
$ZnSO_4•H_2O$	4 µM	0.716	Zn^{2+}

5.3.4. Analytical procedures

5.3.4.1. Lettuce studies

Plants were harvested after 30 days of hydroponic growth. The harvested plants were washed with copious amounts of DI water and the plants were separated into roots and leaves. The roots were washed with 10 mM $CaCl_2$ solution to remove any attached NZVI but the nanoparticles were found difficult to be removed. Moreover, there was algae growth around root surfaces. Therefore, the roots were not analyzed for macro- and micro-nutrients. The fresh weight of leaves was taken. Three plants for each variety grown in the reactors (plastic container) were combined during weighing and the combined mass is reported. The chlorophyll content was measured after 15 days of transplantation and just before harvest (30 days) using a Minolta Chlorophyll Meter SPAD-502 (Minolta, Japan). Total soluble solid (TSS) content was measured immediately after harvesting using a hand refractometer (Model N1; Atoago, Tokyo, Japan). For TSS measurement, 10 g of fresh tissue from a sample was ground and the paste was squeezed through four-layer cotton cloth to extract juice. A few drops of juice were then dropped onto the

refractometer window for reading the Brix value. The results are expressed in degree Brix. The harvested plant material was then dried at 65 ^{0}C until constant weight. Thereafter, the samples were powdered and homogenized using a mortar and pestle.

5.3.4.2. Macro- and micro-nutrient measurements

The powdered plant tissues collected from hydroponic experiments were digested using the protocol by Jones Jr. (2001). Samples (~0.25 g) were weighed into a digestion tube and 5.0 mL of conc. HNO_3 was added. The mouth of the digestion tubes was covered with watch glasses and then the tubes were allowed to stand overnight. The tubes were then placed on a hot plate and digested at 125 ^{0}C for 1 h. The tubes were then allowed to cool to room temperature (22±2 °C) and 3 mL of 30% H_2O_2 was added to the tubes. The contents were again digested at 125 ^{0}C until the digest was clear. The colorless digest was brought to volume adding 1:10 HNO_3 and the solution was analyzed for P, K, Ca, Mg, Na, Zn, Fe, Mn, Cu, B using a Perkin Elmer ICP-OES (5300 DV Model). A control standard was run after every ten samples to check whether the values were within acceptable limits (10% of the expected values). Total N in plant tissue was measured using a nitrogen combustion analyzer (Vario MAX cube, Elementar Americas Inc).

5.3.5. Statistical analysis

All elements are reported in mg/plant or µg/plant. However, the data was transformed where needed using Johnson transformation and Box-cox transformation prior to statistical analysis to increase the homogeneity of variance. One-way analysis of variance (ANOVA) was performed for elements among the treatments, followed by a Tukey's pairwise comparisons. Means of the results are reported. Pearson correlation analysis was also performed between the elements for each treatment. Significant correlations with $r > 0.5$ (therefore explaining 25% of

total variation) was only considered for further discussion. All statistical analysis was performed on Minitab version 17. Significance was determined based on *p*-values <0.05.

5.4. Results and Discussion

5.4.1. Particles characterization

Average particles size of virgin NZVI was 16.24 ± 4.05 nm (Almeelbi and Bezbaruah, 2012). The percentage of oxygen in the virgin NZVI was found to be 12.10%. Krajangpan et al. (2012) reported it as 15.66%. The presence of a very low amount (0.51%) of Na was observed in the virgin NZVI. Sodium (Na) was possibly left behind as a residual from sodium borohydride ($NaBH_4$) used in the NZVI synthesis process.

5.4.2. Plant germination and growth

Lettuce seed germination done on a plug tray was almost 100%. After the harvest, the similar parts (e.g., leaves) from each reactor (three plants each for each variety) were combined together and were weighed immediately. The average weight (g) is reported here. The dry weight is also shown in **Table 5.7**.

5.4.2.1. Leaf growth

The fresh weight of lettuce plants varied depending on the treatment (**Figures 5.3 and 5.4**). In the plants treated with bare NZVI, the average weights of fresh leaves were 82.3, 52.5, and 77 g/plant for Iceberg, Black Seeded Simpson and Parris Island, respectively. The corresponding values for the control (all the nutrient elements) were 71.5, 54.8, and 63.8 g/plant, respectively. Bare NZVI treatment resulted in increase in fresh weight of the lettuce by ~15% and ~21% for Iceberg and Parris Island, respectively compared to the control. However, fresh weight of NZVI-treated Black Seeded Simpson decreased by 4% compared to the control. However, the difference between these two treatments was not statistically significant. In the

plants treated with spent NZVI (phosphate sorbed), the average weights of fresh leaves were 5.3, 0.8, and 4.2 g/plant for Iceberg, Black Seeded Simpson, and Parris Island, respectively. The corresponding values for No Fe and P (all nutrients but Fe and P) were 1.9, 0.3, and 1.4 g/plant. The values for No P (all nutrients without P) were 3.6, 0.5, 1.4 g/plant for Iceberg, Black Seeded Simpson and Parris Island, respectively. The fresh weight of lettuce plants treated with bare NZVI and the control were significantly higher across the varieties than in the plants treated with other treatments (No Fe and P, No Fe, No P and spent NZVI). Spent NZVI, which contains Fe in nano form and sorbed P, did better compared to No Fe and P, and No P, but the differences were not statistically significant. This suggests that the plant nutrients were available for plant uptake, but not to the point of being comparable to the plants grown with all regular nutrient elements (Control).

Figure 5.3. Lettuce biomass under different treatments after 30 days of hydroponic growth. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients.

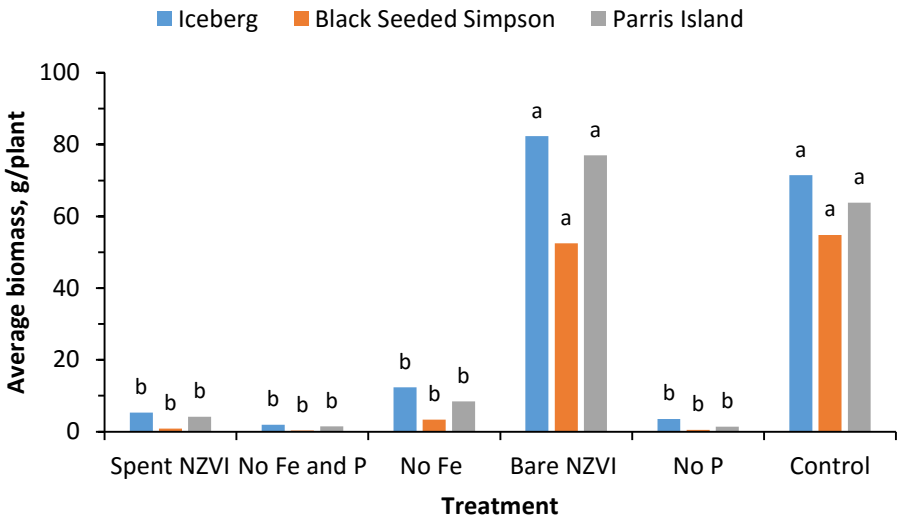


Figure 5.4. Average weights of lettuce leaves biomass after 30 days of hydroponic growth. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients. The values represent the average weight of three plants grown for each variety in each container. Different letters indicate significant differences between different treatments within the same variety. There were significant differences between bare NZVI treatment and No Fe and P, No Fe and No P. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison.

Table 5.7. Average dry weights of lettuce leaves biomass after 30 days of hydroponic growth. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients. The values represent the average weight of three plants grown for each variety in each container.

Treatment	Iceberg	Black Seeded	Parris Island
Spent NZVI + all nutrients but Fe and P	1.13	0.26	0.89
No Fe and P	0.57	0.10	0.53
No Fe	1.16	0.34	0.99
Bare NZVI + all nutrients but Fe	4.62	3.06	3.45
No P	0.63	0.28	0.51
Control	3.54	2.68	2.85

There are conflicting reports in the literature about the effects of NZVI on plant growth. Some researchers demonstrated that NZVI can enhance the growth of plants (Almeelbi and

Bezbaruah, 2014, Kadar, et al., 2012). Other researchers reported the toxic effects of NZVI on plants (Ma, et al., 2013, Wang, et al., 2016). It is evident that in most of the cases these scientists worked with different species of plants (rice, spinach, lettuce, algae, cattail, etc.) and applied different doses of NZVI (10 mg/L, 20 mg/L, 200 mg/L, etc.). Plants exhibit a wide variety of responses towards nanoparticles which accounts for the contradictory reports about the toxicity of nanoparticles (López-Moreno, et al., 2010, Navarro, et al., 2008, Wang, et al., 2016).

In the present experiment, enhanced growth was observed in Iceberg and Parris Island varieties treated with bare NZVI compared to plants treated with the control (containing all the nutrient elements). These findings could be corroborated by the findings of Almeelbi and Bezbaruah (2014). Almeelbi and Bezbaruah (2014) studied the impacts of spent NZVI (phosphate sorbed) on spinach (*Spinacia oleracea*) and revealed that spent NZVI increased the roots and shoots of spinach by ~3.5 times compared to control (which contained all nutrients required for plant growth but no NZVI). The findings of this present experiment are also in line with the findings of Kadar et al. (2012); marine microalgae *Tetraselmis suecica* demonstrated 30% higher growth rate in the presence of NZVI (Kadar, et al., 2012). NZVI was also found to enhance the root elongation of *Arabidopsis thaliana* (Kim, et al., 2014). On the other hand, Trujillo-Reyes et al. (2014) used 10 and 20 mg/L concentrations of core-shell structured nanoscale materials (Fe/Fe_3O_4), and found no positive or negative effects on the length of roots and on the biomass of lettuce. Ma et al. (2013) varied the doses of NZVI in hydroponics and demonstrated that NZVI at lower concentrations (25-50 mg/L) enhanced plant growth in cattail (*Typha latifolia*) biomass. However, it triggered toxicity in plants at concentrations higher than 200 mg/L; NZVI was found to reduce plant growth and biomass in cattail and hybrid poplar (*Populous deltoids* × *Populous nigra*). NZVI at higher concentrations (>200 mg/L) also reduced

transpiration rate in hybrid poplar. The decrease in transpiration was attributed to the formation of iron plaque on the root surface that might have reduced water uptake and to the significantly less number of leaves in plants (Ma, et al., 2013).

In a soil study, Wang et al. (2016) used 0, 100, 250, 500, 750 and 1000 mg/kg of NZVI to investigate its effects on germination, seedlings growth, and physiology as well as toxicity on rice (*Oryza sativa*). They found no effects of NZVI on germination, but seedlings' growth was found to be affected in higher concentrations (>500 mg/kg). The plants were noticeably shorter than the controls and the seedlings exhibited visible signs of chlorosis. At 1000 mg/kg of NZVI, they observed reduction in fresh weight of the root and shoot tissues by 46.8% and 22.8%, respectively. Visible symptoms of iron deficiency in plants were observed at higher concentrations (>500 mg/kg) of NZVI. The deficiency was induced by the apparent blocking of active iron from the root to the shoot as the cortex tissues were seriously damaged by NZVI applied in the soil (Wang, et al., 2016). Wang et al. (2016) also opined that the effect of NZVI on plant growth is dependent on the culture conditions and plant species. From the present experiment and the previous experiments conducted by other researchers it appears that the impact of NZVI is dependent on plant species as well as varieties.

5.4.2.2. Root growth

The fresh weight of lettuce roots also varied depending on the treatment and followed the same trend like leaves (**Figure 5.5**). In the plants treated with bare NZVI, the average weights of fresh roots were 7.04, 4.10, and 6.14 g/plant for Iceberg, Black Seeded Simpson and Parris Island, respectively. The corresponding values for the control, which contained all the nutrient elements, were 5.60, 4.25, 4.84 g/plant, respectively. Bare NZVI treatment resulted in increase in fresh weight of the lettuce roots by ~26% and ~27% for Iceberg and Parris Island, respectively,

compared to the control. However, fresh weight of roots of bare NZVI-treated Black Seeded Simpson decreased by ~3% compared to the control. In the plants treated with spent NZVI, the average weights of fresh roots were 0.41, 0.04, 0.26 g/plant for Iceberg, Black Seeded Simpson, and Parris Island, respectively. The fresh weight of lettuce roots treated with bare NZVI and the control were significantly higher across the varieties than in the plants treated with other treatments (No Fe and P, No Fe, No P and spent NZVI).

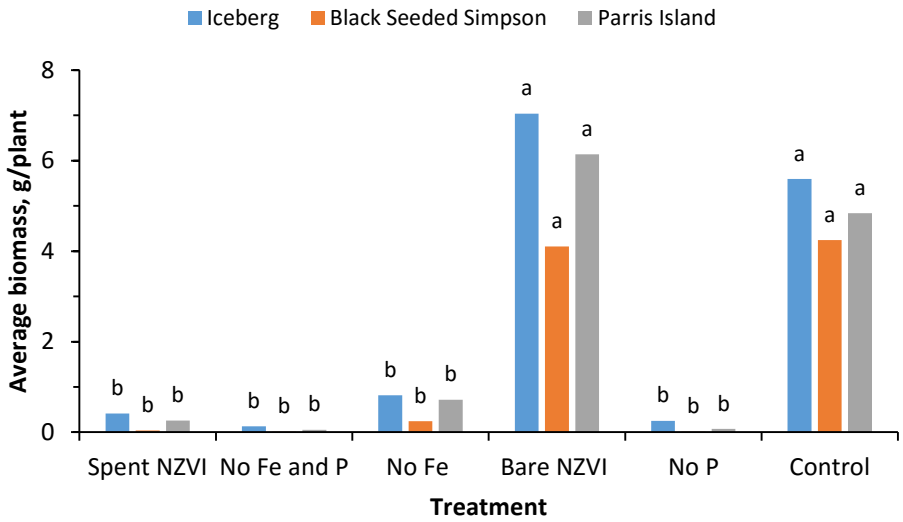


Figure 5.5. Average root biomass weights of lettuce after 30 days of hydroponic growth. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients. The values represent the weight of three plants grown for each variety in each container. Different letters indicate significant differences between different treatments within the same variety. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison.

5.4.2.3. Chlorophyll content

The chlorophyll content of the leaves was measured by Minolta SPAD-502 Chlorophyll Meter (Spectrum Technologies, Plainfield, Ill.) 15 days after transplantation into the hydroponic solution and just before harvest. The SPAD-502 provides an alternative method for the

measurement of chlorophyll. A good correlation was reported between SPAD 502 readings and spectrophotometric chlorophyll content of leaves (Hawkins, et al., 2009, Ling, et al., 2011).

After 15 days of transplantation, the chlorophyll content was higher in Iceberg variety treated with bare NZVI (51.1 SPAD unit) compared to No Fe and P (30.3 SPAD unit), No Fe (16.8 SPAD unit) and the control (33.1 SPAD unit) (**Figure 5.6**). The chlorophyll content in lettuce plants with spent NZVI treatment (42.0 SPAD unit) was comparable with bare NZVI treatment. However, there was a significant difference between spent NZVI and bare NZVI in terms of fresh weight of lettuce leaves. It is likely that the chlorophyll content is more concentrated in the plants treated with spent NZVI that yielded less biomass. No significant differences in chlorophyll content were observed for other two varieties (Black seeded

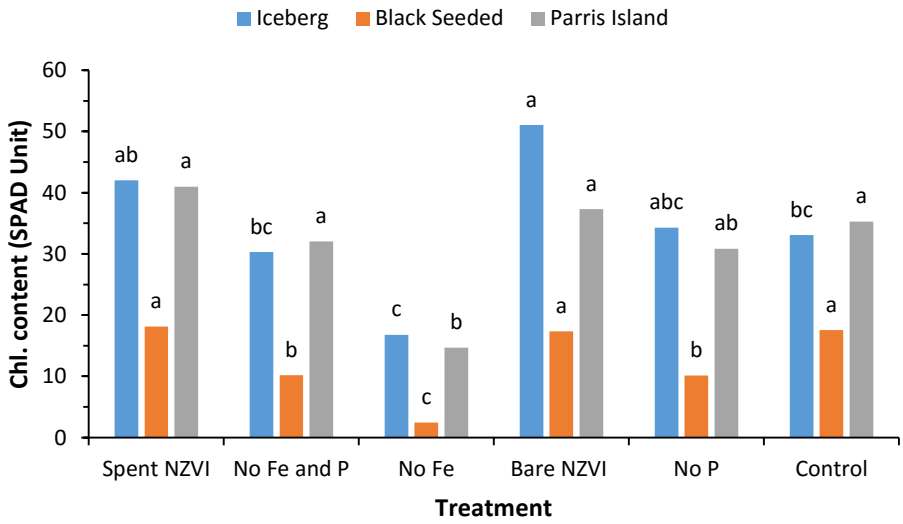


Figure 5.6. Average chlorophyll content of lettuce leaves after 15 days of hydroponic growth. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients. The values represent the average chlorophyll content (SPAD Unit) of three plants grown for each variety in each container. Different letters indicate significant differences between different treatments within the same variety. There were significant differences between bare NZVI treatment and No Fe and P, No Fe and the control for Iceberg variety. No significant differences were observed for other two varieties between bare NZVI and control. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison.

and Parris Island) treated with bare NZVI, spent NZVI and the control. That bare and spent NZVI increased the chlorophyll content in Iceberg variety is in agreement with the findings of others (Almeelbi and Bezbaruah, 2014, Wang, et al., 2016). Almeelbi and Bezbaruah (2014) reported that bare NZVI and spent NZVI (with phosphate sorbed) enhanced the Chlorophyll A (Chl. A) content substantially in *Selenastrum capricornutum* (a common green algae). In a hydroponic study with ginger (*Zingiber officinale*), Siva and Benita (2016) observed that iron oxide nanoparticles at 100 mg/L increased chlorophyll content in ginger leaves compared to plants treated with FeEDTA. On the other hand, Trujillo-Reyes et al. (2014) used 10 and 20 mg/L concentrations of core-shell nanoscale materials (Fe/Fe_3O_4), and observed no effects on the chlorophyll content of *Lactuca sativa*. Comparison of work by Almeelbi and Bezbaruah (2014), Siva and Benita (2016), and the current research leads to the conclusion that iron nanoparticles in zero-valent (Fe^0) form affects chlorophyll content in plants.

