Selenium and Neem oil accumulation in Aquaponics Systems

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Abstract

Aquaponics is a horticulture technique that has been increasing in popularity for industry and household use. It converts the ammonia fish waste to nitrites and nitrates through nitrifying bacteria, Nitrosomonas and Nitrobacter. The treated water is used to fertilize plants on a hydroponic bed. Recirculation and treatment conserves water but can lead to an accumulation of various toxins.

This project focuses on the effects and accumulation of dosing systems with selenium and neem oil. Selenium is an essential nutrient for humans with a narrow daily intake range of 40-400µg. Selenoproteins mediate oxidative stress through use of the encoded amino acid selenocysteine (SeCys), while selenium-containing proteins accumulate selenomethionine SeMet. Neem oil is an effective organic pesticide that is used widely throughout the agriculture industry. It is effective against insects, but the effects on nitrifying bacteria in aquaponics are not known.

Selenium was dossed to the water and later quantified in various foodstuffs and water collected from the systems. Samples were analyzed with Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The accumulation of Se in water was more pronounced from inorganic species; compared with organics that showed more accumulation in foodstuff.

The active molecule of neem oil, azadirachtin, and its degraded form were detected via Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) in foodstuff. Chlorophyll content in leaves was determined with UV spectroscopy and used to determine the health of the systems metabolism.

The nitrification was not affected by any treatment and no toxic levels of Se or neem oil were found in edible parts.

Introduction

Food and water insecurity are becoming an increasing problem as global population grows. 821 million people were estimated to be malnourished worldwide in 2018. (UN, 2020)

As demand for agricultural products increases, the increased environmental impact must be considered. Agriculture alone represents 92% of the global water footprint, and 10% and 11% of current farmland could experience shortages of usable water. (Hoekstra, A. Y., M. M. Mekonnen, et al. 2012) (Fitton, N., P. Alexander, et al. 2019) Conventional methods produce large amounts of wastewater requiring treatment, making them less viable. Fertilized irrigation water leeching into water sources as runoff is a major source of wastewater in agriculture. High concentrations of nitrogen in runoff can stimulate large blooms of phytoplankton leading to disruption of the ecosystem. (Michael Beman, J., K. R. Arrigo, et al. 2005)

Many parts of the world are looking to aquaponics as a green alternative to traditional agriculture and aquaculture techniques. Aquaponics systems are recirculating aquaculture systems that treats wastewater from fish to be used as fertilizer for a hydroponic grow bed (fig. 1). Ammonium contaminated water is pumped from the fish tank to a biofilter where solids are removed, and nitrifying bacteria oxidize ammonium to nitrites and nitrates. Treated water flows to a hydroponic grow bed, fertilizing crops before retuning to the fish tank. No water exchange is necessary apart from replacing that lost from transpiration and evaporation.

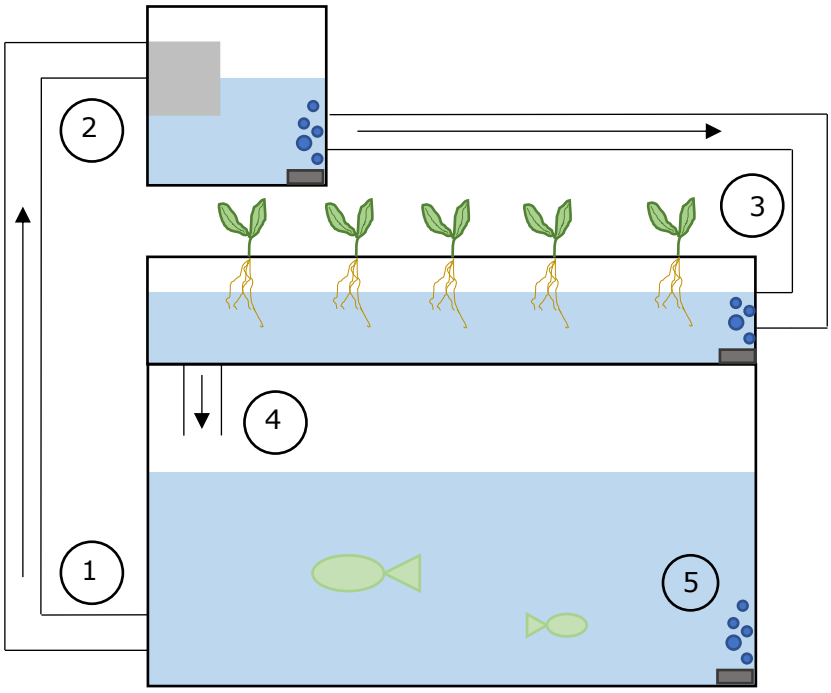


Figure 1. *A diagram of an aquaponics system. 1) Ammonium containing water is pumped out of the fish tank to the biofilter. 2) The biofilter removes solid waste and converts NH4+ to NO3- using nitrifying bacteria. 3) Treated water flows to a hydroponic grow bed. 4) Clean water returns to the fish tank. 5) Nitrification is an aerobic process; therefore, air stones are needed to maintain dissolved oxygen in all parts of the system.*

Aquaponics provides a completely organic alternative to hydroponic farms when paired with organic pesticides. However, a balance of nutrients is necessary for the health of the system. Oxidation of ammonia produces acid and eventually lowers the pH, away from the optimal range of 7.0-9.0. (Rakocy, J., M. P. Masser, et al. 2016) Therefore, it is important to maintain a balance of plants and fish to prevent nutrient deficiency or accumulation. Additives and supplements can also contribute to system toxicity. Because no water change is necessary, any toxicants in feed or pesticides will remain and accumulate in the system. As aquaponics increases in commercial use, more research is being conducted to improve its efficiency.

