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# **The Effects of Single-Walled Carbon Nanotubes On Plant Growth**

By

Matthew Ray

A Thesis  
Submitted to the Faculty of Graduate Studies  
through Environmental Engineering  
in Partial Fulfillment of the Requirements for  
the Degree of Master of Applied Science  
at the University of Windsor

Windsor, Ontario, Canada

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# **The Effects of Single-Walled Carbon Nanotubes On Plant Growth**

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## ABSTRACT

Nanotechnology is not only being used to enhance commercial goods but the research into the use of nanomaterials as soil and groundwater remediation options has been underway for some time. The research to date suggests that once CNTs have been taken up by humans, or other species they may cause inflammation, oxidative stress, cell damage, or adverse effects on cell performance. However, when considering the interactions between CNTs and plants cells or developing plants the outcomes are less certain and may be counterintuitive. Interactions between developing plants seem to show neutral or positive short-term effects. Research conducted thus far shows unfunctionalized single-walled carbon nanotubes, when suspended in an aquatic environment, seem to enhance the growth of plant life in the short term.

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## CHAPTER I

### INTRODUCTION

Nanotechnology is defined by the American National Science and Technology Council (2004) as “...the understanding and control of matter at dimensions roughly 1 to 100 nanometers (nm) where its unique phenomena enable novel applications.” For comparison, the diameter of a single red blood cell is approximately 5,000 nm, whereas a single human hair is approximately 80,000 nm thick (ETC group 2005). The first mention of the concepts used in modern ‘nanotechnology’ were present in Dr. Richard Feynman’s December 1959 Caltech lecture, entitled, “There’s Plenty of Room at the Bottom”. Feynman begins to ask questions such as “*What would happen if we could arrange the atoms one by one, the way we want them?*” (Feynman 1959).

The term “nanotechnology” was not officially defined until a paper published in 1974 by Norio Taniguchi, who stated “ ‘Nanotechnology’ mainly consists of the processing of, separation, consolidation, and deformation of materials by one atom or one molecule.”

Only recently has nanotechnology made its way into the consumer’s market place by enhancing widely used products, such as cosmetics and recreational equipment, as well as being used in research for the industrial and medical sectors. Nanotechnology is not only being used to enhance commercial goods but the research into the use of nanomaterials as soil and groundwater remediation options has been underway for some time. However, nanomaterials being released into the environment in such a manner may have as many drawbacks as benefits. As research into enabling these novel applications progresses research into the environmental impacts of nanotechnology should progress

simultaneously. This research examines how a specific nanomaterial, single-walled carbon nanotubes (SWCNTs), may affect the vegetation in a region where the CNTs have been released in the environment for the remedial purposes.

### Carbon Nanotubes

Fullerenes are one of four naturally occurring allotropes of elemental carbon (British Standards Institute 2007), first identified in the year 1985 by researchers at the University of Sussex in England and Rice University in the United States (Kroto et. al 1985). Classified under the group of fullerenes, Carbon Nanotubes (CNTs) are comprised of sheets of graphene where the hexagonal crystal lattice is rolled into an elongated tubular form (British Standards Institute 2007). CNTs can range in a few nanometers to over a millimeter in length. First discovered in 1991 by Sumio Iijima, CNTs can be found as Single Walled Carbon Nanotubes (SWCNTs) or Multi-Walled Carbon Nanotubes (MWCNTs). SWCNTs are constructed of a single sheet of carbon atoms arranged in a honeycomb or hexagonal lattice (as graphene) and then rolled to form a single seamless tubular object bonded together through triple carbon bonding. SWCNTs are typically only a few nanometers in diameter. MWCNTs employ the same structure as SWCNTs but instead consist of multiple concentric rolled graphene sheets. Carbon nanotubes can be naturally created through various forms of combustion as well as multiple forms of laboratory synthesis. Naturally occurring CNTs lack purity and consistent dimensions, thus the preferred methods for modern production consist mainly of the carbon arc discharge method, laser ablation, and chemical vapor deposition (Nanowerk 2008).

Currently the most widely used method of CNT production is the arc discharge method, where multiple fullerenes can be produced rather simply through arc vaporization of two carbon rods in an enclosure typically filled with an inert gas at a low pressure (Nanowerk 2008). This method produces the necessary product, although hidden in a mixture of soot and other nanoparticles. Cleansing and purification of the CNTs must take place to ensure quality and consistency.