When compared with 15 days data, after 30 days of hydroponic growth, the chlorophyll content in Iceberg (44.4 SPAD unit) and Parris Island (35.7 SPAD unit) varieties decreased but the content increased in Black Seeded Simpson (27.4 SPAD unit) with the bare NZVI treatment in comparison with the control (56.0, 21.4 and 44.2 SPAD unit for Iceberg, Black Seeded and Parris Island varieties), where chlorophyll content increased across the varieties. However, the differences between bare NZVI and the control were not statistically significant (**Figure 5.7**). That chlorophyll content increased in Black Seeded Simpson and decreased in Iceberg and Parris Island varieties with time in the presence of NZVI could possibly be explained by maturity time of the three varieties. Iceberg and Parris Island varieties might have matured earlier than Black Seeded Simpson in the presence of NZVI. Chlorophyll content of certain species increases until maturity and then it decreases (Yang, et al., 2013). It is ascribed to the breaking down of the

proteins of the chloroplasts owing to the shortage of carbohydrates in the plant (Singh and Rao, 1937). That chlorophyll content was high in Iceberg and Parris Island varieties in the middle of the growth period compared to maturity stage is in agreement with the findings of Pinto et al. (2014). In their experiment with lettuce, the chlorophyll content was found to decrease in an age-related manner (Pinto, et al., 2014). In this work, there were no statistically significant differences observed among bare NZVI, spent NZVI and the control treatments across the varieties. Chlorophyll contents in No Fe and P (nutrient solution with no Fe and P) and No Fe (nutrient solution with no Fe) were significantly lower than most of the treatments; plants did not have Fe in the solution which is needed to synthesize chlorophyll and photosynthetic apparatus (Meharg and Marschner, 2012).

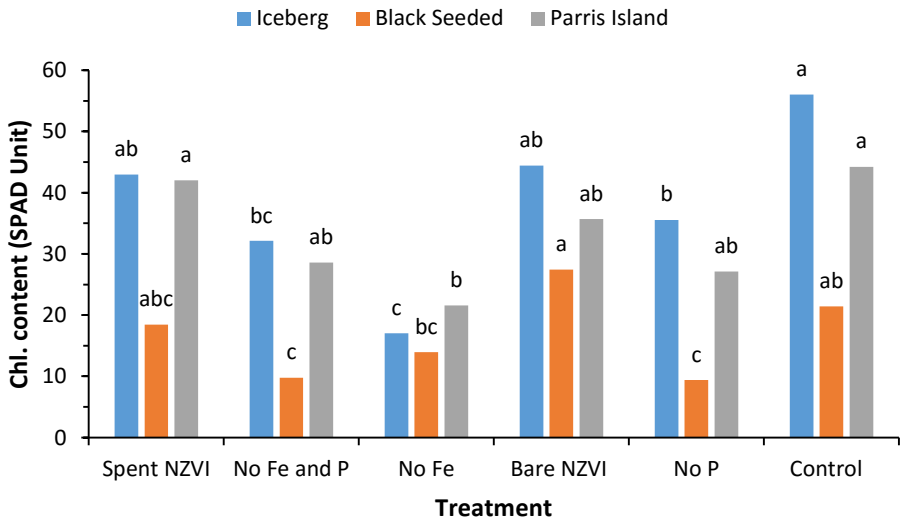


Figure 5.7. Average chlorophyll content of lettuce leaves after 30 days of hydroponic growth. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients. The values represent the average chlorophyll content (SPAD Unit) of three plants grown for each variety in each container. Different letters indicate significant differences between different treatments within the same variety. There were no significant differences between bare NZVI treatment and the control for Iceberg, Black Seeded and Parris Island varieties. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison.

5.4.2.4. Total soluble solids (TSS) content

Total soluble solid (TSS) was measured (as ^{0}Brix) for all the varieties immediately after the harvest for two treatments (bare NZVI and the control) (**Figure 5.8, Table 5.8**). ^{0}Brix values were 4.6±0.8, 4.8±1.0 and 4.7±0.8 for Iceberg, Black Seeded and Parris Island varieties, respectively, treated with bare NZVI. The corresponding values were 4.4±0.6, 6.2±1.0, and 4.2±0.5 for three varieties treated with the control (all nutrients including Fe but no NZVI). The plants treated with other treatments did not produce enough plant tissues which made it impossible for the plants to be tested for TSS. TSS in plant sample (measured as ^{0}Brix) indicate the percentage of dissolved solids contained in the juice prepared from plant biomass and is an indicator of sugar content. A decrease in soluble solids is attributed to sugar consumption through respiration (Moreira, et al., 2006).

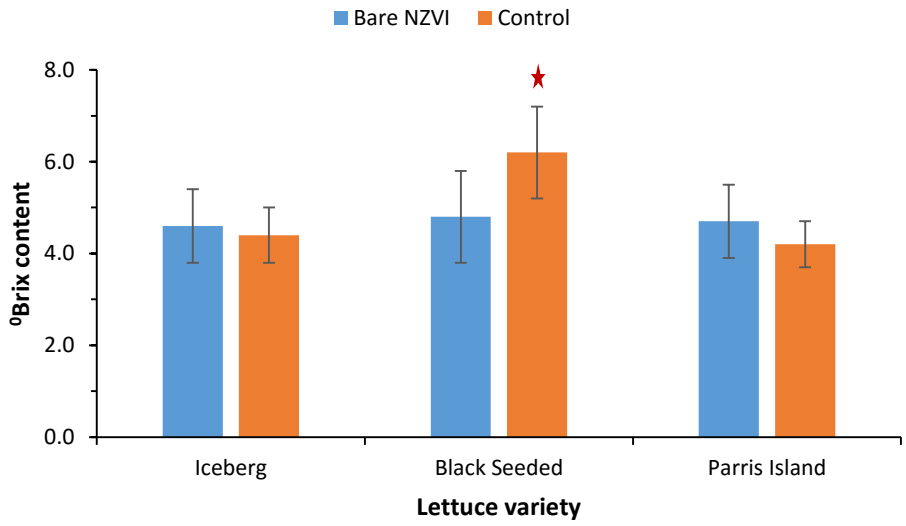


Figure 5.8. Average ^{0}Brix content in lettuce leaves after 30 days of hydroponic growth. Treatments are: (a) Bare NZVI + all nutrients but Fe, (b) Control: all nutrients. Two sample t-tests were performed to see the difference. Statistically significant difference at 5% level of significance was observed only for Black Seeded Simpson variety.

Table 5.8. Total soluble solid (TSS) for three varieties at harvest. Treatments are: (a) Bare NZVI + all nutrients but Fe, (b) Control: all nutrients. Two sample t-tests were performed to see the difference. Statistically significant difference was observed only for Black Seeded Simpson variety.

Sample	TSS (^{0}Brix)		
	Iceberg	Black Seeded	Parris Island
Plant treated with Bare NZVI	4.6±0.8	4.8±1.0	4.7±0.8
Control	4.4±0.6	6.2±1.0	4.2±0.5
Significance	NS	*	NS

*****Significant at 5% level of significance

Higher values of ^{0}Brix indicate the health and expression of the plant's genetic potential (Franquera, 2015). Lettuce having values of 8, 6, and 4 ^{0}Brix are good, average and poor in quality, respectively (Harrill, 1994). The plants tested for ^{0}Brix from three varieties had values falling between average and poor in terms of quality. Statistically significant difference ($p = 0.041$) was observed between bare NZVI and the control (all nutrients but no NZVI) for Black Seeded Simpson Variety. No statistically significant differences were observed between plants treated with bare NZVI and the control (all nutrients) for Iceberg ($p = 0.761$) and Parris Island ($p = 0.169$) varieties (**Table 5.8**). Therefore, it appears that bare NZVI had little effect on the total soluble solids content of lettuce.

5.4.3. Macro- and micro-elements analysis

All the macro- and micro-elements in mature lettuce were analyzed and reported here as mg or µg per unit mass of plant. Uptake of elements was calculated by multiplying the dry weight of plant material by the concentration (mg/kg or µg/kg) of an element of particular interest.

5.4.3.1. Fe uptake

5.4.3.1.1. Iceberg variety

The uptake of iron in Iceberg variety was higher in plants treated with bare NZVI and spent NZVI compared to the control (all nutrients). Iron accumulation was ~56% and ~32% more for bare NZVI and spent NZVI compared to the control. Iron uptake in plants treated with bare NZVI and spent NZVI were 510.3 µg/plant and 432.0 µg/plant, respectively. The uptake of iron by No Fe and P, No Fe, and No P were 52.5, 43.9, and 77.2 µg/plant, respectively (**Table 5.9 and Figure 5.9**). The control had 327.5 µg Fe/plant which was higher than the plants treated with No Fe and P (all nutrients but Fe and P), No Fe (all nutrients but Fe), and No P (all nutrients but P). It is evident that there was a marked difference in Fe uptake between bare NZVI and the control.

Table 5.9. Uptake of elements per plant (Iceberg variety) during the growth period. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients. Data from total N, P, K, S, Ca, Mg, Na, Fe, Cu, and B were Johnson transformed before statistical analysis. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison. Different letters in the same column indicate significant differences between different treatments.

Treatment	Total N (mg)	P (mg)	K (mg)	S (mg)	Ca (mg)	Mg (mg)	Na (mg)	Zn (µg)	Fe (µg)	Mn (µg)	Cu (µg)	B (µg)
Spent NZVI	35±19a	0.76±0.47b	32±21b	1.6±0.9b	5.5±3.7a	2.7±1.6a	1.5±1.2a	60±32b	432±269a	152±83b	3.8±1.3a	100±70ab
No Fe and P	24±12a	0.42±0.19b	38±12ab	3.2±1.4ab	4.7±1.9a	1.8±0.8a	1.2±0.6a	196±64ab	52±1a	359±6ab	6.1±2.6a	33±18b
No Fe	52±52a	8.3±8.6ab	79±67ab	3.7±1.6ab	11.2±6.9a	4.3±2.9a	2.9±1.8a	238±75ab	44±17a	385±128ab	6.3±4.3a	63±43ab
Bare NZVI	236±25a	39.7±2.5a	358±22a	9.9±1.0a	28.8±10.1a	12.5±1.9a	12.2±4.9a	233±22ab	510±15a	309±130ab	27.7±6.6a	2146±1178a
No P	27±14a	0.39±0.20b	32±13ab	2.6±0.7ab	4.5±1.6a	1.8±0.7a	1.1±0.4a	144±64b	77±72a	288±116ab	5.2±2.7a	30±14b
Control	173±38a	23.4±5.3ab	255±18ab	8.7±2.0a	24.4±2.9a	11.0±0.5a	2.4±0.0a	356±11a	328±108a	591±91a	24.8±5.0a	116±2ab

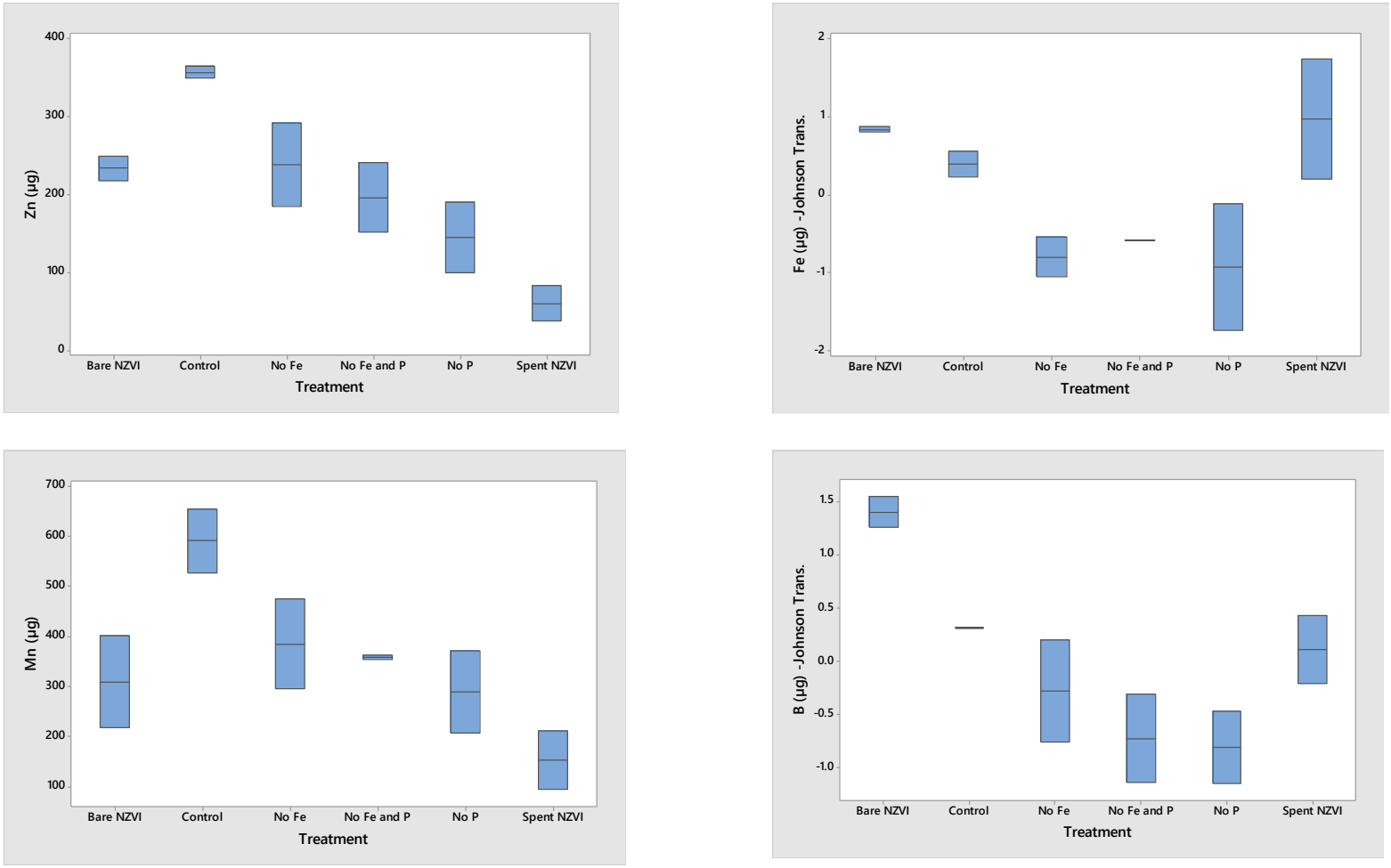


Figure 5.9. Total uptake of Zn, Mn, Fe, and B in Iceberg variety exposed to different treatments. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients.

5.4.3.1.2. Black seeded simpson variety

The uptake of iron in Black Seeded Simpson variety was higher in plants treated with bare NZVI (380.67 µg/plant) compared to the control (all nutrients) (234.09 µg/plant). However, iron uptake was lower in plants treated with spent NZVI (124.53 µg/plant) in comparison with the control. The iron uptake increased by ~63% in plants treated with bare NZVI compared to the control. Total iron accumulation was 31.4, 21.8, and 70.2 µg/plant for No Fe and P, No Fe and No P treatments, respectively (**Table 5.10 and Figure 5.10**).

5.4.3.1.3. Parris island variety

In Parris Island variety, iron accumulation was higher (322.68 µg/plant) in plants dosed with bare NZVI compared to No Fe and P (40.51 µg/plant), No Fe (54.87 µg/plant), No P (58.42 µg/plant), the control (243.71 µg/plant), and spent NZVI (224.01 µg/plant) (**Table 5.11 and Figure 5.11**). There was a ~32% difference in Fe accumulation between bare NZVI treated plants and plants treated with the control (all nutrients). Iron uptake from spent NZVI was comparable with the control (all nutrients).

Plants treated with spent NZVI had more iron than in plants treated with No Fe and P, No Fe, No and the control. It is evident from this study that bare NZVI was not only able to supply required iron to all the varieties of lettuce but also induced an enhanced uptake of iron. Trujillo-Reyes et al. (2014) also observed an enhanced uptake of Fe in lettuce root treated with nanoscale iron oxide compounds (10 and 20 mg/L) which were significantly higher compared to the control (Millipore water). In the present study, spent NZVI supplied plants with iron but the biomass production was significantly lower compared to plants treated with bare NZVI and the control (**Figures 5.9, 5.10 & 5.11**). In a soil study, Wang et al. (2016) found an increased uptake of iron by rice plant treated with 1000 mg NZVI/kg soil compared to control (no NZVI applied in soil).

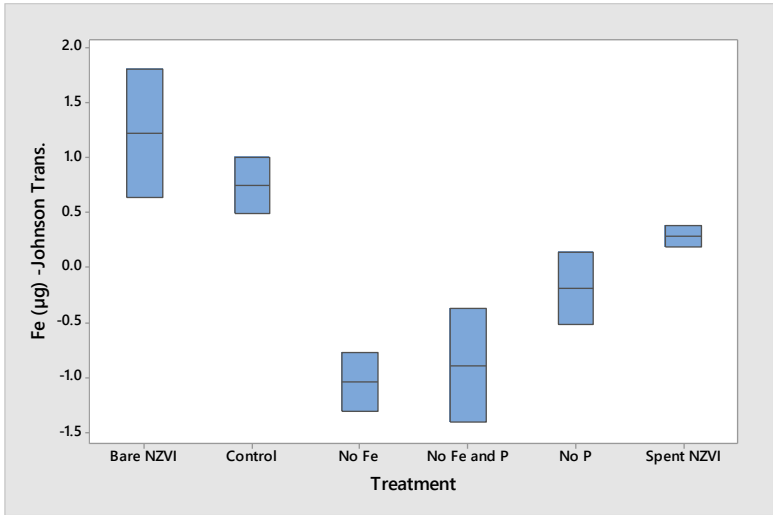
However, the uptake was high in the roots of rice confirming the translocation of iron from soil to roots but not from roots to shoots. It is believed that iron nanoparticles in the root cannot cross the endodermis because of the casparian strip and the deposition of suberin between radial cell walls, which is characteristic of the endothermal cells and vitally important for the protection of the plant (Esau, 1977). In the present experiment, significant uptake was found in the upper part of the plants treated with bare NZVI; this is in contrast with the findings of Wang et al. (2016). That NZVI enhanced the uptake of Fe indicates an alternative route for the acquisition of Fe by lettuce. It is possible that plants just used the route it usually takes to take up iron (as ions) from the hydroponic solution. NZVI dissolution might have given the plant enough ionic iron in the solution and as a result obviated any need for plants taking other routes of iron acquisition.