Selenium is an essential nutrient for human health. Selenoproteins have various functions including redox homeostasis, cell signaling, and thyroid metabolism. (Papp, L. V., J. Lu, et al. 2007) Many of the functions of these proteins are conserved among animals, including fish. (Bryszewska, M. A. and A. Måge 2015) However, at elevated concentrations can be toxic. (Sun, H.-J., B. Rathinasabapathi, et al. 2014), (Selvaraj, V., J. Tomblin, et al. 2013) Plants can be considered accumulators, or non-accumulators of Se, and while accumulators are relatively tolerant, Se shows toxicity in non-accumulators. (Terry, N. Zayed, A. M. et al. 2000)

Fish feed is often enriched with selenium for use in aquaculture to mediate oxidative stress. (Cotter, P. A., S. R. Craig, et al. 2008) High concentrations of Se is appropriate for water that is regularly changed but can lead to unavoidable hyperaccumulation in aquaponics systems. The species of selenium can greatly affect the bioavailability. A study by Sele, V., Ørnsrud, R. et al. (2018) observed higher retention of selenium in Atlantic salmon supplemented with organic selenium opposed to inorganic selenium as selenite. Accumulation of SeMet can lead to toxicity in fish and plants (Gupta, M., & Gupta, S. (2017), (Dörr, A. J. M., Pacini, N. et al. 2008), but the effects on nitrifying bacteria are less well understood.

Neem oil is growing in popularity in the agriculture industry as an organic pesticide. Azadirachtin and other C-seco limonoids in neem oil act as antifeedants and growth regulators in insects, while maintaining relatively little toxicity in animals. (Govindachari, T., Narasimhan, N. et al. 1996), (Deng, Y. X., Cao, M. et al. 2013) Neem oil has been shown to have bacteriostatic and antimicrobial properties as well. (Pu, Z. H., Zhang, Y. Q. et al. 2010), (Mishra, P., S. K. R. S, et

al. 2014) Though its effects on nitrification in aquaponics systems is relatively unknown, neem oil can be used as a nitrification inhibitor in soil, stopping as much as 30.9% of nitrification. (Kumar, R., Devakumar, C. et al. 2007)

Aquaponics farms need to maintain profitability to become a large-scale sustainable method, rather than small personal systems. A survey conducted by Love, D. C., Fry, J. P. et al. (2015) found only 31% of aquaponics farms are profitable within the first year. Though most farms surveyed were considered small, the size of the operation had no effect on this trend. Relatively high system failure rate is an important factor in this. Many farms fail to profit because of unexpected expenses, improper scaling, or lack of pest control. (Walraven, B. C. 2014) Therefore, dosing with selenium and neem oil may prove beneficial to aquaponic systems by preventing loss of fish or crops, and supplementing foodstuff with Se as an essential human nutrient. The aim of this ongoing study is to determine if where in the system neem oil and Se accumulate, how much accumulates, and how it effected the overall nitrification of the system.

Materials

All experiments were done in triplicate unless otherwise noted. NaCl and HPLC-grade Acetonitrile were purchased from Fisher Chemical (Pittsburgh, PA, USA). Tris(hydoxymethyl) aminomethane was obtained from Acros Organics (Pittsburgh, PA, USA). Sodium dodecyl sulfate was purchased form MP Biomedicals, LLC (Solon, OH, USA). PierceTM Protease Inhibitor Tablet were purchased from Thermo Scientific (Pittsburgh, PA, USA), and 100% Ethanol was obtained from Decon Labs, Inc. (King of Prussia, PA, USA). Garden Safe® Neem Oil Extract, mung beans, adzuki beans, and fish feed were purchased at local hardware store (Cincinnati, OH, USA). Se(IV), Se(VI), and Se(Met) were purchased from Fisher Chemical (Pittsburgh, PA, USA).