The laser ablation method for producing CNTs consists of a dual pulsed laser set to vaporize a graphite rod with a 50:50 catalyst mixture of Cobalt and Nickel at 1200 degrees Celsius in flowing Argon. Following this pulse is a heat treatment of 1000 degrees Celsius taking place in a vacuum, to remove unwanted fullerenes, and is followed by a second pulse of the laser to increase the uniformity of the vaporization of the target (Nanowerk 2008).

Chemical vapor deposition has been used to create a number of carbon fibre products for a number of years. However, the creation of carbon nanotubes with this method seems to be less frequently used than the aforementioned processes and is thus only used in special cases when required.

Currently carbon nanotubes have many uses that span from the strengthening of tennis racquets and bicycle frames to use in computer microchips through to soil and groundwater remediation. However, the multiple uses and applications offered by

nanotubes suggests that the health, safety, and environmental uncertainties should be better understood, assessed, and if necessary, controlled.

### Hypothesis

Currently the use of carbon nanotubes in commercial products goes unquestioned by many. New legislation has yet to be created to govern the use of CNTs on the grounds that the use of carbon has already been categorized with existing legislation and thus its use on the nano-scale should fall under the already created regulations. However, carbon on the nano-scale, such as carbon nanotubes, carbon nanowires, and other fullerenes possess significantly different properties when compared to carbon on the micro, and macro scales.

In cases of groundwater and brownfield remediation where carbon nanotubes are used for their sorptive properties to neutralize or bond to contaminants, the nanomaterials are being pumped directly into the contaminate plume and are allowed to interact with the surrounding environment. This interaction between CNTs and the environment has so far remained virtually untested in a non-laboratory scenario and it has yet to be determined how this method of remediation will affect the ecology of the region, and specifically the flora and fauna in the treated region. It is hypothesized that carbon nanotubes, because of their extremely small size and unique properties, will have a detrimental effect on flora in a region treated with CNTs for remedial purposes. This research project examines the effects carbon nanotubes have on plants throughout their development cycle. The testing of this hypothesis will take place through an in depth evaluation of the effects of SWCNT

on the growth and development of pumpkin plants (*Cucurbita maxima*) and the overall well being of these developing plants.

## CHAPTER II

### REVIEW OF LITERATURE

The research published on the topic of the environmental interaction of carbon nanotubes, and nanomaterials in general, is very limited. Nanomaterials are a relatively new discovery in science and only recently have researchers begun to thoroughly investigate their properties and effects on both humans and the environment, and the applications of this new class of materials. The nanomaterial works published most relevant to this experiment start with laboratory health and safety guidelines, which were to be used in labs manufacturing and handling all nanomaterials. With a lack of intimate knowledge of each specific nanomaterial, general procedures were developed for handling and disposing of anything that is on the nano-scale. As more research occurred, this general set slowly developed into more detailed documents for the more common nanomaterials. Each document that has been developed to guide in the safe handling and disposal of manufactured nanomaterials has been published relatively recently, and because of that it is difficult to determine which document is the most 'correct'. For use with this experiment, the document published in 2007 by the British Standards Institution (BSI) will be used (part 2, guide to safe handling and disposal of manufactured nanomaterials). Canada has not yet put forth any guidelines on the subject of nanotechnology. The documents released from the United States National Nanotechnology Initiative (NNI) have been roughly equivalent if not less detailed when compared to the material published by the BSI.

## Toxicity

To date the research conducted on carbon nanotubes has been focused mainly on human interaction with only a few experiments dedicated to interaction with plants because of the priority placed on human health.