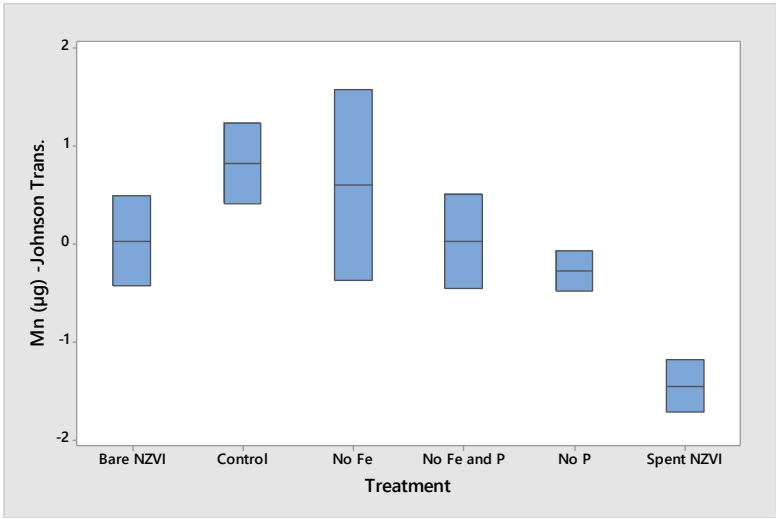
Table 5.10. Uptake of elements per plant (Black Seeded variety) during the growth period. Treatments are: Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients. Data from P, K, S, Ca, Mg, Na, Zn, Fe, Mn, Cu, and B were Johnson transformed before statistical analysis. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison. Different letters in the same column indicate significant differences between different treatments.

Treatment	P (mg)	K (mg)	S (mg)	Ca (mg)	Mg (mg)	Na (mg)	Zn (µg)	Fe (µg)	Mn (µg)	Cu (µg)	B (µg)
Spent NZVI	0.2±0.0bc	9 ± 0 a b	0.53±0.03b	1.6±0.0a	0.99±0.04ab	0.4±0.0bc	1 9 ± 2 b	1 2 4 ± 2 2 a b	3 7 ± 6 a	1.40±0.02a	3 7 ± 0 a b
No Fe and P	0.1±0.0c	7 ± 2 b	0.77±0.22ab	1.0±0.1a	0.46±0.02b	0.2±0.0c	4 2 ± 1 6 a b	3 1 ± 2 4 a b	2 2 7 ± 1 8 9 a	5.63±4.13a	2 4 ± 1 2 a b
No Fe	2.3±2.0abc	2 7 ± 2 6 a b	1.99±1.87ab	2.0±1.5a	1.01±0.68ab	0.4±0.3bc	1 4 5 ± 1 4 5 a b	2 2 ± 9 b	3 5 8 ± 3 5 5 a	3.52±3.61a	1 7 ± 1 0 b
Bare NZVI	27.5±13.1a	2 0 5 ± 7 6 a	9.08±3.58a	17.9±8.1a	9.24±4.45a	7.4±2.7a	2 0 0 ± 1 1 7 a b	3 8 1 ± 2 6 1 a	2 2 6 ± 1 8 3 a	19.19±11.18a	1 4 5 8 ± 8 2 8 a
No P	0.3±0.0abc	1 6 ± 1 a b	1.79±0.42ab	2.2±0.0a	1.14±0.03ab	0.6±0.1bc	7 1 ± 2 0 a b	7 0 ± 4 3 a b	1 2 9 ± 5 5 a	2.92±0.27a	2 2 ± 0 a b
Control	19.2±4.2ab	1 6 8 ± 3 3 a b	7.96±0.76a	18.5±9.4a	8.99±1.88a	1.6±0.7ab	2 8 4 ± 9 8 a	2 3 4 ± 9 8 a b	4 4 6 ± 1 7 2 a	21.06±0.25a	1 0 4 ± 3 8 a b

Table 5.11. Uptake of elements per plant (Parris Island variety) during the growth period. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients. Data from Total N, P, K, S, Ca, Mg, Na, Mn, Cu, and B were Johnson transformed and Zn and Fe were subjected to Box-cox transformation prior to statistical analysis. Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison. Different letters in the same column indicate significant differences between different treatments.

Treatment	Total N (mg)	P (mg)	K (mg)	S (mg)	Ca (mg)	Mg (mg)	Na (mg)	Zn (µg)	Fe (µg)	Mn (µg)	Cu (µg)	B (µg)
Spent NZVI	3 0 ± 1 a b	0.5±0.0ab	1 8 ± 2 b	1.1±0.2c	3.3±0.1ab	1.6±0.2a	1.1±0.1b	3 0 ± 6 c	224±47ab	6 2 ± 1 2 c	4.8±2.9a	72±20abc
No Fe and P	2 3 ± 4 b	0.4±0.1b	26±10ab	2.9±0.9b	3.8±0.9ab	1.4±0.3a	1.0±0.2b	138±69ab	4 0 ± 1 9 b	300±203abc	5.9±1.8a	2 7 ± 4 b c
No Fe	4 3 ± 2 a b	7.8±0.3ab	7 3 ± 3 a b	4.0±0.4ab	10.3±2.1ab	4.5±0.4a	2.3±0.4ab	275±44ab	55±22ab	514±77ab	7.4±0.5a	62±11abc
Bare NZVI	1 9 2 ± 8 6 a	39±23a	267±126a	9 ± 5 a	18.4±5.8ab	10.6±4.2a	11.7±6.3a	209±119ab	323±174a	189±105abc	27.1±15.8a	1120±344a
No P	2 2 ± 9 b	0.4±0.1b	21±7ab	2.4±0.3bc	3.4±0.8b	1.5±0.4a	1.0±0.0b	89±29bc	58±38ab	144±26bc	4.4±1.2a	2 5 ± 8 c
Control	1 3 2 ± 2 3 a	21±6ab	176±24ab	10±2a	25.6±8.0a	11±3a	2.6±1.0ab	390±22a	244±124ab	657±75a	18.6±3.5a	118±45ab





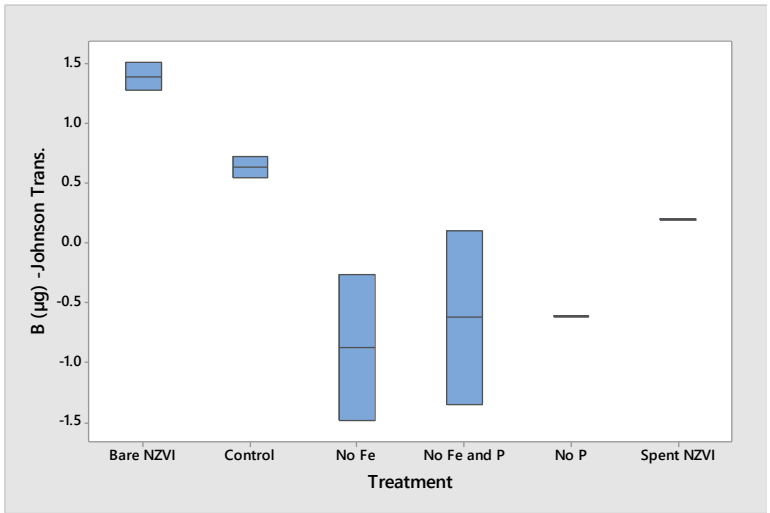


Figure 5.10. Total uptake of Fe, B, and Mn in Black Seeded Simpson variety dosed with different treatments. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients.

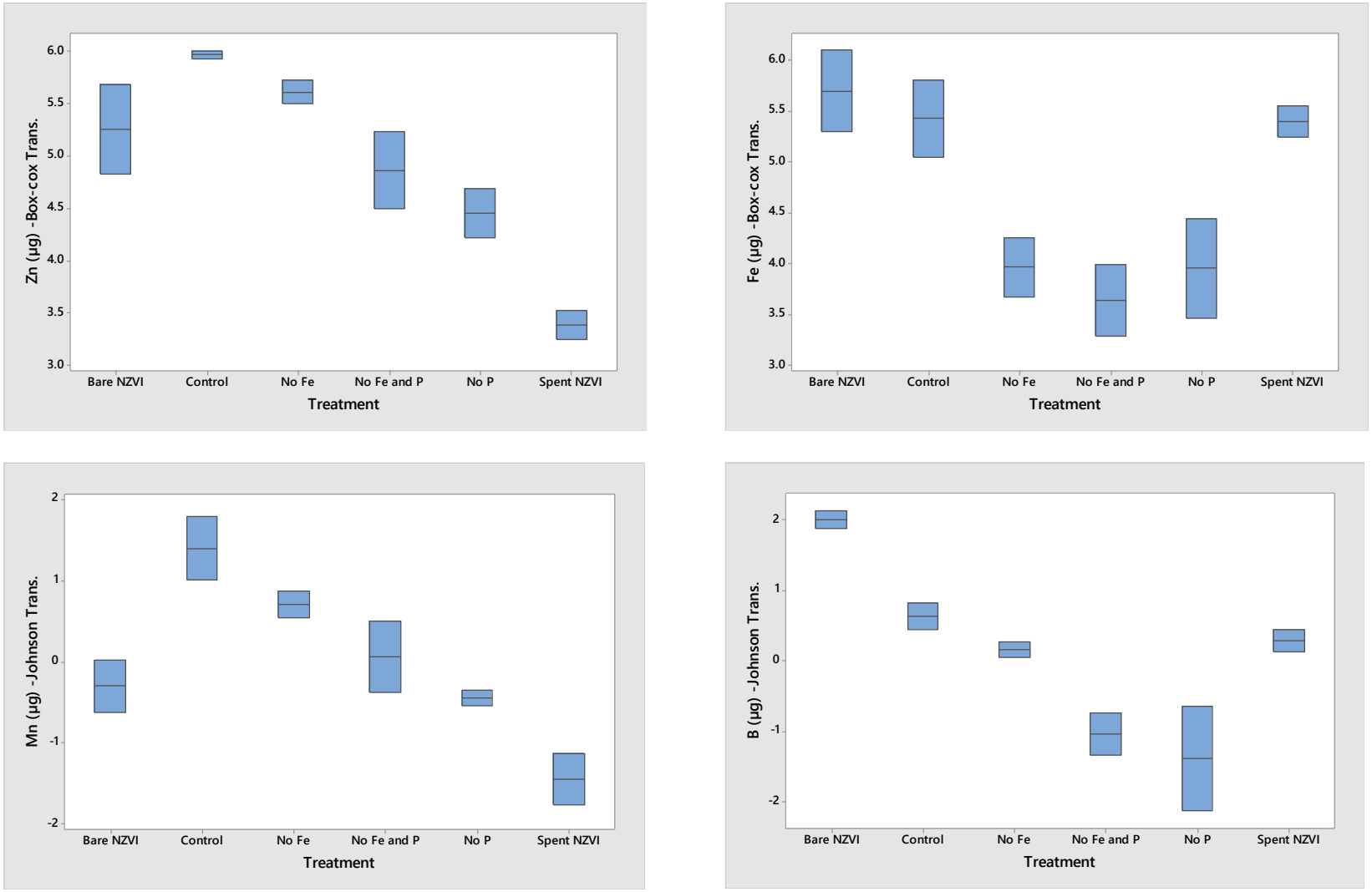


Figure 5.11. Total uptake of Zn, Mn, Fe and B in Parris Island variety dosed with different treatments. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients.

5.4.3.2. Element uptake

While high Fe uptake was observed when plants were exposed to NZVI, it would be prudent to analyze whether the presence of NZVI affects plant uptake of other nutrients. Uptake patterns were analyzed for different elements in the present study (**Tables 5.9, 5.10 and 5.11)**.

5.4.3.2.1. N, P, and K uptake

In Iceberg variety, plants treated with bare NZVI exhibited higher uptake of some of the elements compared to plants treated with all nutrients (Control) and other treatments (No Fe and P, No Fe, No P and spent NZVI) (**Figures 5.12, 5.13 and 5.14**). Bare NZVI treatment enhanced the uptake of total N, P, and K in the plants. Total N, P, and K accumulation was ~36%, ~69%, ~40% higher in plants treated with bare NZVI compared to the control (all nutrients).

Black Seeded Simpson variety also behaved like Iceberg variety in terms of uptake of some minerals. NZVI application modulated the elemental uptake by plants significantly. Bare NZVI was found to improve the uptake of P and K. The increase in uptake was ~43% and ~22% for P and K, respectively.

The total uptake of N, P, and K was positively affected in Parris Island variety treated with bare NZVI. The increase in mineral uptake due to the application of bare NZVI was ~46%, ~84%, and ~52% for total N, P, and K, respectively.

5.4.3.2.2. Zn and Mn uptake

Bare NZVI treatment reduced the total uptake of Zn and Mn in Iceberg variety. The uptake of Zn and Mn in Iceberg variety was reduced by ~34% and ~48% compared to the control (all nutrients) (**Figures 5.9, 5.10 and 5.11**).

In Black Seeded Simpson variety, the uptake of Zn and Mn was negatively affected by the application of NZVI. The decrease in Zn and Mn uptake was ~30% and 49%, respectively, compared to the control.

In Parris Island variety, the uptake of Zn and Mn was reduced due to the application of NZVI. The decrease in the uptake of Zn and Mn was of the order of ~46% and ~71% compared to the control in the variety.

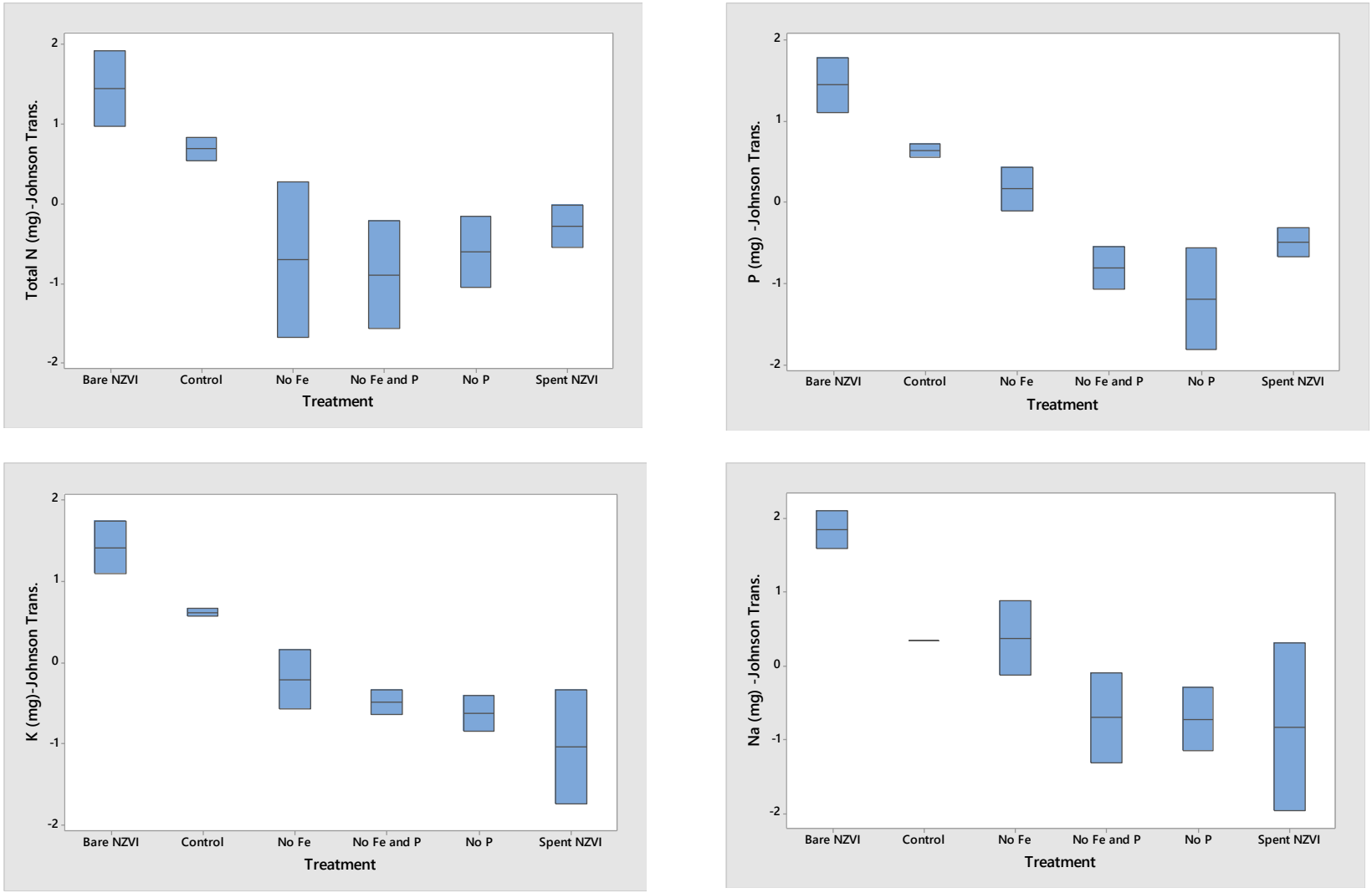


Figure 5.12. Total uptake of N, P, K and Na in Iceberg variety exposed to different treatments. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients.