Methods

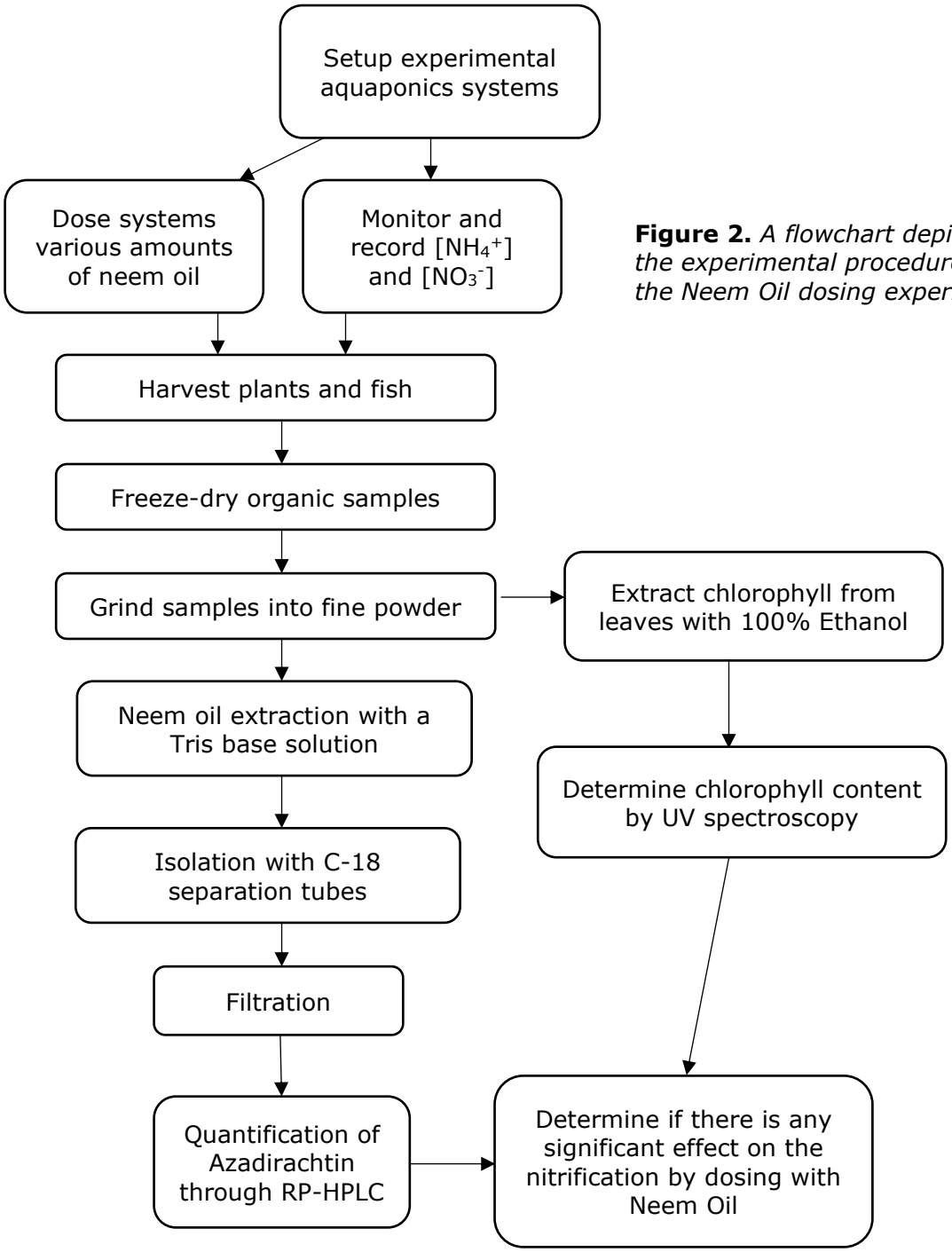
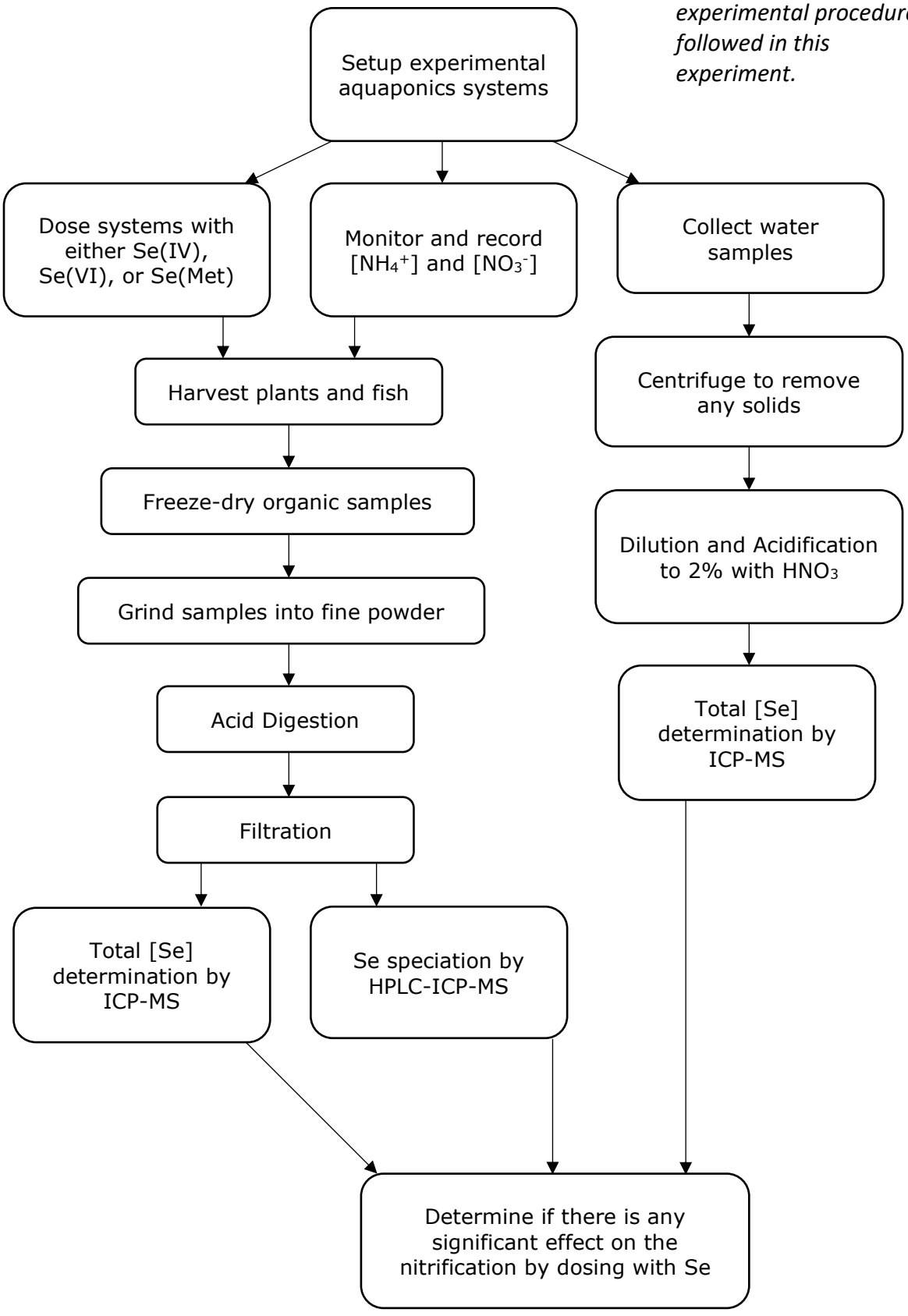


Figure 2. *A flowchart depicting the experimental procedure for the Neem Oil dosing experiment.*

Figure 3. *A flowchart depicting the experimental procedures followed in this experiment.*



Experimental Aquaponics Systems for Se dosing

Four aquaponics systems were set up in the Crosley Tower greenhouse, University of Cincinnati, Cincinnati, OH, from 1/14/2020 to 2/11/2020. The systems contained ~100L of water, hydroponic grow beds, biofilters, water pumps, and aerators. Biofilters were inoculated with nitrifying bacteria and dosed with ammonium nitrite one week before the experiment. Mung and adzuki beans were grown and each tank contained 4 freshwater crayfish. 1.0g of feed containing 1ppm of Se was given to each tank every other day between 11 a.m. and 1 p.m. Water was added to replenish loss from evaporation at the same time as feeding.