### Pulmonary Toxicity

Early studies conducted on rats showed that intratrachelly instilled SWCNTs produced transient inflammation, cell injury effects, and a subsequent dose dependent series of multifocal granulomas (Warheit et al. 2004). In 2005 Muller et al. compared the pulmonary toxicity of ground and unground MWCNTs in rats, using carbon black and asbestos (*Rhodesian chrysotile*) as references. Ground nanotubes were reduced in length from 5.9 to 0.7 $\mu\text{m}$  and provided much better dispersion in lung tissue. After 60 days there were indications of a higher degree of pulmonary inflammation (Muller et al. 2005). The authors therefore concluded from this research that the results point to a specific toxicity related to the unique properties of CNTs (Muller et al. 2005). Furthermore, the results of a pilot study released on 20 May 2008 concluded that long straight carbon nanotubes may be as dangerous as asbestos fibres, potentially causing cancer in cells lining the lung (Van Noordan 2008). In this pilot study, MWCNTs and asbestos fibres were injected between the membranes lining the lungs and abdominal organs in mice. A comparison revealed that long straight nanotubes caused inflammation and lesions in membrane cells of the sort that have been shown to lead to cancer (Van Noordan 2008). From the tests researchers found that CNTs under twenty micrometers and tangled CNTs did not cause the 'asbestos-like' health issues.



### Dermal Toxicity

The handling of raw CNTs in a laboratory setting has raised early questions regarding the dermal toxicity of the material; however early testing has shown that dermal toxicity is on a scale that can be easily dealt with. A 2001 study was conducted to determine the risks of direct contact with CNTs and various fullerenes. The study allowed forty volunteers to participate in a patch test, which would use a soot containing CNTs. From the results of the experiment the authors concluded that no significant risks are associated with short-term direct contact (Huczko and Lange 2001). However, as more and more risks are becoming associated with fullerene contact researchers are now beginning to question tests previously conducted. More research is needed in this area to allow for any final conclusions regarding dermal toxicity.

### Translocation

The current literature dealing with the translocation of nanomaterials presents some contradictory findings. Each individual material carries with it unique structural, chemical, and electrical properties that affect the translocation of that particular material; only the relevant studies documenting the translocation of carbon nanotubes are reviewed.

A 2004 study conducted by Wang et al. showed that functionalized hydroxylated SWCNTs with radioactive iodine-125 atoms behaved very closely to small molecules when injected into mice. These nanotubes were said to have easily passed through a number of compartments and were seen to accumulate in bone while being distributed

throughout the entire body of the mouse with exception of the brain (Wang et al. 2004). After a period of eleven days, eighty percent of the total dosage injected was excreted by urine and feces (Wange et al. 2004). However, a study conducted two years later by Singh et al. (2006) found no accumulation of functionalized chelated diethylenetriaminepentaacetic and indium-111-labeled SWCNTs or MWCNTs in mice after 24hr. From these two examples it can be said that the largest difference is the functionalization of the CNTs. Functionalization of CNTs is a form of surface modification that adds specific chemical functional groups into the molecular structure of the CNTs. The addition of these functional groups allows the surface of the CNTs to undergo a more specific and predictable chemical reaction. The functionalized nanotubes were seen to be removed from the blood through renal excretion, with no toxic side effects or mortalities (Singh et al. 2006).

From the evidence presented to the research community regarding the translocation of CNTs obtained by *in vivo* studies, researchers are now investigating the potential for a CNT 'medical drug delivery system'. A May 2008 study published online demonstrates the potential use of a small molecule delivery system using 'carbon nanotube test tubes' as carriers. Researchers essentially created nano-scale test tubes using a CNT with one end capped, and one end left open. A polystyrene polymer trapping a coloured dye inside sealed the open mouths of the 'test tubes' (Ittisanronnachai et al. 2008). When placed in the correct solvents the polymer would be dissolved thus allowing the dye to escape the tube (Ittisanronnachai et al. 2008). With this method of delivery, medicines can be delivered directly and more efficiently to the source. However, the toxicity of the

nanotubes used and functionalized coatings will ultimately be the deciding factor when the body processes this new system.

### Environmental Interaction

The studies published surrounding CNT interaction have focused mainly on mammalian interactions. To date only three studies have focused on environmental interactions, and only two of these studies have been conducted with Single-Walled Carbon Nanotubes. However, preliminary studies have concluded CNTs to be bioavailable to a variety of organisms in addition to being biopersistent: CNTs may have the ability to accumulate up the food chain.