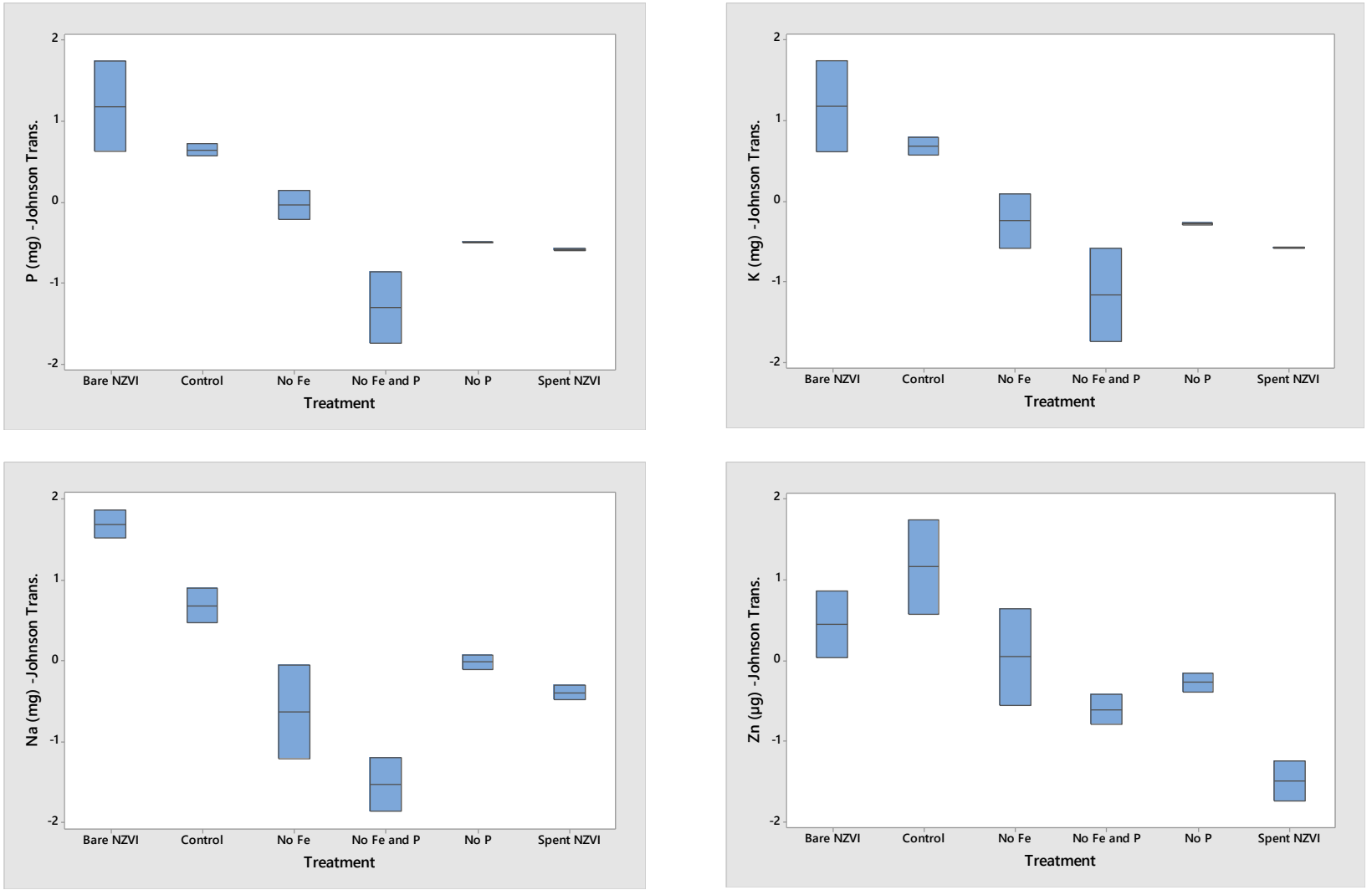


Figure 5.13. Total uptake of P, Na, K, and Zn in Black Seeded Simpson variety dosed with different treatments. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients.

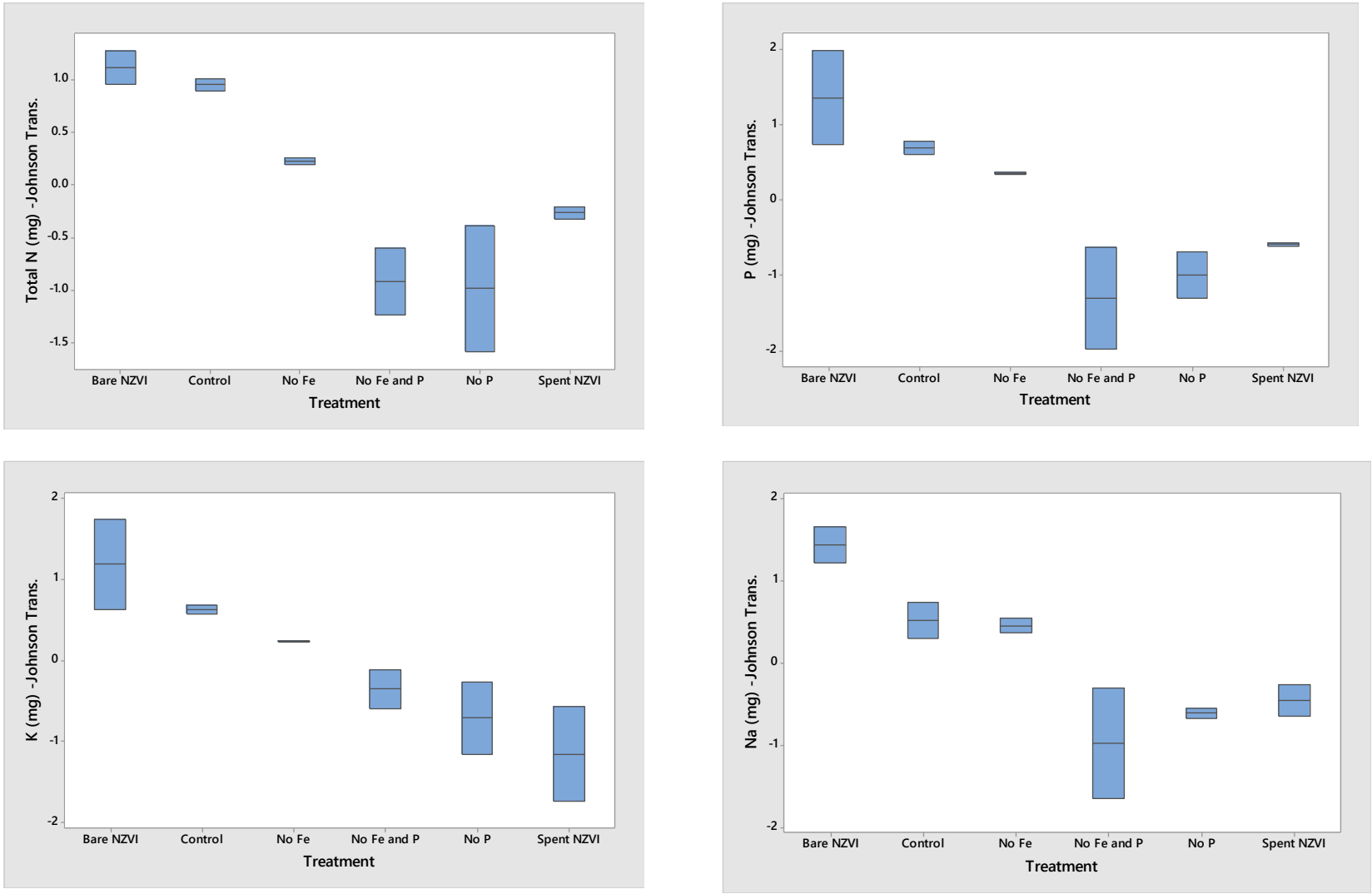


Figure 5.14. Total uptake of total N, K, P, and Na in Parris Island variety dosed with different treatments. Treatments are: (i) Spent NZVI + all nutrients but Fe and P, (ii) No Fe and P: all nutrients but Fe and P, (iii) No Fe: all nutrients but Fe, (iv) Bare NZVI + all nutrients but Fe, (v) No P: all nutrients but P, and (vi) Control: all nutrients.

5.4.3.2.3. Na and B uptake

For all three varieties, the uptake of Na, and B was significantly higher in the presence of bare NZ

VI (**Figures 5.9-5.14**). Na and B accumulation was ~408% and ~1744% higher in Iceberg variety plants treated with bare NZVI compared to the control (all nutrients). In Black Seeded Simpson variety, the increase in uptake was ~348%, and ~1309% for Na and B, respectively. The uptake of Na and B in Parris Island variety was ~346% and ~846% more compared to the control. Boron (B) is an ingredient of the human diet. Boron intakes by humans range from 1.7-7 mg/d with fruits, nuts and vegetables as the major sources (Penland, 1994). Boron is associated with bone, mineral and lipid metabolism, energy utilization, and immune function (Penland, 1998). Boron deprivation might result in poorer performance in human body, particularly tasks of motor speed and dexterity, attention and short-term memory (Penland, 1998). Therefore, the presence of boron in high amounts could be beneficial with respect to human nutrition. Sodium is also vital for fluid balance and cellular homeostasis. About 500 mg Na/day is required to maintain homeostasis in adults (Farquhar, et al., 2015). The uptake of boron and sodium by lettuce can help meet up the demand of humans. The strikingly higher uptake of Na and B could be attributed to the residuals of sodium borohydride used for the synthesis of NZVI. Almeelbi et al. (2014) characterized bare NZVI with X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy with energy dispersive spectroscopy (SEM/EDS) and they indicated a significant presence of sodium (Na) and boron (B). They reported 0.51% of Na in virgin NZVI.

In the present study, an antagonistic relationship was observed between Fe and Zn and Fe and Mn across the varieties (**Tables 5.9-5.12**). In Iceberg variety, bare NZVI-treated plants were

found to take up 510.3 μg/plant of Fe, 233.2 μg/plant of Zn, and 309.3 μg/plant of Mn, whereas the corresponding values for the control were 327.5, 356.4, and 591.2 μg/plant, respectively. In Black Seeded Simpson variety, the uptake of Fe, Zn, and Mn was 380.7, 199.9, and 226.5 μg/plant, respectively, in the plants treated with bare NZVI. The corresponding values were 234.1, 284.1, and 446.1 μg/plant for plants treated with the control. In Parris Island variety, the plants treated with bare NZVI took up 322.7 μg/plant of Fe, 209 μg/plant of Zn, and 188.8 μg/plant of Mn. On the other hand, the plants treated with the control had 243.7, 390.5, and 657 μg/plant of Fe, Zn and Mn taken up. These findings are in agreement with previous findings by other scientists. A group of researchers carried out a study with tomato (*Lycopersicon esculentum* L.) and reported that Fe suppressed plant uptake of Mn and Zn and there is an antagonistic relationship between Fe and Mn (Gunes, et al., 1998). Fe was also found to inhibit Mn translocation from root to shoot (Ghasemi-Fasaei, et al., 2005). The antagonistic effect of Fe with Mn is chiefly via the restriction of absorption stage (Heenan and Campbell, 1983). Increasing iron level was also found to decrease Zn translocation in plants (Brar and Sheklon, 1976). Antagonistic effect of Fe with Mn and Zn in sorghum was demonstrated by (Singh and Yadav, 1980). Proteins (plasma membrane transporters) embedded in root membranes participate in nutrient uptake. The transport of nutrient elements across the plasma membrane is catalyzed by plasma membrane transporters. Similar cations and similar anions compete for binding to specific carrier proteins. Because of their valence, Fe, Zn and Mn compete for the same carrier proteins. Therefore, if one is taken up more, others are taken up less and vice versa (Rietra, et al., 2015).

Table 5.12. A summary of increased or decreased uptake of elements in the presence of NZVI compared to the control, which contained all the nutrients. Uptake of Fe, Na, and B is not shown here. Increase/decrease was denoted by (↑) and (↓), respectively.

Variety	P	K	S	Ca	Mg	Zn	Mn	Cu
Iceberg	↑	↑	↑	↑	↑	↓	↓	↑
Black Seeded Simpson	↑	↑	↑	↓	↑	↓	↓	↓
Parris Island	↑	↑	↓	↓	↓	↓	↓	↑

From this study, it is clear that plants treated with bare and spent NZVI had more iron than in plants treated with No Fe and P, No Fe, No P and the control. Bare and spent NZVI were able to supply iron to all the varieties of lettuce. The nanoparticles also induced an enhanced uptake of iron. Other researchers working with iron nanoparticles also observed enhanced uptake of iron in plants (Trujillo-Reyes, et al., 2014). However, some scientists (Wang, et al., 2016) reported that the uptake of Fe is less in leaves which is in contrast with the findings of present study. It is possible that lettuce in the present study used an alternative route for the acquisition of Fe. NZVI dissolution might have given the plant enough ionic iron in the solution for uptake. Core-shell Fe/Fe_3O_4, which is similar to bare NZVI used in this experiment, was found to release 1.7 mg/L of iron ions in aqueous phase at 250 mg/L (Trujillo-Reyes, et al., 2014). The oxidation of NZVI and subsequent dissolution might have provided lettuce sufficient Fe^{2+}/Fe^{3+} needed for growth (Kadar, et al., 2012).

Other researchers working with nanoparticles revealed that nanoparticles could modify uptake patterns of minerals by plants. A group of researchers worked with cerium oxide nanoparticles and observed an enhanced uptake of P, K, Ca, Mg, S, Fe, Zn, Cu and Al in the plants treated with 250 mg/kg (soil) of cerium oxide nanoparticles (Rico, et al., 2015). Jacob et al. (2013) carried out an experiment with TiO_2 nanoparticles and observed significant changes in the uptake pattern of nutrient elements by *E. Canadensis*; uptake of Mg and Mn was altered by

the application of TiO_2 nanoparticles. In the present study, with few exceptions bare NZVI was found to increase the uptake of N, P, K which are macroelements for plants and decreased the uptake of Zn and Mn which are microelements. It is not clear how bare NZVI caused an increase in nutrient uptake for other elements in lettuce. However, in a previous study with *Arabidopsis thaliana*, Kim et al. (2013) revealed that NZVI enhanced root elongation by triggering OH radical-induced cell wall loosening (degradation of polysaccharides occurred as a result), and that in turn increased endocytosis in root cells. This could be an avenue for enhanced uptake of elements in lettuce and spinach. In another study, Kim et al. (2014) demonstrated that NZVI triggered high plasma membrane H^+-ATPase activity in *Arabidopsis thaliana*, which led to a decrease in apoplastic pH, an increase in leaf area, and also wider stomatal aperture. They attributed these phenomena to a gene called AHA2 (Plasma Membrane H^+-ATPase gene), which exhibited enhanced expression in the roots and leaves of *Arabidopsis thaliana*. In the current experiment, it is believed that a combination of these factors might have facilitated the enhanced uptake of the nutrient elements.

5.5. Conclusions

In this study, the feasibility of using NZVI as a biofortificant was tested using lettuce. The experimental results suggest that NZVI can be used as a biofortificant for plants. Lettuce varieties responded well to the application of NZVI. The iron content and yield was positively affected by NZVI application. Iron uptake in Iceberg variety treated with bare NZVI and spent NZVI were 510.3 µg/plant and 432.0 µg/plant, respectively, where the accumulation of Fe was ~56% and ~32% more compared to the control (327.5 µg Fe/plant). Likewise, the uptake of iron in Black Seeded Simpson variety and Parris Island variety was higher in plants treated with bare NZVI (380.67 µg/plant and 322.68 µg/plant for Black Seeded Simpson and Parris Island,

respectively) compared to the control (all nutrients) (234.09 µg/plant and 243.71 µg/plant for Black Seeded Simpson and Parris Island, respectively). The iron uptake increased by ~63% and ~32% in Black Seeded Simpson and Parris Island varieties treated with bare NZVI compared to the control (all nutrients). Iron uptake by Black Seeded Simpson and Parris Island from spent NZVI was comparable with the control (all nutrients). It is evident from this study that not only Bare NZVI was able to supply required iron to all the varieties of lettuce but also induced an enhanced uptake of iron and some other elements. Spent NZVI supplied plants with iron but the biomass production was significantly lower compared to the plants treated with bare NZVI and the control (all nutrients). Plants treated with spent NZVI might have suffered because of phosphorus deficiency rather than iron deficiency as phosphorus was not possibly bioavailable. The higher uptake of Fe in plants could be due to NZVI dissolution which furnished the plants enough ionic iron in the solution for uptake. The oxidation of NZVI and subsequent dissolution might have provided Fe^{2+}/Fe^{3+} needed for lettuce growth. However, conclusive statements cannot be made about NZVI application as some of the findings are contradictory to previous findings by other scientists. Bare NZVI was found to affect total soluble solids content of lettuce in Black Seeded Simpson variety. Therefore, optimum dose of NZVI should be determined for each species and variety by carrying out an extensive study with that particular variety of a species. The combination of plant species, type and dose of nanoparticles is likely to determine how a given plant will respond to a given nanoparticle. Further research is required to consolidate the findings and to determine how to apply NZVI as a biofortificant in agricultural fields.

5.6. References

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6. CORRELATION BETWEEN *LS*HA2 EXPRESSION AND THE AVAILABILITY OF IRON (NANO VS NON-NANO)

6.1. Abstract

An experiment was conducted to see the change in the expression level of the *Lactuca sativa* H^+-ATPase2 (*Ls*HA2) gene, which is an Arabidopsis ortholog of H^+-ATPase2 (AHA2) gene, because of the availability of iron from nanoscale zero-valent iron (NZVI) and other sources of iron, namely micro zero-valent iron (MZVI), iron sodium EDTA (FeNaEDTA) and ferrous sulfate ($FeSO_4$) as a control. The current investigation found that exposure of *Lactuca sativa* to NZVI (at 100 mg/L), MZVI (1000 mg/L) and FeNaEDTA triggered *Ls*HA2 gene in the leaves of lettuce, which was demonstrated by the higher biomass in the plants of those treatments. The differences in the gene expression level between nanoparticles (NZVI) and non-nano sources (MZVI, FeNaEDTA and $FeSO_4$) were governed by the availability of iron in solution.