Se dosing and Collection of water samples

Every other day, at the time of feeding, 5mL of water was collected form each system for Se analysis. Experimental systems were then dosed with ~20 ppm selenium as either Se(IV), Se(VI), or Se(Met). After 10 minutes another 5mL sample of water was collected. Ammonium and nitrate concentrations were measured following the API® Master Freshwater Test Kit protocol.

Sample preparation for Se analysis by ICP-MS

Foodstuff was harvested from the systems on 2/11/2020 after the final water sample was collected, including crayfish, and roots, beans leaves, and stems of beans. All samples were weighed before and after freeze drying to determine both wet and dry weight. Dried foodstuff was ground and homogenized with an agate pestle and mortar. Acid microwave digestion was used to breakdown organic molecules, then each sample was filtered for analysis by ICP-MS.

Water Sample preparation for Se analysis by ICP-MS

A theoretical Selenium concentration was calculated to determine an appropriate dilution ratio for each sample. Samples were centrifuged, and the supernatant was diluted and acidified to 2% with concentrated nitric acid.

Plant Material Sample preparation for Neem Oil Analysis by RP-HPLC.

Plant material used for neem oil analysis was harvested from experimental aquaponics systems setup throughout the month of April 2019. Systems were dosed with various amounts

of Neem Oil by spraying directly on leaves of the plants. Freeze-dried, ground leaves (.1g) and beans (.1g) and wet roots (2g) muddled with an extraction solution containing Tris, SDS, NaCl, and protease inhibitor [3mL; 8.8mM Tris(hydoxymethyl) aminomethane, 2.5mM Sodium Dodecyl Sulfate, 15mM, 1 protease inhibitor tablet/30mL] in an agate pestle and mortar. The extraction mixtures were transferred into 10ml centrifuge tubes and separated by centrifugation using a Clay Adams™ Dynac ∞ Centrifuge. .5mL of the supernatant was then purified further by centrifugation filtering through a 2mL, .45µm filter centrifuge tube with a Fisher Scientific accuSpin Micro 17 at 10,000*g. The active was isolated from more polar molecules by passing the solution through a C-18 SPE cartridge and rinsing twice by centrifugation with DDI and ACN [1mL; 97% DDI water, 3% Acetonitrile]. Azadirachtin and its degraded form were recovered with and 80% ACN solution [1ml; 20% DDI Water, 80% Acetonitrile] and transferred into 2mL brown HPLC vials.

Neem Oil Standard Preparation.

All the centrifugation done for the standard preparation was done using the Clay Adams™ Dynac ∞ Centrifuge. Four replicates of the Azadirachtin standards were prepared through liquid-liquid of Garden Safe® Neem Oil Extract with Acetonitrile [1mL Garden Safe® Neem Oil Extract/ 3mL Acetonitrile]. Samples were centrifuged and the supernatant was diluted to a 3% ACN solution. Azadirachtin was further isolated from more polar molecules by passing the solution through a C-18 SPE cartridge and rinsing twice by centrifugation with DDI and ACN [1mL; 97% DDI water, 3% Acetonitrile]. The target molecule was recovered with and 80% ACN solution [1ml; 20% DDI Water, 80% Acetonitrile] and transferred into 2mL brown HPLC vials.

Chlorophyll Extraction and Quantification.

Leaf tissue (.05g) was muddled with 100% Ethanol (1.5mL) to extract chlorophyll. Solids were separated by centrifugation then diluted 50X. Absorbance was measured at 642nm and 662nm using a ThermoSpectronic Genesys 20 spectrophotometer and quartz cuvets.

Isolation of Azadirachtin by RP-HPLC.

Isolation of azadirachtin was achieved through gradient elution, RP-HPLC. The high-performance liquid chromatography system (HPLC) included an Aglient 1100 series (Agilent Technologies, Santa Clara, CA, USA), equipped with a binary HPLC pump, and autosampler, vacuum degasser system, a temperature column compartment, and a diode array detector. UV absorbance was measured at 280nm. A Phenomenex Kinetex® XB-C18 100 Å [2.6µm, 100 x 4.6mm] reverse-phase column was used. The column was equilibrated with 20% ACN for two minutes before injection of 5µL samples. Azadirachtin was eluted between 8-10 minutes. Peaks were identified by overlying chromatograms of Neem Oil standards with those of each sample as seen in figure 1. Complete parameters for the RP-HPLC separation are given in Table 1.

Table 1. *Operating conditions for RP-HPLC*

HPLC Parameters	Column	Phenomenex Kinetex® XB-C18 [2.6µm, 100 x 4.6mm]
	Flow Rate	.8ml/min
	Mobile Phases	A, DDI Water B, ACN
	Gradient	2min 20% B 13min 100% B 15min 100% B 18min 20% B 20min 20% B
	Injection Volume	5µL
	Column Temperature	60°C

Results and Discussion

Analysis of selenium in water samples and foodstuff is underway, but incomplete. This section will focus on Neem Oil accumulation and chlorophyll content analysis by RP-HPLC and UV/VIS spectroscopy.

It is known that azadirachtin is UV reactive and will degrade over time when exposed to sunlight, so two target peaks were observed and quantified for each chromatogram (Fig. 4). The first peak eluted at 8.8mins was assigned to be active azadirachtin and the second the degraded

molecule at 9.6mins. UV absorbance was quantified using OriginPro 8.5 integration over a manually plotted baseline.