The Journal of Environmental Monitoring (2008) reports the first case of uptake, translocation and accumulation of manufactured iron oxide nanoparticles by plant life (Zhu et al. 2008). Using pumpkin plants (*Cucurbita maxima*), grown in aqueous media containing magnetite ( $\text{Fe}_3\text{O}_4$ ) nanoparticles, the research team verified uptake, translocation and accumulation of the particles by detection of the magnetization signals produced by the particles. Strong magnetic signals were present in all leaf tissue tested while the signals weaken throughout the stem except where the stem connects to the roots (Zhu et al. 2008). The distribution of signal strength shows that the particles have been taken up through the roots, translocated through the stem and deposited in the leaves. Preliminary testing originally began growing both pumpkin plants and lima bean plants hydroponically in the  $\text{Fe}_3\text{O}_4$  particle suspension; however, no magnetic signals were detected from the lima bean plants (Zhu et al. 2008). This shows that certain plants have

the capacity to absorb nanoparticles and translocate them; these actions are not something every plant will do. With the accumulation of particles in leaves it does raise the question of these particles bioaccumulating up the food chain, and thus this may be reason for concern.

A May 2009 study entitled '*Carbon Nanotubes as Molecular Transporters for Walled Plant Cells*' presents the first visual evidence that SWCNTs have the ability to penetrate the cell walls of living plant cells. Using *Nicotiana tabacum L.cv.* cells and oxidized SWCNT's noncovalently labeled with fluorescein isothiocyanate (FITC) researchers were able to show, through the use of fluorescent microscopes, the bundles of SWCNTs within the borders of the living cells. This experiment shows in the correct conditions, certain plant cells will uptake the CNTs with out cell death occurring.

Also in the fall of 2009 an experiment took place at the University of Arkansas to describe the effect of penetration of plant seed coats by carbon nanotubes. This study was the first that would allow CNTs to engage plant seeds during germination. Researchers placed sterile tomato seeds (*cv. Micro-Tom*) on a standard agar medium supplemented with varying concentrations of multi-walled CNTs. The addition of MWCNTs to the agar medium was found to accelerate the process of seed germination and significantly shortened the germination time. The germination percentage for seeds that were placed on regular medium averaged 32% in 12 days and 71% in 20 days, while germination percentage of the seeds placed on medium supplemented with MWCNTs averaged 74–82% in 12 days and 90% in 20 days.

In addition to shorter germination periods, the fresh weight of total biomass of the nanotube-supplemented seeds was on the scale of 2.5 fold over the control group, as well as increased seed coat permeability. Dry seeds measured a moisture level of 18.4%; seeds exposed to MWCNTs measured approx. 57.6%, and seeds unexposed kept only 38.9% of moisture.

The results of these environmental studies show that nanomaterials have the ability penetrate cell walls, as well the seed coats of germinating plants.

In summary, the research to date suggests that once CNTs have been taken up by humans, or other species they may cause inflammation, oxidative stress, cell damage, adverse effects on cell performance, and in the long term, may cause pathological effects such as granulomas, fibrosis and wall thickening as well as more serious problems on a case by case basis. All of the aforementioned negative attributes have been observed through a variety of studies and all appear to be time and dose dependent. It should also be noted that the functionalization of the CNT plays a large role in how the tubes interact with the body, cells and the environment. However, when considering the interactions between CNTs and plants cells or developing plants the outcomes are less certain and may be counterintuitive. Interactions between plants seem to show, at least in early stages of plant development, a neutral or “no-effect” outcome, or in some species, positive effects on growth and development.

## CHAPTER III

### METHODOLOGY AND APPROACH

The experiments will demonstrate how developing plants interact with carbon nanotubes, not on a cellular level, but on a level that should be visible in any natural environment. Specific measurements taken in regular intervals will quantify any noticeable increase or decrease in growth rates, water consumption, and the general quality of the plants aqueous medium. By observing a plant in a favorable and controlled environment, data can be collected that will demonstrate the plants' rates of development. When a new solution is added, the new data can be compared to determine whether that additional solution enhances or hinders plant development. In these experiments, the initial aqueous medium used is a basic solution of Milli-Q water and growth nutrients. After the plants become acclimated to this initial solution, the SWCNTs solution is added.

The plants grown in this experiment are done so in three cycles to provide repeatability with both a '*Control Group*' and an '*Experimental Group*' found in each cycle. After a period of monitoring the plants growth, water consumption and medium quality, the CNTs are added into the each of the *Experimental Groups*, and the same observations continue to be recorded. The measurements remain constant throughout each cycle so that they can be compared to assess any changes the plant may have undergone after the addition of the nanotubes.