6.2. Introduction

Engineered nanomaterials (ENMs) have found their way into various applications because of their unique physical, chemical, thermal, magnetic and optical properties. During the last decade, there has been widespread interest in the application of nanomaterials for agronomic purposes. Nanomaterials have been used as smart delivery systems of fertilizers, herbicides, pesticides and plant growth regulators (Perez-de-Luque and Hermosin, 2013). Nanoparticles have also been used to modify genetic constitution of plants. Novel genes have been introduced or delivered with the help of nanoparticles with pinpoint accuracy, which led to an enhanced growth of crops (Torney, et al., 2007, Yashveer, et al., 2014). Scientists have used starch-nanoparticles as plant-transgenic vehicle to bind the green fluorescence protein (GFP) gene and

then transport it across the cell wall of *Dioscrea Zigberensis G H Wright plant* by readily creating pore channels in the cell wall, cell membrane and nuclear membrane (Liu, et al., 2008). Surface-functionalized mesoporous silica nanoparticles have facilitated the precise manipulation of gene expression at single cell level by delivering DNA and its activators in a controlled manner by penetrating through the plant cell wall (Torney, et al., 2007).

Scientists have also started to explore the possibility of using nanoparticles to biofortify plants. It is basically a form of agronomic intervention where nanoparticles are applied to enhance the growth of plants, thereby increasing the uptake of mineral elements. In a hydroponic study, Almeelbi and Bezbaruah (2014) used nanoscale zero-valent iron (NZVI) for phosphate removal and subsequently used the spent (phosphate-sorbed) NZVI as a source of phosphorus and iron for spinach and algae. They found increased growths of algae and spinach when spent NZVI was used as a sole source of iron and phosphorus. Fresh NZVI also showed enhanced growth. The iron content increased significantly in all plant parts (roots, stems and leaves) when spent NZVI was used as a source of iron compared to control where no iron was applied. Iron content increased by 7 and 11 times in the stem and leaves of the plant of spinach as compared to the control. A group of scientists reported that superparamagnetic iron oxide nanoparticles (SPIONs) can be taken up and translocated by hydroponically-grown soybean plant and as a result SPIONs can increase chlorophyll levels in the plants with no trace of toxicity (Ghafariyan, et al., 2013). In a study with lettuce and spinach (in this research), NZVI was found to supply plants with needed iron where NZVI was the sole source of iron. Moreover, it enhanced the uptake of other essential elements (See **Chapter 5**).

Plants take up iron following two phylogenetically distinct uptake strategies. Strategy II plants (graminaceous monocot plants, such as barley, maize and rice) rely on the excretion of

phytosiderophores which can chelate iron; subsequently the resulting Fe-loaded complexes are imported by the roots (Santi and Schmidt, 2009). When grown under Fe deficiency, Strategy I plants use multiple approaches. Several morphological and physiological responses in their roots occur which help them to enhance Fe mobilization and uptake. Some of these responses include development of subapical swelling with abundant root hairs, development of transfer cells, enhancement of ferric reductase activity (due to enhanced expression of *Arabidopsis thaliana* ferric reductase (AtFRO2)-like genes), enhancement of Fe^{2+} uptake capacity (due to enhanced expression of *Arabidopsis thaliana* iron transporters (AtIRT1)-like genes), acidification of the extracellular medium (due to enhanced expression of H^+-ATPase genes), and release of flavins and phenolics (Hell and Stephan, 2003, Romheld and Marschner, 1986). In the model plant *Arabidopsis thaliana*, the reduction-based strategy (Strategy I) for iron uptake involves the dissolution of iron by rhizosphere acidification through the action of the H^+-ATPase AHA2, and then reduction of iron from ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron by the reductase FRO2, and finally bivalent Fe ions are imported into the root cell by the metal transporter IRT1 (Ivanov, et al., 2012). The soil acidification activity of AHA2 seems to be regulated independently of the iron reduction and transport. In spite of the altered soil acidification in the *aha2* mutants, the reductase activity remained unchanged (Santi and Schmidt, 2009). In a similar manner, in the *frd1-1* mutant (defective for FRO2 gene), the soil acidification activity corresponds to that of wild-type (Yi and Guerinot, 1996). However, expression of the AHA2 gene is dependent on transcription factor FIT. FIT is necessary but not sufficient for AHA2 expression. Another potentially important AHA isoform is *Arabidopsis thaliana* H^+-ATPase7 (AHA7), which is also induced under iron deficiency and its upregulation is also dependent on FIT (Colangelo and Guerinot, 2004). Mutant plants lacking a functional AHA7 gene still could induce acidification

activity under iron deprivation in the same manner like the wild type. However, *aha7* mutant roots showed less root hair density, which was more pronounced under iron deficiency (Santi and Schmidt, 2009). This phenomenon suggests that AHA7 might be involved in iron deficiency as a regulator of root developmental responses. Therefore, AHA2 is the main gene triggered in the absence of available Fe. AHA2 belongs to the P-type ATPase superfamily of cation-transporting ATPases and it pumps protons out of the cell, generating a proton gradient that drives the active transport of nutrients by proton symport (TAIR website https://www.arabidopsis.org/). This AHA2 gene in leaves is an essential component of stomatal opening (Wang, et al., 2014).

Nanoscale zero-valent iron (NZVI) has unique redox reactivity. Because of their strong reducing capacity, NZVI gets oxidized and insoluble iron oxy-hydroxides, such as FeOOH and $Fe(OH)_3$ form rapidly on their surface. Moreover, NZVI can raise pH by water decomposition following electrochemical/corrosion reactions (**Reactions 1 and 2**), which results in thermodynamically less soluble Fe (Zhang, 2003). The combined effect is the reduced Fe availability in the rhizosphere. Citing these reasons, Kim et al. (2014) demonstrated that the presence of NZVI enabled the operation of proton pumps in plants. Plants dosed with NZVI had lower apoplastic pH. They opined that exposure of *Arabidopsis thaliana* to NZVI required plants to acidify their rhizosphere. Plants needed to activate plasma membrane (PM) H^+-ATPase to extrude protons and acidify their rhizosphere. They also revealed that high plasma membrane H^+-ATPase activity is accompanied by an increase in leaf area, which promotes CO_2 assimilation (Kim, et al., 2015, Kim, et al., 2014).

$$2Fe^0_{(s)} + 4H^+_{(aq)} + O_{2(aq)} \rightarrow 2Fe^{2+}_{(aq)} + 2H_2O_{(l)} \quad (1)$$

$$Fe^0_{(s)} + 2H_2O_{(aq)} \rightarrow Fe^{2+}_{(aq)} + H_{2(g)} + 2OH^-_{(aq)} \quad (2)$$

In a previous experiment with lettuce (*Lactuca sativa*), enhanced uptake of Fe and some other elements (e.g., P, K, S, Mg, and Cu) was seen when plants were grown with NZVI as a source of Fe (**Chapter 5**). That particular work was the motivation to undertake this study. Therefore, this study was carried out to delve into the mechanism of Fe acquisition in lettuce plants, particularly the role of NZVI on the expression of AHA2 gene involved in Fe acquisition, which encodes an H^+-ATPase that was shown to be expressed in the root epidermis in the model plant *Arabidopsis thaliana* (Luc Moriau et al., 1999). To date no work had been done on the effect of nanoscale zero-valent iron (NZVI) on the AHA2-like gene in lettuce (*Lactuca sativa*). The hypothesis is that the presence of NZVI in rhizosphere would increase plasma membrane (PM) H^+-ATPase activity in the roots due to the upregulation of AHA2-like gene in lettuce which will increase the uptake of Fe. It is also hypothesized that overexpression of AHA2-like gene in plant leaves will increase stomatal opening, thereby enhancing the photosynthetic activities of plants. And finally there will be a difference in the activity of plasma membrane (PM) H^+-ATPase activity based on whether the plants are exposed to nanoparticles or non-nano sources of iron.

6.3. Materials and Methods

6.3.1. Chemicals

Iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$, 99 % pure, Alfa Aesar), micro zero-valent iron powder (<10 micron, 99.9+%, SIGMA-ALDRICH), sodium borohydride ($NaBH_4$, 98 %, SIGMA-ALDRICH)), sodium hydroxide (5 N NaOH, Alfa Aesar), HNO_3 (68 %, J.T. Baker), methanol (production grade, BDH), ethanol (ACS grade, Mallinckrodt Chemicals), calcium nitrate tetrahydrate ($CaNO_3 \cdot H_2O$, Alfa Aesar), potassium nitrate (KNO_3, Mallinckrodt Chemicals), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, Mallinckrodt Chemicals),

magnesium nitrate hexahydrate ($Mg(NO_3)_2 \cdot 6H_2O$, Alfa Aesar), ammonium dihydrogen phosphate ($NH_4H_2PO_4$, Alfa Aesar), sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$, amresco), copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, BDH), manganese sulfate monohydrate ($MnSO_4 \cdot H_2O$, Mallinckrodt Chemicals), sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$, BTC), zinc sulfate monohydrate ($ZnSO_4 \cdot H_2O$, J.T. Baker) and iron(III) monosodium salt ($FeNa(O_2CCH_2)_2NCH_2CH_2N(CH_2CO_2)_2$, ACS Grade, Alfa Aesar) were used as received unless otherwise specified.

6.3.2. Synthesis and preparation of NZVI

NZVI particles were synthesized using sodium borohydride reduction method (Almeelbi and Bezbaruah, 2012). Iron (II) sulfate heptahydrate (10 g) was dissolved in 100 mL of 30% of methanol (30 mL methanol + 70 mL deoxygenated de-ionized (DI) water) (Solution A). The pH of the solution was then adjusted to 6.1 adding 5 N NaOH drop by drop. In the meantime, 3.94 g of sodium borohydride was dissolved in 100 mL of deoxygenated DI water in a 100 mL volumetric flask (Solution B). Once the pH reached 6.1, Solution A was immediately added dropwise to Solution B using a burette under vigorous stirring conditions (using a magnetic stirrer). The combined solution was then allowed to stand for 20 min. The resultant black precipitates (NZVI) were centrifuged and washed with ethanol. The NZVI in slurry form was then dried in a vacuum oven under nitrogen environment. Finally, the dried NZVI particles were ground using a mortar and pestle and stored in 20 mL vials (headspace flushed with nitrogen) for later use.

6.3.3. Plant growth conditions

6.3.3.1. Germination and plant preparation for hydroponic experiment

Lettuce seeds of Parris Island variety (*Lactuca sativa*, Burpee, Warminster, PA) were purchased from a local outlet. The lettuce seeds were germinated in plug trays with nonabsorbent cotton as media (**Figure 6.1**). The seeds were kept moist using automated misting nozzles in a greenhouse. The germinated seeds were then moved to another room and allowed to grow for ten days. The seedlings were fed intermittently with half strength Hoagland solution. The seedlings were provided with cool-white fluorescent light (14 h light/10 h dark cycle). The light intensity was ~100 µmol/m^2/s.

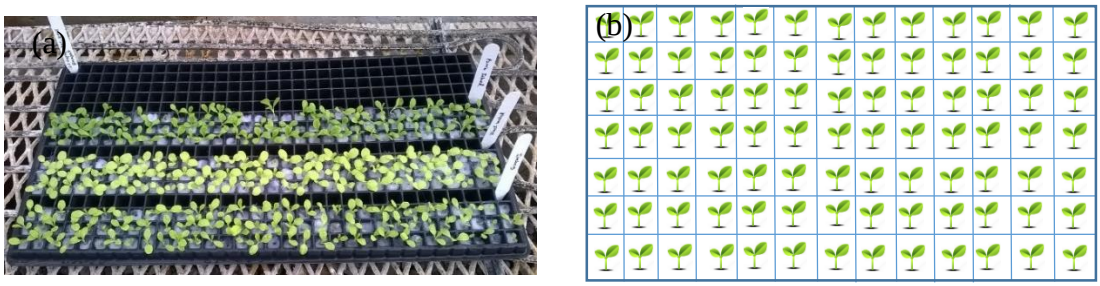

Figure 6.1. (a) Lettuce seeds were germinated in a plug tray (b) Schematic of the plug tray.

6.3.3.2. Hydroponic studies

After plants developed a root system, healthy plants of similar sizes were carefully removed from the plug trays and the roots of the plants were rinsed with copious amount of deionized water, keeping the roots unharmed. Plants were then anchored in nutrient solutions contained in 1 L plastic tumblers. First of all, one lettuce seedling was placed into a Styrofoam disc float (a hole was made in the disc float using a hole punch) with their roots below the disc and the shoots supported above with a wrap of non-absorbent cotton (Jacob, et al., 2013). The seedlings were held erect by plugging the gaps with non-absorbent cotton. The Styrofoam disc was cut in a way so that it snugly fitted the opening of the plastic tumbler; this was done to

reduce the light entering the nutrient solution beneath the Styrofoam. The discs were then placed on the surface of 750 mL Hoagland nutrient solution without iron added and with iron added (**Tables 6.1 and 6.2**) in a tumbler ensuring continuous root contact with the nutrient solution (Hoagland and Arnon, 1950). The nutrient solutions had already been treated with nanoparticles and other treatments before the seedlings were transplanted. The experiment was a completely randomized design with three treatments, namely NZVI (100 mg/L), micro zero-valent iron (MZVI) (100 mg/L), MZVI (1000 mg/L), and FeNaEDTA (1.69 mg/L) and a control containing $FeSO_4$ as a source of iron. There was a total of 20 tumblers with 4 replicates per treatment. The nutrient solution was aerated with bubblers (at a rate of ~2 cc/minute) to provide oxygen to the roots and also to keep the nanoparticles in suspension (Trujillo-Reyes, et al., 2014), and the solution was replaced every five days. The plants were grown 30 days in hydroponics. Light was provided in 14 h light/10 h dark cycles with cool-white fluorescent plant bulbs with a light intensity of ~100 µmol/m^2/s.

Table 6.1. Modified Hoagland solution (Hoagland and Arnon, 1950).

Chemicals	Final concentration		Important ions
	mM or µM	mg/L	
$Ca(NO_3)_2•4H_2O$	2 mM	472	Ca^{2+}, NO_3^-
KNO_3	6 mM	606	K^+, NO_3^-
$MgSO_4•7H_2O$	0.5 mM	123	Mg^{2+}, SO_4^{2-}
$Mg\ (NO_3)_2•6H_2O$	0.5 mM	128	Mg^{2+}, NO_3^-
$NH_4H_2PO_4$	2 mM	230	NH_4^+, $H_2PO_4^-$
$Na_2B_4O_7•10H_2O$	20 µM	3.81	$B_4O_7^{2-}$
$CuSO_4•H_2O$	0.5 µM	0.089	Cu^{2+}
$MnSO_4•3H_2O$	10 µM	2.05	Mn^{2+}
$Na_2MoO_4•2H_2O$	0.5 µM	0.12	MoO_4^{2-}
$ZnSO_4•H_2O$	4 µM	0.716	Zn^{2+}
$FeSO_4•7H_2O$/FeNaEDTA	10 µM	1.69	Fe^{2+}

Table 6.2. Modified Hoagland solution containing no iron (modified for this research).

Chemicals	Final concentration		Important ions
	mM or µM	mg/L	
$Ca(NO_3)_2•4H_2O$	2 mM	472	Ca^{2+}, NO_3^-
KNO_3	6 mM	606	K^+, NO_3^-
$MgSO_4•7H_2O$	0.5 mM	123	Mg^{2+}, SO_4^{2-}
$Mg\ (NO_3)_2•6H_2O$	0.5 mM	128	Mg^{2+}, NO_3^-
$NH_4H_2PO_4$	2 mM	230	NH_4^+, $H_2PO_4^-$
$Na_2B_4O_7•10H_2O$	20 µM	3.81	$B_4O_7^{2-}$
$CuSO_4•H_2O$	0.5 µM	0.089	Cu^{2+}
$MnSO_4•3H_2O$	10 µM	2.05	Mn^{2+}
$Na_2MoO_4•2H_2O$	0.5 µM	0.12	MoO_4^{2-}
$ZnSO_4•H_2O$	4 µM	0.716	Zn^{2+}

6.3.4. Primer design (*Arabidopsis* ortholog in *Lactuca sativa*)

Actin was selected as a housekeeping gene as described in Borowski et al. (2014). The HA2 gene was the experimental gene chosen based on *Arabidopsis thaliana* data showing that the upregulation of the AHA2 gene was putatively involved in the prolific growth after exposure to NZVI particles (Kim, et al., 2014). To identify the *Lactuca sativa* orthologous gene the *A. thaliana* AHA2 cDNA sequence was mined from the TAIR website (https://www.arabidopsis.org/). The *A. thaliana* AHA2 cDNA sequence (AT4G30190.1) was used in BLAST searches against the expressed sequence tag (EST) database in the national center for bioinformatics information (NCBI) database limited to *Lactuca sativa* (taxid: 4236). The *Lactuca sativa* ESTs with the highest significance were assembled into a unigene contig using the Vector-NTI software. The resulting unigene sequence was assembled into a *Lactuca sativa* orthologous HA2 unigene sequence. Three primer pairs were designed from the *Lactuca sativa* HA2 unigene sequence, designated *Ls*HA2, that were expected to yield 151, 201, and 221 bp amplicons (**Table 6.3**). The primer pairs were tested on cDNA and the PCR amplicons of the

expected size were sequenced on an ABI777 (Genscript) utilizing the original primers used to generate each amplicon.

Table 6.3. Primer sequences designed to amplify the *Lactuca sativa Ls*HA2 ortholog and the actin housekeeping gene. The amplicon size and the melting temperature of the primers are given.