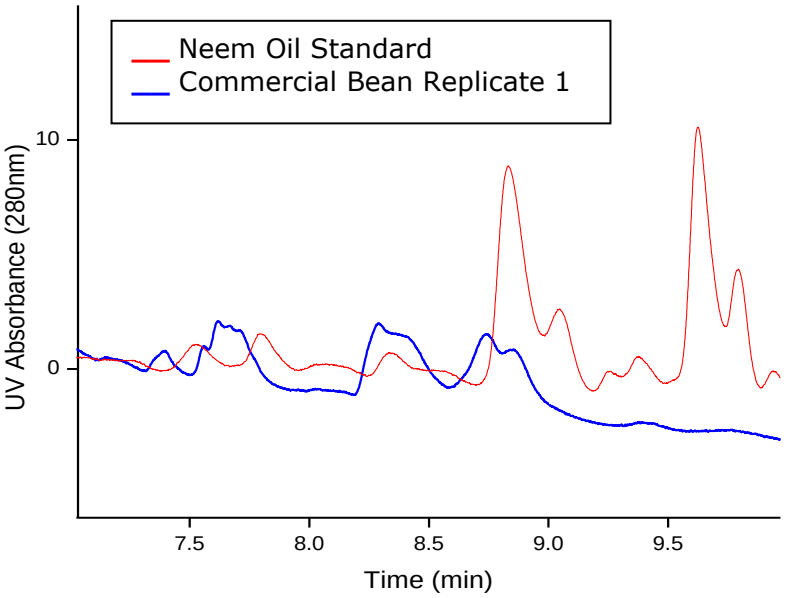
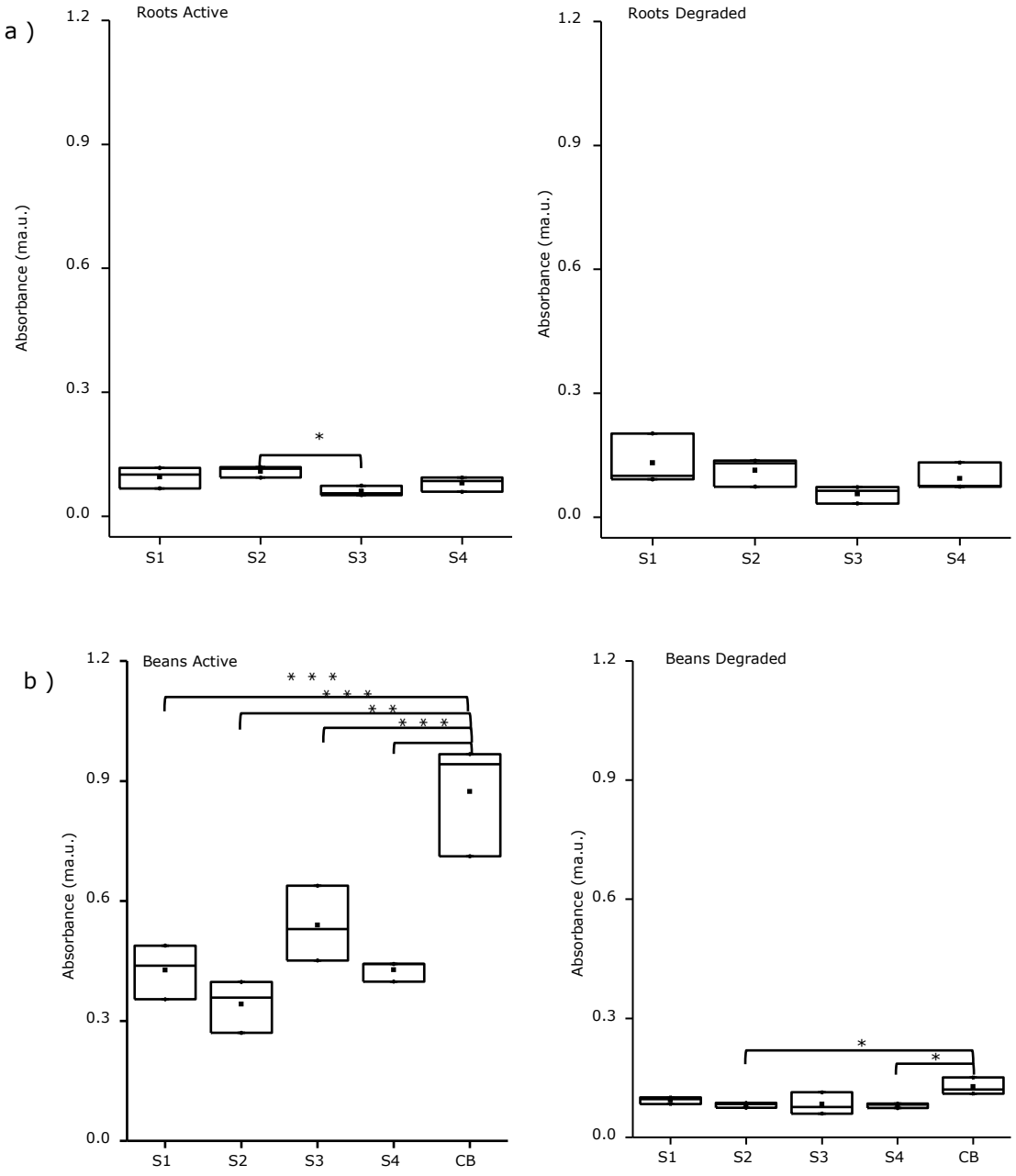


Figure 4. *Reverse phase chromatograms of azadirachtin and its degraded form for a neem oil standard and commercially produced beans. Water damage to the column caused a slightly lowered retention time in peaks of commercial beans compared to the standard. The neem oil standard was analyzed before the column was damaged.*