Each cycle contains twelve plants: six in the '*Control Group*' and six in the '*Experimental Group*'. Along with the two groups of plants there are universal controls

that are monitored to provide baseline readings throughout each cycle. These universal controls consist of a gauge that measures the evaporation of the plants aqueous medium, as well as a container that holds the pure medium where no plant is grown. This medium-only control provides the baseline reading of the medium quality, which will show if the aqueous medium containing the plants is of better or worse quality. The quality of the medium is determined by '*Total Organic Carbon*' (TOC) readings. TOC readings are most widely used to determine the purity of drinking water through the detection of carbon, and thus will be used to monitor the amount of carbon suspended in the plant medium. The aqueous medium used for each plant consists of a mixture of Milli-Q water and specific plant growth nutrients.

Each cycle runs for twenty-one days and is broken into two phases. The first phase lasts fourteen days in which both groups of plants grow in only the aqueous medium of Milli-Q water and nutrients. The second phase begins with the addition of the SWCNT solution into the '*Experimental Group*' and lasts for seven days. The universal controls undergo observation throughout each cycle's entirety to obtain necessary data used to compare one cycle against the other cycles. Comparing the data and observations from each cycles first phase against the same cycles second phase will then determine the effect the SWCNT solution had on the growth of the plants, as well as the effect on their observed condition and development.

Due to an unforeseen equipment error, experiments using a now removed stage in the methodology and approach had to be halted. This stage was to occur as a precursor to the

experiment that has been described above, and would show the interactions between a single plant cell and the SWCNTs. This stage of experimentation included culturing cells through the use of cell suspensions and allowing these cells to interact with fluorescently labelled SWCNTs. The experiment design details and the progress that was made can be found in Appendix A.

### Expected Results

The results of this experiment may vary considerably. Based on the literature reviewed, the addition of nanomaterials would be expected to impair the development of the plants causing unmistakable changes such as moderate to severe slowed development or even death of the plant after the SWCNT solution has been administered. Conclusive evidence during plant uptake may be obtained through observed alteration of the plant/root physiology or termination of the host plant. The alteration of the plant/root physiology will show that the CNTs are contacting the roots in a manner similar to the plants natural nutrient uptake procedure. However, because of reasons yet to be determined the attempted absorption of the nanomaterial may cause damage to the plant/roots either resulting in termination or impairment of the normal functioning. Through the control plants we can verify normal plant/root physiology to determine the damages that occur from the CNT solution.

However, some of the reviewed literature suggests that the SWCNT solution may boost plant development: this would imply that the SWCNT solution is interacting favorably with the plants. Quickened development can be measured by an increased growth rate of



the plants after the addition of the solution, as well as a higher fresh weight of the plants at the end of the experimental phase. Both of these changes would demonstrate that the SWCNT solution is interacting with the plant/root physiology in a positive manner and allowing the plants to absorb more water/nutrients and thus aiding development.

## CHAPTER IV

### TESTING PROTOCOL

The method used for completion of this experiment will be comprised of three repeated cycles. Each cycle is broken into two phases at which observations throughout must be recorded. Both phases are compared within each cycle and then each cycle will be assessed against the other cycles. Pumpkin (*Cucurbita maxima*) will be grown in the aqueous medium in each of the three cycles. The experimental protocols are presented in this Chapter. Each protocol has been adapted from 'Plant Cell Culture' by D.E Evans, J.O.D Coleman and A. Kearns.

#### Single-Walled Carbon Nanotube Solution

The single-walled carbon nanotubes used in this experiment were created using the catalytic chemical vapor deposition (CCVD) method and acid purified with an outer diameter of 1-2nm, and an inner diameter of 0.8-1.6nm with a length between 5-30um. No functionalization took place. The SWCNT solution used in this experiment is a mixture of 2 drops of Polysorbate 80, or more commonly known as "Tween 80", which is a surface active substance or surfactant, with 0.65 grams of SWCNTs dispersed into 1.35L of nutrient solution already present as each plants aqueous growth medium. The dispersion process used involves the ultrasonic emulsification of the SWCNTs combined with Tween 80 into Milli-Q water, which is then evenly distributed into each of the plants listed in the Experimental group, at the appropriate time.