Name	Primer sequence 5´-3´ (forward)	Primer sequence 5´-3´ (reverse)	Amplicon length (bp)	Ta (^{0}C)
SSAHA2Q1	TGCAGTTTCTATCACTATCCGTATT	AGATGGCTTCACTCTATCCTTTG	151	62
SSAHA2Q2B	ATTGTGCTTACTGAACCTGGTCTCAG	ATCGTTAAGGATTGCGATAATC	201	62
SSAHA2Q2A	AGAAGGCAGACATTGGAATTGC	ATCAAACTTCCATATCAATGCGATG	221	62
Actin	AGGGCAGTGTTTCCTAGTATTGTTG	CTCTTTTGGATTGTGCCTCATCT	106	62

6.3.5. Total RNA extraction

Leaf and root tissue (~30 mg) were collected from *Lactuca sativa* (Parris Island variety) plants 7 days after exposure to nanoparticles and at maturity (30 days after exposure to nanoparticles) dosed with NZVI, MZVI, FeNaEDTA and $FeSO_4$. Total RNA was isolated from the samples using Qiagen RNA extraction kit following the manufacturer's standard protocol (**Protocol A1**). The concentration (or quantity) of extracted RNA was measured at 260 nm using a Qubit 2.0 Fluorometer. The measurement of RNA concentration was done using 199 µL buffer (Qubit ®RNA BR Buffer), 1 µL 200X dye (Qubit RNA BR Reagent) and 1 µL RNA. Purity of the total RNA extracted was determined as the 260/280 nm ratio and the integrity of total RNA was checked on an agarose gel stained with ethidium bromide. Before running on the gel, the RNA samples were prepared using 9 µL H_2O, 10 µL dye (Ambion gel loading dye buffer II) and 1 µL RNA.

6.3.6. cDNA synthesis

~640 ng of RNA was used as template in the reverse transcription reaction to synthesize cDNA using the GoScript Reverse Transcription System (Promega) (**Protocol A2**). The resulting 20 µL of cDNA was diluted with the addition of 40 µL ultrapure water (1:3) (Ambion).

6.3.7. PCR Reaction

All the PCR reactions were performed using Go Taq DNA polymerase (M3001) and buffer, 2.5 mM dNTP stock solution and 10 µM working concentrations of primers. The final conc. of dNTP and primers were 75 µM and 500 nM, respectively. Briefly, 6 µL of 5X Go Taq Buffer (PCR Master mix), 0.5 µL of dNTP, 0.2 µL of Taq polymerase, 1 µL of forward and reverse primer each, 1 µL of cDNA, and 20.3 µL of H_2O were used in a total reaction volume of 30 µL. The thermocycler program was one initial cycle of denaturation at 95 °C (3 min), followed by 35 cycles of 95 °C (30 s, denaturation), 62 °C (45 s, annealing), 72 °C (1 min, elongation) and a final elongation at 72 °C (5 min). The PCR amplicons were purified using Wizard Gel purification kit (A9281) following manufacturer's protocol (**Protocol A3**).

6.3.8. qRT PCR

Quantitative real-time PCR (qRT-PCR) reactions for LsHA2 were carried out using the BIO-RAD SsoAdvanced Universal SYBR® Green Supermix using the manufacturer protocol on a CFX-96 Real Time PCR detection system (BIO-RAD) (**Protocol A4**). The *Lactuca sativa* actin gene (Genbank acc. # AY260165.1) was used as a housekeeping gene (endogenous control) to normalize the *Ls*AHA2 gene expression. Samples treated with $FeSO_4$ were used as control for expression analysis at 7 day and at maturity. Three biological replicates were analyzed using three experimental replicates per sample and the resulting data was analyzed using the BIORAD CFX Manager software. The real-time PCR efficiency was determined for each gene with the slope of a linear regression model.

6.3.9. Data acquisition

Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of PCR reaction (C_T). The C_T values were

transformed into starting quantities using PCR efficiencies on BIORAD CFX Manager Software (BIORAD, USA).

6.3.10. Statistical analysis

C_T values were analyzed using BIORAD CFX Manager 3.1 software. Four biological replicates were used and for qPCR three technical replicates were run from each of the biological replicate. The values were manually checked for inconsistencies, if any. A p-value less than 0.05 indicated that difference in variation of expression could be deduced.

6.4. Results and Discussion

6.4.1. Characterization of nanoscale zero-valent iron (NZVI)

Average particles size of virgin NZVI was 16.24 ± 4.05 nm (Almeelbi and Bezbaruah, 2012). NZVI particles were characterized using scanning electron microscopy with energy dispersive spectroscopy (SEM/EDS). Elemental composition of virgin NZVI was determined using SEM/EDS (JEOL JSM-6300, JEOL, Ltd.). The percentage of oxygen in the virgin NZVI was found to be 12.10%. (Krajangpan, et al., 2012) reported it as 15.66%. The presence of a very low amount (0.51%) of Na was observed in the virgin NZVI. Sodium (Na) was possibly left behind as a residual from sodium borohydride ($NaBH_4$) used in the NZVI synthesis process.

6.4.2. Plant biomass

The fresh weight of lettuce plants (measured after the harvest) varied depending on the treatment (**Figure 6.2 & Table 6.4**). In the plants treated with NZVI 100 mg/L, the average weight of fresh leaves and root were 33.07 g/plant and 2.52 g/plant which were significantly different than in the plants treated with the control (9.52 g/plant and 0.85 g/plant) and MZVI 100 mg/L (16.73 g/plant and 1.19 g/plant). The average weight of fresh leaves (29.42 g/plant) and roots (2.46 g/plant) treated with MZVI 1000 mg/L were significantly different than

in the plants treated with the control and MZVI 100 mg/L but were not significantly different than the plants treated with NZVI 100 mg/L. The average weight of fresh leaves (30.25 g/plant) and roots (2.68 g/plant) treated with FeNaEDTA were significantly different than in the plants treated with the control ($FeSO_4$) and MZVI 100 mg/L but were not significantly different than the plants treated with NZVI 100 mg/L and MZVI at 1000 mg/L.

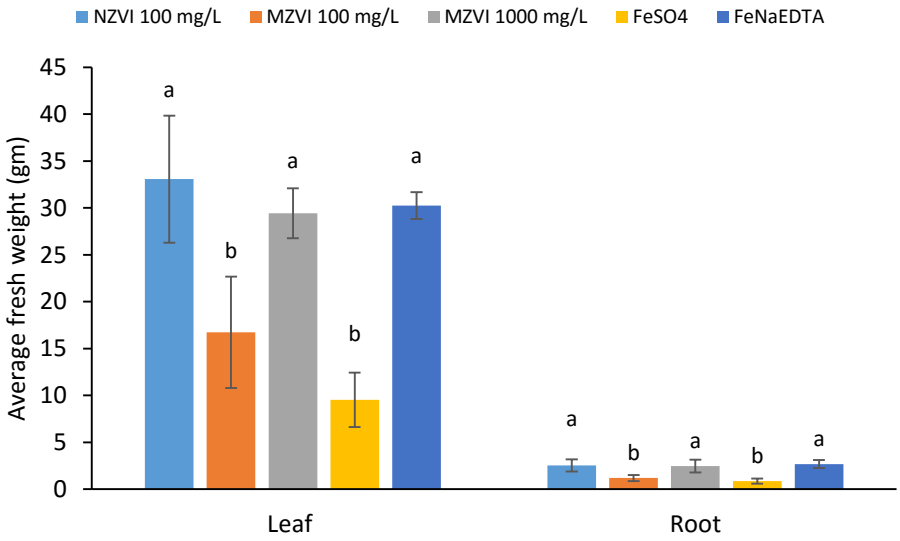

Figure 6.2. Average fresh weights of lettuce leaves and roots treated with NZVI, MZVI, FeNaEDTA and $FeSO_4$. Treatments are (a) NZVI at 100 mg/L, (b) MZVI at 100 mg/L, (c) MZVI at 1000 mg/L, (d) FeNaEDTA at 1.69 mg/L, and (e) Control ($FeSO_4$). Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison ($p<0.05$). Different letters above bars indicate significant differences between different treatments.

The dry weight of leaves and roots followed the same trend (**Figure 6.3 & Table 6.4**). The dry weight of plant leaves and roots with NZVI treatment, i.e., NZVI at 100 mg/L (2.74 g/plant and 0.23 g/plant for leaves and roots, respectively) were statistically different than the control (0.80 g/plant and 0.09 g/plant for leaves and roots, respectively) and MZVI treatment at 100 mg/L (1.41 g/plant and 0.13 g/plant for leaves and roots, respectively). Plants treated with the higher dose of MZVI, i.e., MZVI at 1000 mg/L (2.39 g/plant and 0.22 g/plant for leaves and

roots, respectively) yielded significantly more dry biomass than the plants dosed with MZVI at 100 mg/L and the control.

Plants treated with FeNaEDTA yielded significantly higher amount of dry biomass (2.51 g/plant and 0.22 g/plant for leaves and roots, respectively) than the plants dosed with MZVI at 100 mg/L and the control ($FeSO_4$). Plant biomass from NZVI at 100 mg/L, MZVI at 1000 mg/L and FeNaEDTA were not significantly different from one another. Plants performed well in the presence FeNaEDTA which is used as a source of iron in hydroponic solution (Hoagland and Arnon, 1950). Plant biomass being comparable at 100 mg/L of NZVI application and 1000 mg/L of MZVI application could be attributed to the comparable surface area of NZVI and MZVI particles. The NZVI particles used in this experiment had a surface area of ~25 m^2/g, whereas MZVI had a surface area of ~2 m^2/g (Almeelbi and Bezbaruah, 2012, Bezbaruah, et al., 2009). The higher reactivity of NZVI particles due to the higher surface area might have facilitated the dissolution of iron (Filip, et al., 2014) more compared to MZVI particles. Consequently, the hydroponic solution contained almost the same amount of ionic iron at 100 mg/L of NZVI and 1000 mg/L of MZVI.

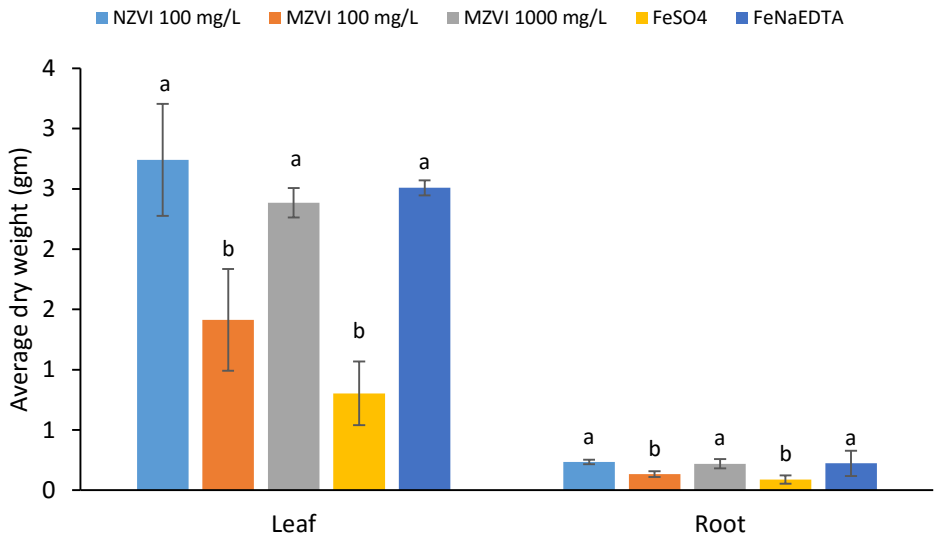

Figure 6.3. Average dry weights of lettuce leaves and roots treated with NZVI, MZVI, FeNaEDTA and $FeSO_4$.Treatments are (a) NZVI at 100 mg/L, (b) MZVI at 100 mg/L, (c) MZVI at 1000 mg/L, (d) FeNaEDTA at 1.69 mg/L, and (e) Control ($FeSO_4$). Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison ($p<0.05$). Different letters above bars indicate significant differences between different treatments.

6.4.3. Chlorophyll content

The chlorophyll content of the plants treated with different Fe sources was measured shortly before harvest. The chlorophyll content varied depending on the treatment (**Table 6.4**). However, there were no significant differences among the values.

Table 6.4. Average fresh and dry weights of lettuce leaves and roots and chlorophyll content of leaves were shown with standard deviation. Treatments are (a) NZVI at 100 mg/L, (b) MZVI at 100 mg/L, (c) MZVI at 1000 mg/L, (d) FeNaEDTA at 1.69 mg/L, and (e) Control ($FeSO_4$). Differences were determined by one-way ANOVA followed by Tukey's pairwise comparison ($p<0.05$). Different letters in the same column indicate significant differences between different treatments.

	Fresh (g)		Dry (g)		
Treatment	Leaf	Root	Leaf	Root	Chlorophyll (SPAD Unit)
(a) NZVI 100 mg/L	33.07±6.77a	2.52±0.64a	2.74±0.46a	0.23±0.02a	38.8±0.87a
(b) MZVI 100 mg/L	16.73±5.94b	1.19±0.32b	1.41±0.02b	0.13±0.02b	42.2±3.14a
(c) MZVI 1000 mg/L	29.42±2.66a	2.46±0.69a	2.39±0.12a	0.22±0.04a	38.8±3.93a
(d) FeNaEDTA	30.25±1.44a	2.68±0.43a	2.51±0.06a	0.22±0.10a	37.3±1.9a
(d) Control ($FeSO_4$)	9.52±2.89b	0.85±0.26a	0.80±0.26b	0.09±0.04b	41.0±2.29a

6.4.4. *Ls*HA2 gene expression

Lettuce (*Lactuca sativa*) leaves and roots were collected 7 days and 30 days post exposure to nanoparticles and other treatments. Total RNA was extracted from the leaf tissue and reverse transcribed to cDNA. The cDNA was used as templates in quantitative real-time polymerase chain reactions (qPCR). All PCRs displayed efficiencies between 93% (Actin) and 97% (*Ls*HA2).

6.4.4.1. Comparison between two time points

The qPCR data was used to analyze the levels of *Ls*HA2 and thus specifically evaluate plasma membrane (PM) H^+-ATPase expression at the transcriptional level. Actin housekeeping gene was used to normalize the *Ls*HA2 gene expression. Samples treated with $FeSO_4$ were used as control for expression analysis at 7 day and at maturity. Relative expression was determined using $FeSO_4$ expression as 1. Expressions for NZVI and other treatments at 7 day and maturity was compared against $FeSO_4$ expression (taking it as 1) at 7 day and maturity, respectively. The expression level of the *Ls*HA2 gene varied between two time points (7 days and 30 days post exposure) across the treatments. The expression level of the *Ls*HA2 gene in plant leaves decreased invariably at 30 days post exposure compared to 7 days post exposure to nanoparticles and other treatments (**Figures 6.4 and 6.5**). Compared to 7 days post exposure expression level, there was 18.66-fold downregulation in the expression level of *Ls*HA2 in plants treated with FeNaEDTA at 30 days after exposure. The gene was downregulated 11.93-fold in the plants treated with nanoparticles (NZVI) at 30 days post exposure compared to 7 days post exposure. The expression level was 4.99, 3.56 and 3.27-fold lower in the plants treated with MZVI 1000 mg/L, MZVI 100 mg/L and the control ($FeSO_4$) at 30 days post exposure compared to 7 days post exposure.

In the root, the changes in the expression of the *Ls*HA2 gene between two time points was not statistically significant. Compared to 7 days after exposure, at maturity (after 30 days of exposure to nanoparticles), the expression level went down by 1.26, 1.27 and 1.09-fold in the plants treated with NZVI 100 mg/L, MZVI 1000 mg/L, and MZVI 100 mg/L, respectively. On the other hand, the expression level for the *Ls*HA2 gene increased 1.35 and 1.26-fold in the plants dosed with $FeSO_4$ (Control) and FeNaEDTA.

Target	Sample	Ctrl	Expression	Expression SEM	Corrected Expression SEM	Mean Cq	Cq SEM
Actin-leaf	MZVI100-Mday		N/A	N/A	N/A	23.86	0.18563
Actin-leaf	NZVI100-7day		N/A	N/A	N/A	22.60	0.04050
Actin-leaf	NZVI100-Mday		N/A	N/A	N/A	22.88	0.13065
Actin-root	FeNaEDTA-7day		N/A	N/A	N/A	21.08	0.21810
Actin-root	FeNaEDTA-Mday		N/A	N/A	N/A	19.49	0.11872
Actin-root	FeSO4-7day	*	N/A	N/A	N/A	20.55	0.11391
Actin-root	FeSO4-Mday		N/A	N/A	N/A	20.26	0.06933
Actin-root	MZVI1000-7day		N/A	N/A	N/A	21.80	0.43170
Actin-root	MZVI1000-Mday		N/A	N/A	N/A	21.00	0.19555
Actin-root	MZVI100-7day		N/A	N/A	N/A	22.13	0.10059
Actin-root	MZVI100-Mday		N/A	N/A	N/A	20.49	0.04351
Actin-root	NZVI100-7day		N/A	N/A	N/A	22.33	0.12834
Actin-root	NZVI100-Mday		N/A	N/A	N/A	20.05	0.21564
AHA-leaf	FeNaEDTA-7day		2.26415	0.25878	0.25878	20.24	0.12936
AHA-leaf	FeNaEDTA-Mday		0.12132	0.03458	0.03458	23.79	0.37200
AHA-leaf	FeSO4-7day	*	1.00000	0.10451	0.10451	22.06	0.10746
AHA-leaf	FeSO4-Mday		0.30605	0.06308	0.06308	23.49	0.30038
AHA-leaf	MZVI1000-7day		2.16757	0.50759	0.50759	21.08	0.25089
AHA-leaf	MZVI1000-Mday		0.43396	0.05600	0.05600	22.69	0.16035
AHA-leaf	MZVI100-7day		0.95965	0.08982	0.08982	22.60	0.11666
AHA-leaf	MZVI100-Mday		0.26934	0.03829	0.03829	24.13	0.18880
AHA-leaf	NZVI100-7day		1.83418	0.14318	0.14318	21.58	0.09771
AHA-leaf	NZVI100-Mday		0.15378	0.03993	0.03993	24.27	0.35810
AHA-root	FeNaEDTA-7day		0.66820	0.14869	0.14869	18.82	0.30988
AHA-root	FeNaEDTA-Mday		0.84368	0.21813	0.21813	17.71	0.32716
AHA-root	FeSO4-7day	*	1.00000	0.32771	0.32771	18.84	0.47625
AHA-root	FeSO4-Mday		1.34715	0.35161	0.35161	18.09	0.38210
AHA-root	MZVI1000-7day		0.36419	0.19336	0.19336	20.49	0.73533
AHA-root	MZVI1000-Mday		0.35435	0.07434	0.07434	19.76	0.29195
AHA-root	MZVI100-7day		0.91354	0.14993	0.14993	19.45	0.22455
AHA-root	MZVI100-Mday		0.83554	0.11564	0.11564	19.24	0.18261
AHA-root	NZVI100-7day		0.38001	0.10955	0.10955	20.68	0.42077
AHA-root	NZVI100-Mday		0.48235	0.20957	0.20957	19.36	0.62626

Figure 6.4. Expression data of LsHA2. Treatments are (a) NZVI100-7 day and NZVI100-Mday: NZVI at 100 mg/L, (b) MZVI100-7 day and MZVI100-Mday: MZVI at 100 mg/L, (c) MZVI1000-7 day and MZVI1000-Mday: MZVI at 1000 mg/L, (d) FeNaEDTA-7 day and FeNaEDTA-Mday: FeNaEDTA at 1.69 mg/L, and (e) $FeSO_4$-7day and $FeSO_4$-Mday: Control ($FeSO_4$).