Mean Comparison of Azadirachtin and its degraded form in dosed Aquaponics Systems

Average absorbance was compared via a one-way Anova with the Bonferroni and Tukey test. Fig. 5-A shows mean absorbance for root samples across each system. Only one significant difference was observed between systems 2 and 3. The bean comparison showed the most significant difference, seen in fig. 5-B. Commercially produced beans consistently contained more active form of azadirachtin than any other sample at $p < .01$. The commercial beans were the only source of significant difference for the degraded form. At $p < .05$, commercial beans had a greater mean absorbance than systems 2 and 4. Fig. 5-C shows the mean absorbance for leaf samples. A significant difference was observed between systems 3-4 and 1-2 for active compounds with $p < .005$, but no difference was observed for the degraded compound. Leaf samples tend to have lower reproducibility because they are directly sprayed with neem oil while dosing the systems.


a)
Roots Active
Roots Degraded
Absorbance (ma.u.)
1.2
0.9
0.6
0.3
0.0
S1
S2
S3
S4
*
b)
Beans Active
Beans Degraded
CB
* * *
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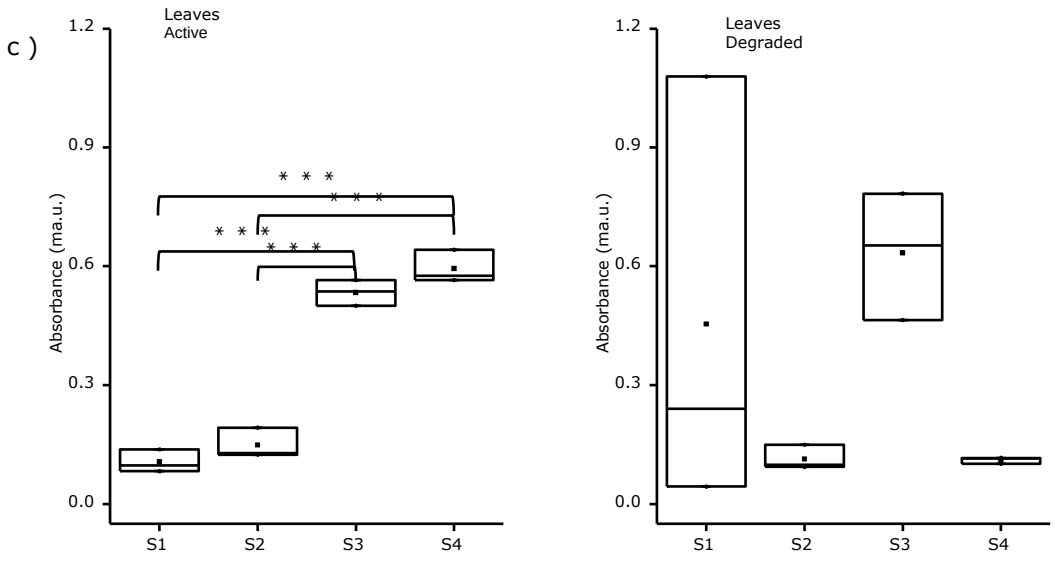
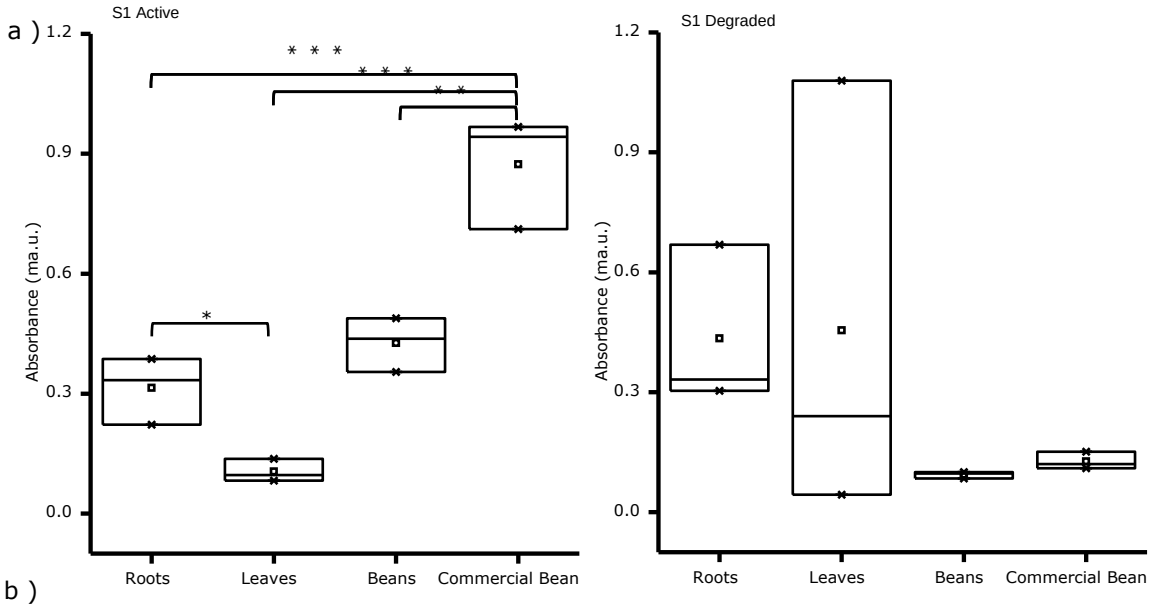


Figure 5. *a) A box chart showing average absorbance (ma.u.) of root tissue samples. b) Average absorbance of bean tissue. c) Average absorbance of leaf tissue. All were subjected to a one-way anova test. A bracket indicates significant difference where * → p < .05 ** → p < .01 *** → P < .005*