Ultrasonic emulsification is an essential part of the process as it hastens the dispersion of the SWCNTs using ultrasonic sound waves to break intermolecular forces in the mixture. This in turn allows the SWCNTs to quickly bond to the Tween, increasing the kinetic stability of the emulsion, and disperse them into the water forming a near homogeneous emulsion. Without functionalization of the CNTs it is impossible to create a completely stable emulsion. The ultrasonic emulsification for each batch of SWCNT solution lasts 24 hours to ensure homogeneity and consistency. Once created and dispersed evenly among the plants listed in the *Experimental* group, each of the 6 plants will receive approximately 108mg of SWCNTs, which will be suspended in the aqueous medium. This leaves each plant in a concentration of 0.0815mg/L.

### Cycle Creation

Cycle creation in this experiment refers to the procedure that includes the preparation and germination of seeds for growth in an aqueous medium. Plant growth using hydroponics is beneficial in this experiment due to its ability to eliminate soil interaction and microbial activity that may interfere with or complicate plant growth. An aqueous medium allows the nutrients necessary for proper growth to be readily available as well as easy to manipulate. It is also easier to supplement the nutrient solution with the CNT solution, thus minimizing errors that may occur.

Each cycle started with seed preparation and seedling growth. When the seedlings reach the appropriate age (between 2-3 weeks depending on development rates of the plant) the SWCNT solution is added to the nutrient medium. After adequate time has elapsed for

uptake to occur, plants will then be removed from the solutions and evaluated in a final record. A control set will show pure growth with only the effect of the nutrient solution. The test set will be grown in a nutrient solution and supplemented with a SWCNT solution to be added after the first 14 days in the system has taken place.

### Cycle Creation Materials

To follow the experimental protocol, each cycle requires the following materials and apparatus. Because there are three cycles, the following lists are essentially “repeated” three times in succession. These include:

- Conical flask (250ml), sealed with aluminum foil
- Petri Dish
- Tea strainer, or muslin squares, autoclaved in foil packets
- Sterile containers for sterilizing plant material
- Forceps
- 2x ‘Perfect Starts’ #4 block
- 2x 10”x20” flat
- 2x 72 cell Perfect Starts inserts
- 2x 12”x20” Hydrofoam Heat Mat
- 2x 6” Humidity Dome
- 1 Gallon Liquid Earth ‘Vigor’ Formula
- 1 Gallon Liquid Earth ‘Grow’ Formula
- Hydroponic System

The hydroponic system used for growing a single plant consists of:

- 1.5L wide mouthed mason jar, painted black (a small, lightly painted strip is left vertically to allow visibility of the water level when a light is projected from behind)
- 4” Diameter black plastic basket
- Clay pellets
- Aquatic air piping (Length varies with distance from air supply)
- Elite 802 Aquatic Air Supply (One 802 supply will feed 6 jars)
- UV light replicating the spectrum of early summer sunlight (1 light feeds 12 plants)
- Power bar, and light timer

These elements are arranged into a full working system capable of supporting 24 plants. As the plants develop and their root systems expand, they can outgrow the capacity of the system. However, given the limited growing time anticipated for the experiment, the system should be more than capable of supporting the range of necessary plant growth. Figures 4.1 and 4.2 display the complete system used in this experiment.



Figure 4.1 – Jars and Air Supply Tubes Used in the Hydroponic System



Figure 4.2 – Full System Used

In Figure 4.2 the blue aquatic bubblers are located along the right hand side. The jars, baskets and air supply piping are also clearly visible. Figure 4.3 shows plants growing in this system.



Figure 4.3- Growth in the Hydroponic System (Cycle 1)

This system, once assembled, remains in operation throughout the 3 cycles. Between each cycle the jars are thoroughly washed and each part of the system is checked to ensure its correct operation before the next cycle is begun. During the length of the cycles the UV lights are set for a total of 16 hours on and 8 hours off to ensure that each plant is receiving an exposure to simulated sunlight to develop properly.

### Cycle Creation Protocol

The cycle creation consists of germinating the seedlings in a controlled environment to grow in an aqueous medium. Growth mediums will be commercially purchased to ensure controlled, uniform growth among the plants, and the experimental plant group will then be supplemented with a SWCNT solution. The following procedure will prepare seeds for growth through the aid of root cubes.