It is evident that *Ls*HA2 was expressed more in the leaves at 7 days post exposure to nanoparticles compared to 30 days post exposure. It implies that the *Ls*HA2 gene was activated in leaves after 7 days of exposure to nanoparticles; *Ls*HA2 is the major gene responsible for stomatal opening (Wang, et al., 2014). Stoma is a key organ for CO_2 uptake for photosynthesis in plants and as a result stomatal aperture is a limiting factor in photosynthesis and plant growth (Kim, et al., 2015, Wang, et al., 2014). Therefore, the higher expression of *Ls*HA2 in leaves at 7 days post exposure was possibly because of plants heightened photosynthetic activities at that point in time.

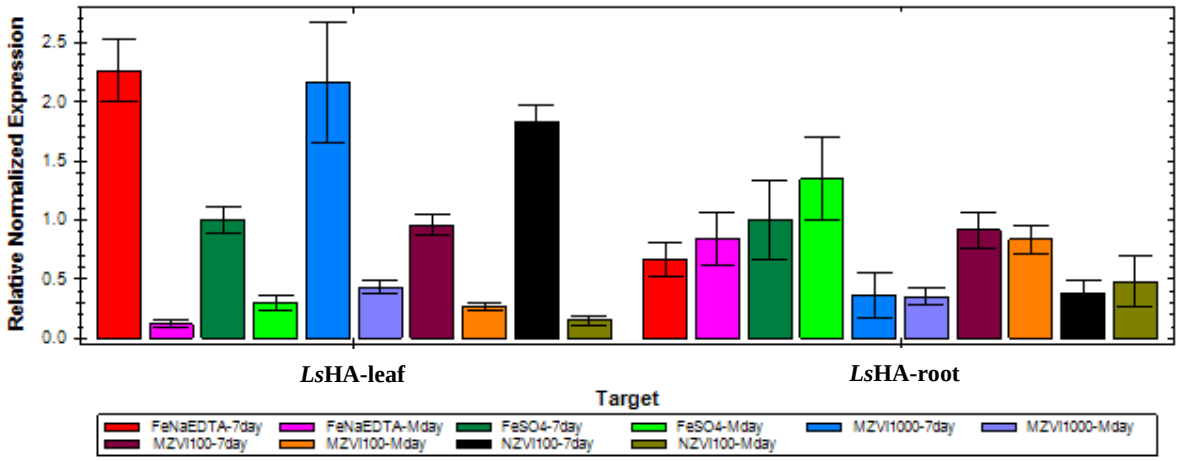

Figure 6.5. Expression of *Ls*HA2 in lettuce leaves and roots at 7 days and 30 days after exposure to nanoparticles and other treatments. Treatments are (a) NZVI100-7 day and NZVI100-Mday: NZVI at 100 mg/L, (b) MZVI100-7 day and MZVI100-Mday: MZVI at 100 mg/L, (c) MZVI1000-7 day and MZVI1000-Mday: MZVI at 1000 mg/L, (d) FeNaEDTA-7 day and FeNaEDTA-Mday: FeNaEDTA at 1.69 mg/L, and (e) $FeSO_4$-7day and $FeSO_4$-Mday: Control ($FeSO_4$). Data are mean and SE of three technical treatments which were taken from three biological replicates.

6.4.4.2. Comparison between the treatments

The expression level of the *Ls*HA2 in lettuce plant leaves and roots were also compared among the treatments. Actin housekeeping gene was used to normalize the *Ls*HA2 gene expression. Samples treated with $FeSO_4$ were used as control for expression analysis at 7 day and

at maturity. Relative expression was determined using $FeSO_4$ expression as 1. Expressions for NZVI and other treatments at 7 day and maturity were compared against $FeSO_4$ expression (taking it as 1) at 7 day and maturity, respectively. Expression level of the *Ls*HA2 gene also differed among the treatments (**Figures 6.4 and 6.5**). Here, the changes in the expression level of the *Ls*HA2 gene with different treatments are compared with the change in the expression level of the gene in the plants treated with the $FeSO_4$ control. After 7 days of exposure, the gene expression level increased 1.83-fold in the leaves of NZVI treated plants, compared to $FeSO_4$ at the same time point. The change was statistically significant at that time point. On the other hand, in the roots of lettuce plants expression level decreased 2.63-fold in 7 days post exposure plants compared to $FeSO_4$ control.

Similar kind of trend was observed in the gene expression level of the plants treated with MZVI 1000 mg/L, which was included in the experiment for a comparison purpose. MZVI at 1000 mg/L has comparable surface area to NZVI at 100 mg/L. After 7 days of exposure, the gene expression level increased 2.16-fold in the leaves of NZVI treated plants compared to $FeSO_4$ at the same time point and the change was statistically significant.

In the plants treated with MZVI 100 mg/L, after 7 days of exposure, the level of gene expression in the leaves and roots were 1.04-fold and 1.09-fold lower compared to $FeSO_4$, which were not statistically significant.

In the plants treated with FeNaEDTA, which is used as a source of Fe in hydroponic solution, the level of expression in the leaves of the plants increased (2.26-fold) after 7 days of exposure compared to $FeSO_4$. The differences in the expression level was statistically significant. On the other hand, at the same time point the expression level was 1.49-fold lower in the roots of the plants compared to $FeSO_4$.

The original hypothesis in this study was that the *Ls*HA2 gene will overexpress in the roots and leaves of lettuce in the presence of NZVI. Data from *Ls*HA2 expression profile suggests that the *Ls*HA2 gene was upregulated in the plant leaves treated with NZVI 100 mg/L, MZVI 1000 mg/L, and FeNaEDTA compared to $FeSO_4$ after 7 days of exposure (**Figures 6.4 and 6.5**). This data correlates with the higher biomass in the plants treated with NZVI 100 mg/L, MZVI 1000 mg/L and FeNaEDTA compared to the control ($FeSO_4$) and MZVI 100 mg/L (**Figure 6.2**). On the other hand, the gene LsHA2 in the roots downregulated in all the treatments compared to the control, where plants were dosed with the regular nutrient solution and $FeSO_4$ (used as a source of Fe) (**Figures 6.4 and 6.5**). It indicates that the *Ls*HA2 gene in the roots of the lettuce were affected in the presence of NZVI (100 mg/L), MZVI (1000 mg/L), MZVI (100 mg/L) and FeNaEDTA. The mRNA accumulation of *Ls*HA2 gene was less in these plants (treated with NZVI, MZVI, and FeNaEDTA) compared to $FeSO_4$. When there is a low Fe availability, plants activate *Ls*HA2 gene or plasma membrane (PM) H^+-ATPase to extrude protons out of the cell and acidify the rhizosphere and a proton gradient is generated which drives the active transport of nutrients. The activation of the this gene could occur across the whole plant due to cross-talk between PM H^+-ATPase and auxin (Hohm, et al., 2014). The mRNA of LsHA2 being less accumulated means plants did not need to acidify the rhizosphere to take up iron from a readily unavailable source (in this case NZVI and MZVI). From the profuse growth of the plants, it is obvious that plants had sufficient Fe in the solution to take up from the medium dosed with NZVI, MZVI (at higher concentration) and also from FeNaEDTA, which is a readily available source. The findings from the current research are partly in agreement with the findings of Kim et al. (2015). They reported that the levels of the AHA2 (H^+-ATPase) increased almost 2-fold not only in leaves but also in roots in 2-week-old *Arabidopsis thaliana*

exposed to NZVI. The findings from the current research indicates that the regulation of *Ls*HA2 gene in lettuce plants was controlled by the availability of iron. In the hydroponic solutions dosed with NZVI, MZVI (1000 mg/L) and FeNaEDTA, plants had sufficient Fe and the vegetative growth was good. The availability of Fe from NZVI is substantiated by the availability of Fe in core-shell Fe/Fe_3O_4, which is similar to bare NZVI used in this experiment; core-shell Fe/Fe_3O_4 was found to release 1.7 mg/L of iron ions in aqueous phase at 250 mg/L (Trujillo-Reyes, et al., 2014). The oxidation and subsequent dissolution of NZVI and MZVI might have provided plants with sufficient Fe^{2+}/Fe^{3+} needed for lettuce growth (Kadar, et al., 2012). Because of the higher growth, plants needed to photosynthesize more which in turn activated the *Ls*HA2 gene in the lettuce plants. LsHA2 gene is responsible for stomatal opening which controls the photosynthesis process. At maturity, plants did not need to accelerate the photosynthesis process and stomatal opening was not an issue. This is probably the reason, the gene was downregulated at maturity in the plants treated with NZVI, MZVI and $FeSO_4$. On the other hand, even though the LsHA2 gene was expressed in the roots of plants treated with $FeSO_4$, MZVI 100 mg/L, the cross-talk between auxin and *Ls*HA2 gene was not that effective (Hohm, et al., 2014). As a result, the expression level of the gene was not that high in those plants.

Therefore, the original hypothesis formulated in this experiment is rejected. The hypothesis was that the presence of NZVI in rhizosphere would increase plasma membrane (PM) H^+-ATPase activity and thus enhance the stomatal opening due to the overexpression of HA2-like gene in plants, thereby enhancing the uptake of Fe and other elements in plants. It is not the nanoparticles, rather the availability of Fe in solution which controls the expression of *Ls*HA2. The plants dosed with NZVI at 100 mg/L and MZVI at 1000 mg/L had sufficient ionic iron in

the solution for uptake. Other Fe-regulating genes [e.g., AHA7 (Colangelo and Guerinot, 2004) of *Arabidopsis*, CsHA1 of cucumber (Santi, et al., 2005), AtFRO2-like genes, AtIRT1-like genes] should be investigated to see their roles in Fe acquisition from not a readily available source like NZVI.

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7. CONCLUSIONS AND FUTURE DIRECTIONS

7.1. Conclusions

In this research, novel iron (Fe) cross-linked alginate (FCA) beads were synthesized and used for aqueous phosphate removal. Batch experiments were conducted with different concentrations of phosphate, including an environmentally relevant concentration of phosphate. The beads were found to remove lower concentrations of phosphate very fast from the solution. Phosphate removal was not affected by the presence of competing ions and compounds. The FCA beads was also used in real life situation (e.g., in eutrophic lakes), and the beads were found to remove 81-100% of phosphate from lake waters. The maximum sorption capacity and the point of zero charge (PZC) of the beads make the beads an ideal candidate for eutrophic lakes. The cost analysis showed that the beads are also very affordable.

Used or spent FCA beads were used for plant studies to see the bioavailability of P and Fe and also to evaluate the potential use of spent FCA beads as slow-release phosphorus and iron fertilizers. Spent FCA beads were found to support biomass production to a certain extent indicating that the phosphorus and iron were available for plant uptake. This research indicates that these FCA beads are promising as a phosphate remover and also as a slow-release non-conventional phosphate fertilizer. While these spent FCA beads were not meant for the primary source of phosphate and iron fertilizer, they can certainly be used as supplementary sources of phosphorus and iron. Because of the biodegradable nature, the phosphate-laden FCA beads could be applied directly to soil as a fertilizer.

In the present study, the feasibility of using nanoscale zero-valent iron (NZVI) as a biofortificant was tested using lettuce and spinach. Lettuce varieties responded well to the application of NZVI. The iron content and yield was positively affected by NZVI application.

The uptake of some macro and micronutrient elements were also enhanced because of the presence of NZVI. The experimental results suggest that NZVI can be used as a biofortificant for plants. However, the dose of NZVI appeared to be an important factor for biofortification. Spinach did not respond in the same manner when the plants were dosed with 110 mg NZVI/L (which is close to 100 mg/L used for lettuce study). NZVI at 55 mg/L of application produced the maximum biomass in spinach. Recommendation on the optimum dose of NZVI should be made for each plant species by carrying out extensive studies with specific species. The availability of iron from NZVI particles was evident from the total uptake and concentration of Fe in plants. That iron from NZVI was bioavailable could be explained by the oxidation and subsequent dissolution mechanism of NZVI.

The availability of iron from NZVI was further proved by the genetic study with lettuce. The presence of NZVI did not trigger the *Ls*HA2 gene which is putatively responsible for rhizosphere acidification when there is a shortage of Fe in the root zone. The gene of interest was rather upregulated in the presence of available Fe from NZVI, MZVI and FeNaEDTA. Therefore, it is not the nanoparticles, rather the availability of Fe in solution which controls the expression of *Ls*HA2. The expression of *Ls*HA2 in the presence of these iron sources indicates the plants dosed with NZVI and MZVI had sufficient ionic iron in the solution for uptake.

The concept and findings from this research can be used to develop new technologies to combat eutrophication of waterbodies, to form a supplementary source of phosphorus. The enhanced uptake of Fe and different mineral elements in the presence of NZVI is exciting considering the importance of plant fortification research. Further research is warranted to investigate the role of NZVI as a fortificant in different plants. Research is also required to determine the application method of NZVI and other nanomaterials in agricultural fields. Fe-

regulating genes should also be investigated to see their roles in Fe acquisition from not a readily available source like NZVI.

7.2. Future Directions

The FCA beads were synthesized in the lab and used for phosphate removal from phosphate-spiked artificial water and also from eutrophic lake water and wastewater. But the efficacy of these FCA beads in *in situ* condition is not proven yet. Therefore, the future challenge would be to produce these beads on a large scale and then use the beads in eutrophic lakes and phosphate-rich waterbodies for phosphate reclamation. That will prove the viability of this technology. Extensive studies are also needed to see the bioavailability of reclaimed phosphate. NZVI particles have been used for iron fortification in lettuce and spinach. While there was promising results from these studies, more studies with different species and varieties of plants are required to understand the iron acquisition strategy by plants from a not readily available source of iron. That research will pave the way for fortifying plants with nanoparticles in the future. To strengthen the iron fortification research, researchers also need to conduct in-depth studies with iron-regulating genes in different plant species.

The biodegradability of the FCA beads is likely to allow the spent FCA beads to release adsorbed phosphorus to soils. Soil properties will determine whether released phosphorus will be readily available for plant uptake or not. Soil studies are needed with the spent beads to see the immediate availability of adsorbed phosphate to plants.

APPENDIX. TABLES, FIGURES, AND PROTOCOLS

Table A1. Reaction rate constants calculated based on the obtained results.

C_0	C_e	Zero Order		First Order		Second Order	
mg/L		K_{obs}	R^2	K_{obs}	R^2	K_{obs}	R^2
5	0.16	-0.002	0.642	-0.0091	0.954	0.076	0.936
50	11.48	-0.0017	0.696	-0.0034	0.851	0.0078	0.954
100	49.6	-0.0003	0.932	-0.0004	0.964	0.0006	0.985
100*	20	-0.0401	0.966	-0.0828	0.987	0.2027	0.922

*****Concentration is in µg/L

Units: K_{obs} Zero Order (mg/L/min), K_{obs} First Order (/min), K_{obs} Second Order (L/mg/min)

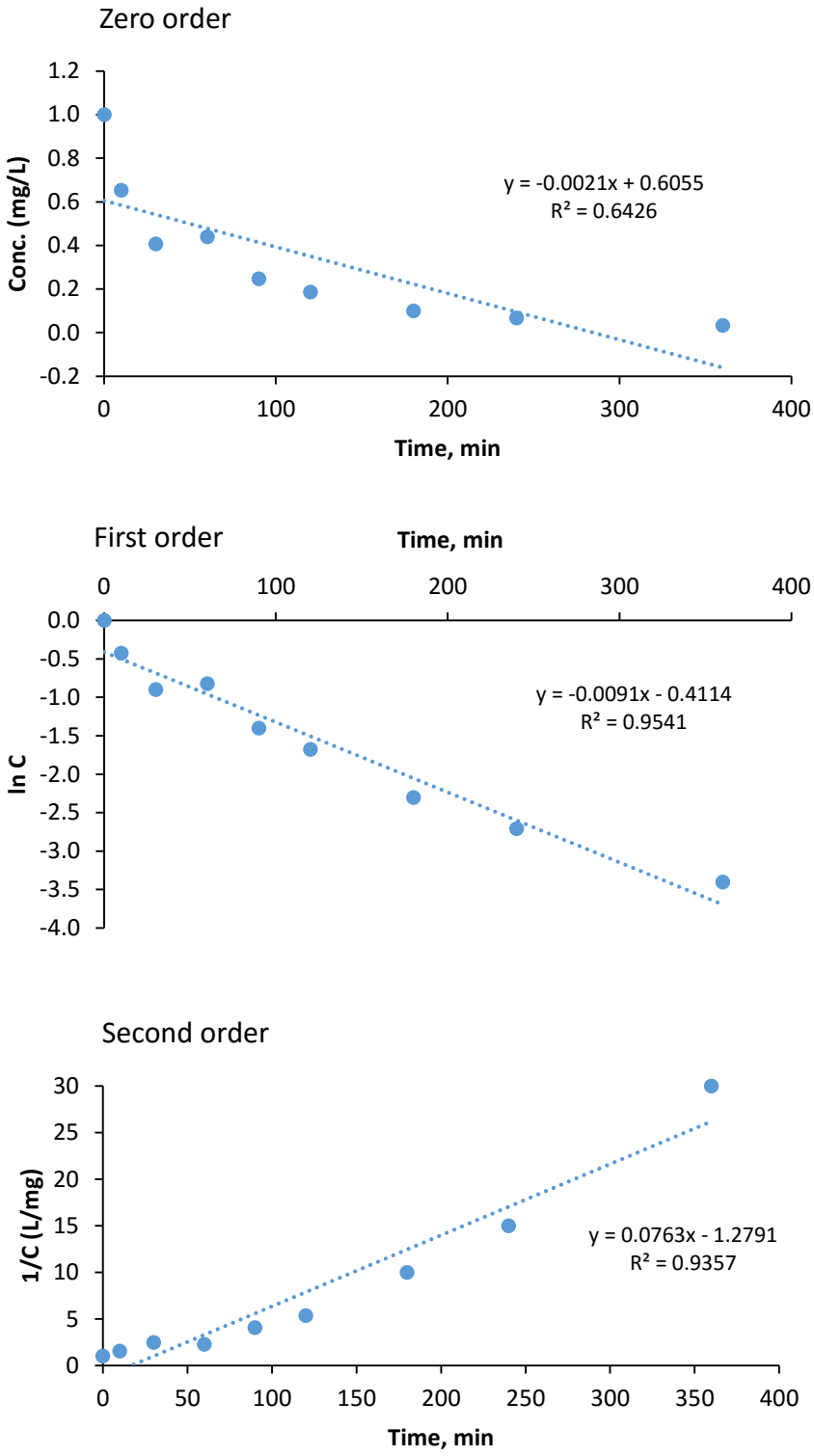

Figure A1. Zero, first and second order rate equations for 5 mg PO_4^{3-}-P/L removal by FCA beads.