Commercial beans were tested against each system using the same method (Fig. 6) and were found to contain significantly more active azadirachtin than samples from the systems, p < .05. The aquaponic systems on average contained more degraded compound than the commercial beans. Fig. 6-B and fig. 6-D show roots of systems 2 and 4 with significantly more degraded compound than the rest of the system. Fig. 6-C shows leaves in system 3 retaining the most degraded azadirachtin.

a)


S1 Active
S1 Degraded
Absorbance (ma.u.)
1.2
0.9
0.6
0.3
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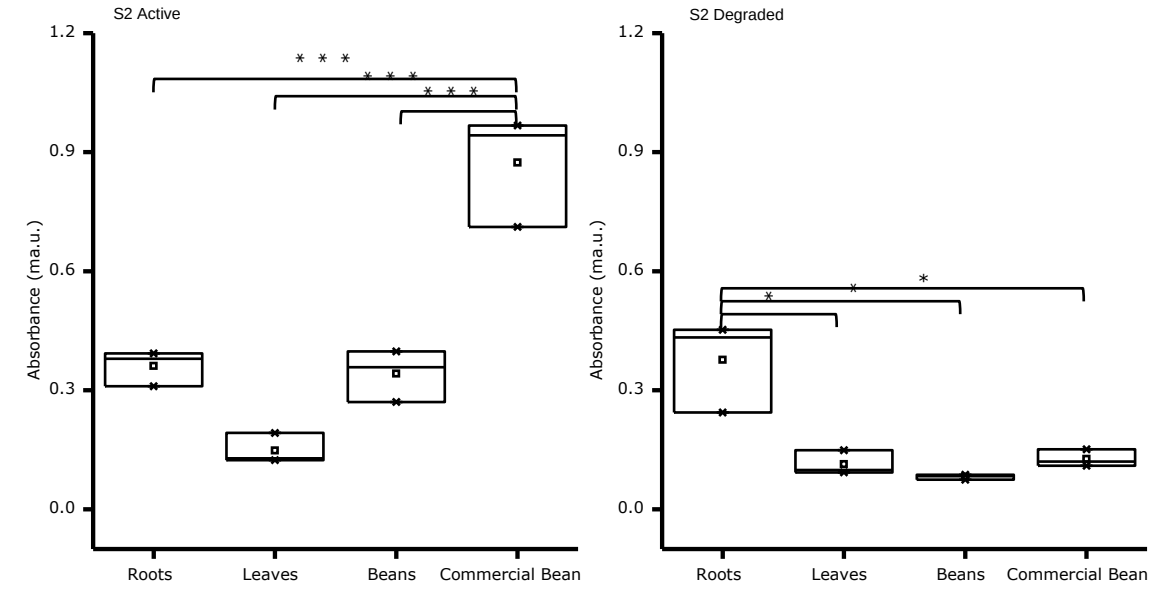
* * *
* * *
* *
*
Roots
Leaves
Beans
Commercial Bean

b)


S2 Active
S2 Degraded
Absorbance (ma.u.)
1.2
0.9
0.6
0.3
0.0
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Roots
Leaves
Beans
Commercial Bean