#### **Protocol – Germination of Seedlings Using Root Cubes** (Evans et al. 2003; Roberto 2003)

1. Place selected seeds in a tea strainer, make a muslin bag to hold them and place directly into a 250ml conical flask
2. Submerge the seeds in absolute alcohol for a few seconds (3-5s). Pipette off alcohol and discard.
3. Immerse the seeds in hypochlorite with a few drops of surfactant (ie. Tween 80) for 15-20 minutes (the duration of the immersion can be varied – less for more sensitive seeds and longer if infection is a problem.)
4. Rinse the seeds 3x5 minutes with 250ml of sterile distilled water. If seeds float to the surface, either pipette off liquid or try a low-speed centrifugation step at 1000g for 5 minutes.
5. Pre-moisten ‘Perfect Starts #4’ growing cubes with a ½ strength nutrient solution, pH at 6.0. Place seeds into starting hole.
6. Place ‘Perfect Starts’ into incubation tray, and place incubation tray atop heat mat set to 22-25 degrees C.



7. Maintain and air temperature between 21-26 degrees C, and humidity of 70-90%, and soft lighting (20W/ft<sup>2</sup>) until seedlings have sprouted, then increase. Continue feeding at ½ strength until light level has been increased.
8. Allow seedlings to grow until the roots have outgrown the ‘Perfect Starts’ cube and have extended beyond the cube allowing easy uptake from an aqueous medium.
9. Transplant to hydroponic system.

Once the seedlings have outgrown the root cubes, they are transplanted to the hydroponic system. The nutrient solutions used are commercially purchased, which makes growth of plants consistent as the formulas have been tested and are known to provide positive results.

Plants will grow in the hydroponic system will take place for approximately twenty-one days. A control set will be grown in the nutrient solution alone, with no SWCNT supplement, while the second experimental set is supplemented with a SWCNT solution on day 14 of the cycle experiment. The experimental set will therefore grow for seven days in the SWCNT solution.

Following the completion of the growth period (twenty-one days from transplantation) the plants will be removed from the aqueous medium and their roots examined through microscopy and comparison to the control set grown in the nutrient solution alone. In addition, upon completion of the development cycles each plant will be photographed

and a fresh weight shall be recorded. After the completion of any one cycle, the data can then be compared between groups to determine if any changes become evident based on the addition of the SWCNT solution.

## CHAPTER V

### RESULTS AND ANALYSIS

#### *Results and Analysis of Plant Growth Data*

The data collected regarding plant growth has been analyzed and the results have been organized into the following Tables 5.1 and 5.2.

Table 5.1 begins by showing the rate of growth of each plant throughout the entire cycle, which is represented as column 1, '*Overall Growth Rate*'. The following column 2 displays the rate of growth for each plant prior to the addition of SWCNTs solution, which is shown as "*Daily Growth Rate Before CNTs*". Also displayed is column 3 for the '*Growth Rate After CNTs*' and finally the remaining column 4 represents the difference between the rates of growth recorded for before the addition of CNTs and after the addition of CNTs. This column is presented as the "*Difference in Growth Rates (Before & After)*" and measures the change in growth rates within a group, in the data sets labeled "*Control*" and "*Experimental*" for all cycles (it should be noted that the *Control* groups in each cycle were never given the SWCNT solution, yet the columns use that event as a temporal marker).

The values listed under the *Control* and *Experimental* sections are compared in the third section of data listed as "*Percent Difference (%)*". The percent difference shows that during the first 14 days where all plants (in both the *Control* and *Experimental* groups) grew only in the Milli-Q water and nutrient solution, the growth rates are relatively close. These data are shown in the first two data sections (*Control* and *Experimental*), in the first

two columns. However, when the SWCNT solution is added to the *Experimental* groups only, the plant growth data shows that these groups begin to grow at an accelerated rate. This increase is shown in the comparisons between the data in the third column, and first two data sections. From this increase in growth rate, the SWCNT solution appears to play a positive role in the plants development in the tested time frame.