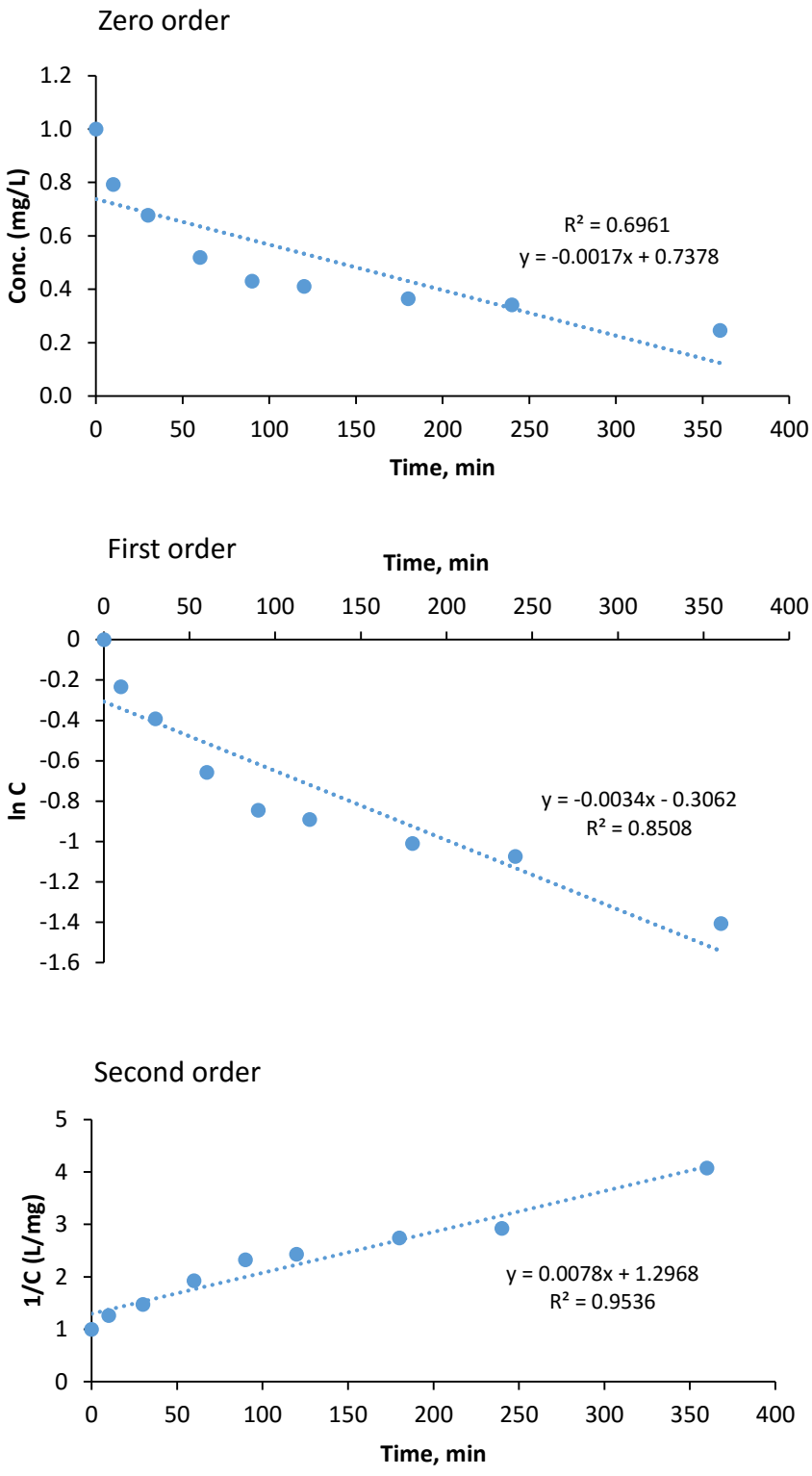

Figure A2. Zero, first and second order rate equations for 50 mg PO_4^{3-}-P/L removal by FCA beads.

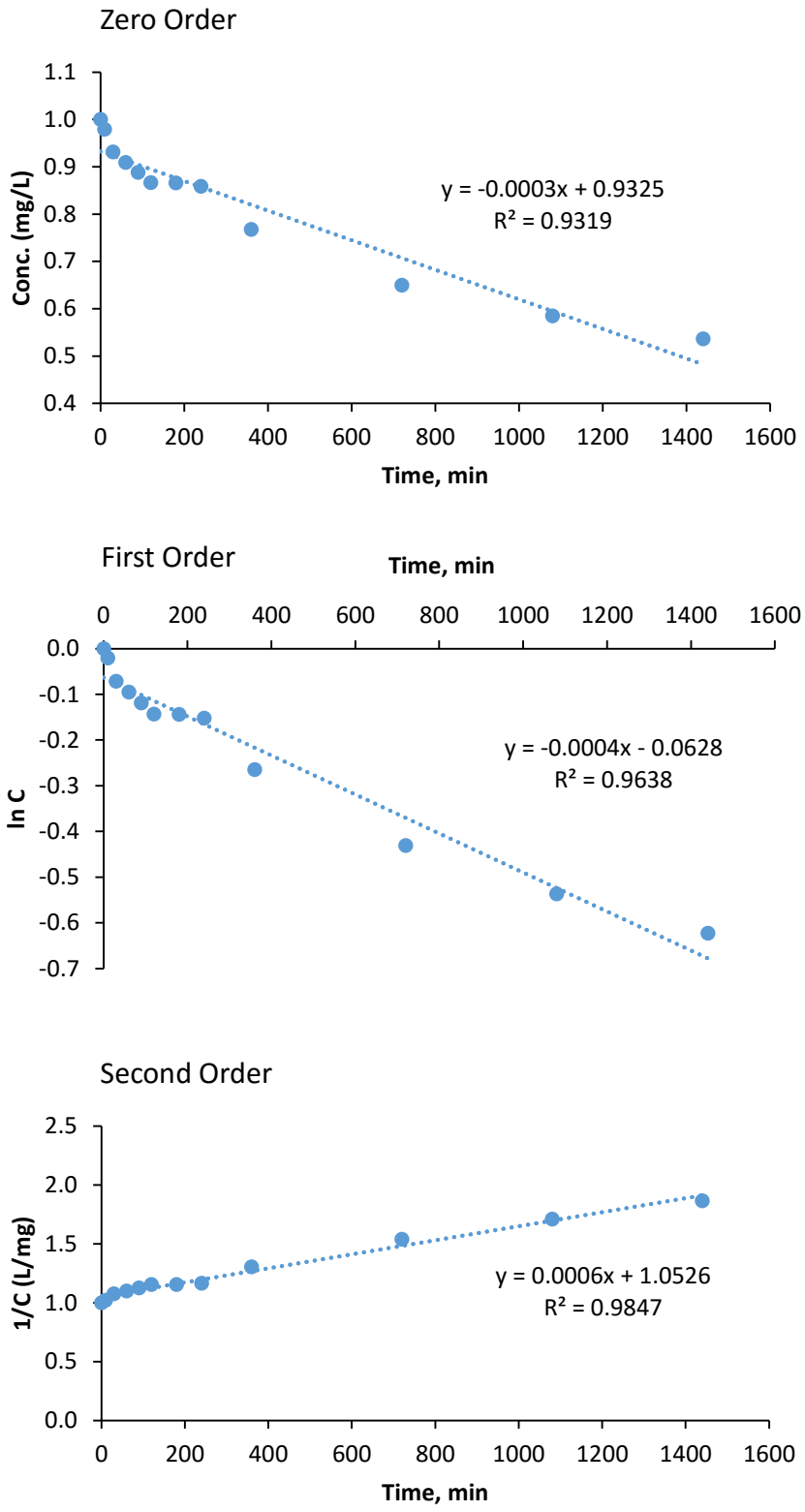

Figure A3. Zero, first and second order rate equations for 100 mg PO_4^{3-}-P/L removal by FCA beads.

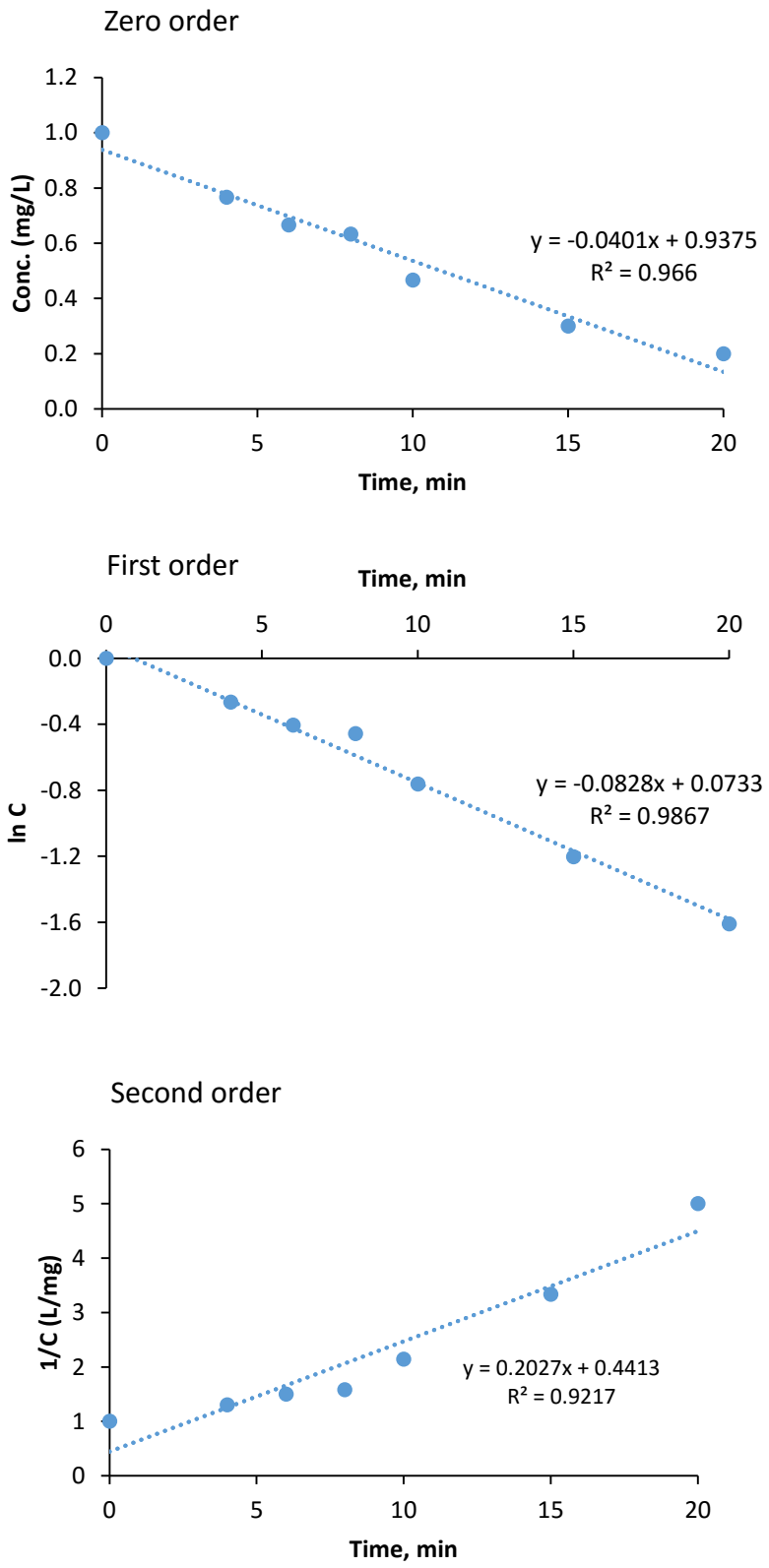

Figure A4. Zero, first and second order rate equations for 100 µg PO_4^{3-}-P/L removal by FCA beads.

Protocol A1. RNA isolation

1. ~30 mg of plant tissue was disrupted and 380 µL of RLT buffer was added to homogenize the lysate. The lysate was centrifuged for 3 min at maximum speed. The supernatant was carefully removed by pipetting.
2. 1 volume of 70% ethanol was added to the lysate (from step 1) and mixed well by pipetting.
3. 700 µL of the sample (from step 2) was transferred to an RNeasy Mini spin column placed in a 2 mL collection tube. The lid was closed and the spin column was centrifuged for 15 s at ≥8000 ×g. The flow-through was discarded.
4. 700 µL of the Buffer RW1 was added to the RNeasy spin column. The lid was closed and the column was centrifuged for 15 s at ≥8000 ×g. The flow-through was discarded.
5. 500 µL of Buffer RPE was added to the RNeasy spin column. The lid was closed and the column was centrifuged for 15 s at ≥8000 ×g. The flow-through was discarded.
6. 500 µL of Buffer RPE was added to the RNeasy spin column. The lid was closed and the column was centrifuged for 2 min at ≥8000 ×g.
7. The RNeasy spin column was placed in a new 1.5 mL collection tube, and 50 µL of RNase-free water was added directly to the spin column membrane. The lid was closed and the column was centrifuged for 1 min at ≥8000 ×g to elute the RNA.

Protocol A2. cDNA conversion

1. Extracted RNA from different samples, Oligo Primer and nuclease-free water were mixed into a final volume of 5 µL.
2. The reaction was heated in a 70 ^{0}C heat block for 5 minutes. The reaction was immediately chilled in ice water for at least 5 minutes. The reaction was then centrifuged

for 10 s in a microcentrifuge. The reaction was stored on ice until reverse transcription mix was added.

3. The reverse transcription reaction mix was prepared, 15 µL for each cDNA reaction, combining the following components: GoScript™ 5X Reaction Buffer (4 µL), 1.5 to 5.0 mM $MgCl_2$ (4 µL), PCR Nucleotide Mix (final conc. 0.5 mM each dNTP) (1 µL), Recombinant RNasin® Ribonuclease Inhibitor (0.5 µL), GoScript™ Reverse Transcriptase (1 µL), and Nuclease-Free Water (4.5 µL). The mixing was done on ice.
4. 15 µL of reverse transcription mix was combined with 5 µL of RNA and primer mix.
5. The RNA with reverse transcription mix and primer mix was annealed in a heat block at 25 ^{0}C for 5 minutes.
6. It was then extended in a heat block at 42 ^{0}C for half to one hour.
7. The reverse transcriptase was inactivated in a heat block at 70 ^{0}C for 15 minutes.

Protocol A3. Purification of cDNA using Promega kit

1. 4 volumes of Binding Buffer (B2) was added to 1 volume of PCR reaction. The reaction was mixed well
2. A PureLink® Clean-up Spin Column in a Wash Tube was removed from the package.
3. Sample in Binding Buffer from Step 1 was added to the PureLink® Spin Column.
4. The PureLink® Spin Column was centrifuged at room temperature at 10,000 ×g for 1 minute.
5. The flow through was discarded and the PureLink® Spin Column was replaced into the Wash Tube.
6. 650 µL Wash Buffer with ethanol was added to the PureLink® Spin Column.

7. The PureLink® Spin Column was centrifuged at room temperature at 10,000 ×g for 1 minute. The flow-through was discarded from the Wash Tube and the PureLink® Spin Column was replaced into the tube.
8. The PureLink® Spin Column was centrifuged at maximum speed at room temperature for 2-3 minutes to remove any residual Wash Buffer. The Wash Tube was discarded.
9. The PureLink® Spin Column was placed in a clean 1.7-mL PureLink® Elution Tube.
10. 50 µL Elution Buffer was added to the center of the PureLink® Spin Column.
11. The PureLink® Spin Column was incubated at room temperature for 1 minute.
12. Then the PureLink® Spin Column was centrifuged at maximum speed for 1 minute.
13. The elution tube contained the purified PCR product. The PureLink Spin Column was removed and discarded. The recovered elution volume is ~48 µL.

Protocol A4. qPCR

1. In a 20 µL reaction, 10 µL of SsoAdvanced™ Universal SYBR® Green Supermix (Catalog #172-5271), 1 µL forward and reverse primers, and 4 µL of cDNA was used to do qRT PCR.
2. Amplification conditions were as follows: denaturation at 95 °C for 30 s followed by 40 cycles at 95 °C for 15 s, 62 °C for 30 s.