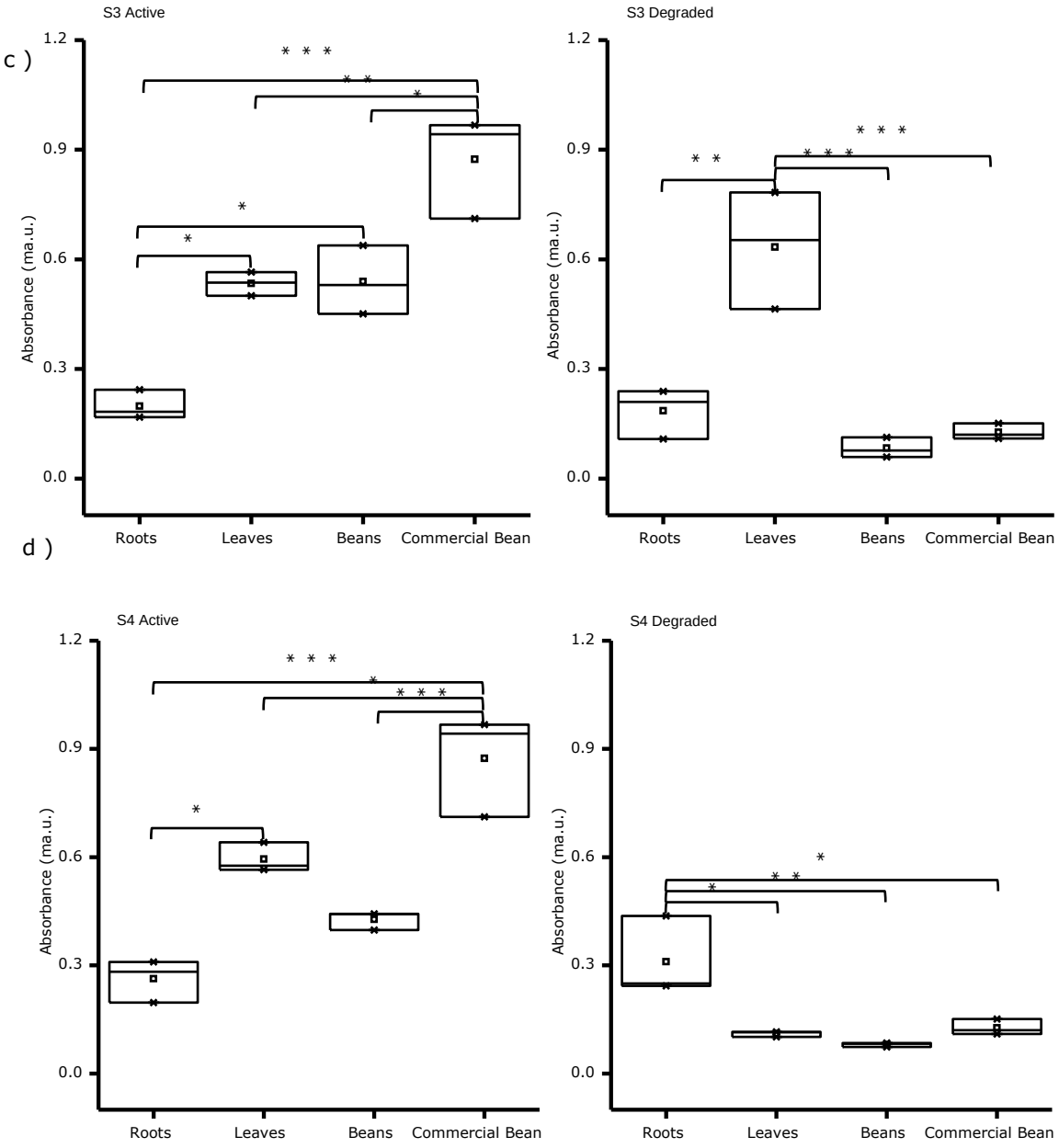

Figure 6. *A) A box chart showing average absorbance of each sample from system 1. B) Average absorbance taken from system 2. C) Average absorbance from samples in system 3. D) Average absorbance from system 4. Each system was compared to the commercial bean as a control for both active and degraded forms of azadirachtin.*

Chlorophyll Comparison in Leaf Tissue

UV absorbance at 662nm and 642nm were tested using the same method as the mean azadirachtin absorbance. Fig. 7 shows box charts for mean absorbance at both wavelengths. System 1 had significantly more chlorophyll than S2-4, but there is no evidence dosing the systems had any effect on the health of the plants.

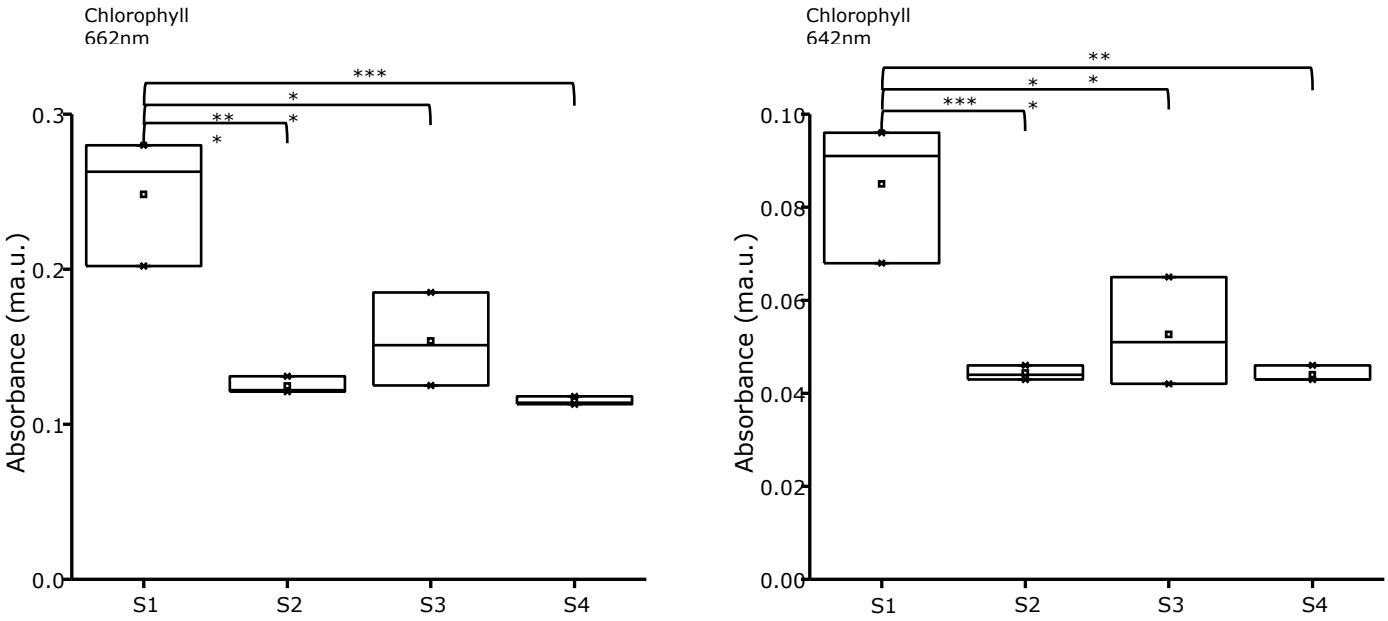

Fig. 7 *Box charts showing chlorophyll absorbance at 662nm and 642nm for leaf tissue in each aquaponic system.*

Conclusions

With these experiments, we observed the retention of azadirachtin in aquaponics systems and their effect on the overall health of the system. Plants of the control system produced more chlorophyll than those dosed with neem oil, suggesting a slight inhibition of plant metabolism. However, healthy plants were produced regardless of the neem oil dosing and contained significantly less active azadirachtin than commercially produced beans. There

were no adverse effects observed on the activity of the biofilter or health of the crayfish at these concentrations. Absorbance of azadirachtin was measured through RP-HPLC with UV-vis detection successfully for root and bean tissue, however showed lower reproducibility for leaves. We were able to determine any residual neem oil in the system had no adverse effects on the health of the plants.

Quantification and speciation of selenium in foodstuffs and water samples have yet to be completed, but some conclusions can still be drawn. Plant growth was observed in all systems during the experiment, suggesting little to no effect on nitrification by Se, regardless of the species. Future experiments will determine how Se is metabolized in an aquaponics system. Quantification of various Se species will help determine if dosed selenium was incorporated into various selenoproteins or accumulated as a toxin. Metabolism of selenium varies by species; therefore, more experiments are needed to properly regulate it as a nutrient in aquaponics systems.

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