When making comparisons within the *Control* group, and within the *Experimental* group, the column labeled “*Difference in Growth Rates (Before & After)*” should be noted. This column depicts the average change within a group, and within each cycle. This column is the difference between the growth rates before the SWCNTs solution is added and after the SWCNT solution is added, within a group, in each cycle. When looking at this column under the ‘*Percent Difference*’ data section the values show the percent difference between the changes in the growth rates between the *Control* group and the *Experimental* group within a given cycle. The numbers in this “*Difference in Growth Rates (Before & After)*” column in the *Percent Difference* data section show rather large differences in the changes of growth rates between groups when the SWCNT temporal event occurs. These results demonstrate that the plants in the *Experimental* groups underwent an increase in their growth rates after the addition of the SWCNT solution. The *Control* groups did not receive the SWCNT solution and their change in growth at this point in time is relatively small. The percent difference in change is clearly noticeable.

Table 5.2 shows the percent difference between the average fresh weights recorded in each cycle. After the completion of each cycle the plants were removed and weighed to

produce a fresh weight, then the fresh weights in each group were compared between the *Control* and *Experimental*.

<b>Plant Growth Summary</b>				
<i>Cycle</i>	<i>Daily Growth Rate Overall</i>	<i>Daily Growth rate Before CNTs</i>	<i>Daily Growth Rate After CNTs</i>	<i>Difference In Growth Rates (Before&amp;After)</i>
<b>Control</b>				
<i>Cycle 1</i>	2.556	2.281	3.104	0.823
<i>Cycle 2</i>	2.549	2.385	2.875	0.490
<i>Cycle 3</i>	1.308	0.944	8.750	7.806
<b>Experimental</b>				
<i>Cycle 1</i>	2.716	2.011	4.125	2.114
<i>Cycle 2</i>	2.514	2.229	3.083	0.854
<i>Cycle 3</i>	1.712	1.120	11.333	10.213
<b>Percent Difference</b>				
<i>Cycle 1</i>	6.071	12.601	28.242	87.927
<i>Cycle 2</i>	1.372	6.772	6.993	54.264
<i>Cycle 3</i>	26.752	17.040	25.726	26.721
<b>Students Two Tail T-Test</b>				
<i>Cycle 1</i>	0.768	0.587	0.277	0.172
<i>Cycle 2</i>	0.937	0.794	0.750	0.691
<i>Cycle 3</i>	0.151	0.388	0.133	0.118

Table 5.1 – Plant Growth Summary

	<b>Cycle 1</b>	<b>Cycle 2</b>	<b>Cycle 3</b>	<b>Average % Diff</b>
<i>Fresh Weight % Difference</i>	13.33	30.81	8.19	17.44

Table 5.2 - Percent Difference of Fresh Weights Between Groups

Table 5.2 shows that in all three cycles the fresh weights of the *Experimental* groups outweigh the *Control* groups on an average percent difference of 17.4%. This weight difference is in keeping with the data presented displaying growth rates, particularly the accelerated growth of the plants in the *Experimental* groups. Examining both the fresh weight comparisons and the accelerated growth rates discovered in each cycle, it appears that the addition of a SWCNT solution is increasing plant growth.

The final section of data in Table 5.1 is labeled “*Students Two Tail T-Test*” which aims to provide statistical significance to the data collected through statistical methods. Constructing a ‘*Students Two Tail T-Test*’ using the collected data from growth measurements recorded on 48-hour intervals begins with the calculation of standard deviations, presented below in Table 5.3.

<b>STD Deviation Summary</b>			
	<i>Daily Growth rate Before CNTs</i>	<i>Daily Growth Rate After CNTs</i>	<i>Difference In Growth Rates (Before&amp;After)</i>
<i>Control</i>			
Cycle 1	0.854	1.152	0.862
Cycle 2	0.890	1.512	1.746
Cycle 3	0.370	3.101	2.756
<i>Experimental</i>			
Cycle 1	0.818	1.842	1.624
Cycle 2	1.118	0.385	1.304
Cycle 3	0.301	2.314	2.076

Table 5.3 – Standard Deviation Summary

It can be seen that a standard deviation for the *Overall Growth Rate* column has not been calculated because this category encompasses both phases of growth, before and after the SWCNT solution is added. This will greatly affect the standard deviation, as well as the rest of the statistical models. The *Experimental* group undergoes a period of accelerated growth, and thus the data cannot be compared between both groups, as the results produced become skewed. Once standard deviation is calculated the variances for each data set is found.

The variances are compared, and for the samples used in calculation, the variances can be taken as equal through the use of a standard F-Test. The F-Test conducted takes into account the variances and produces a result that allows them to be regarded as equivalent

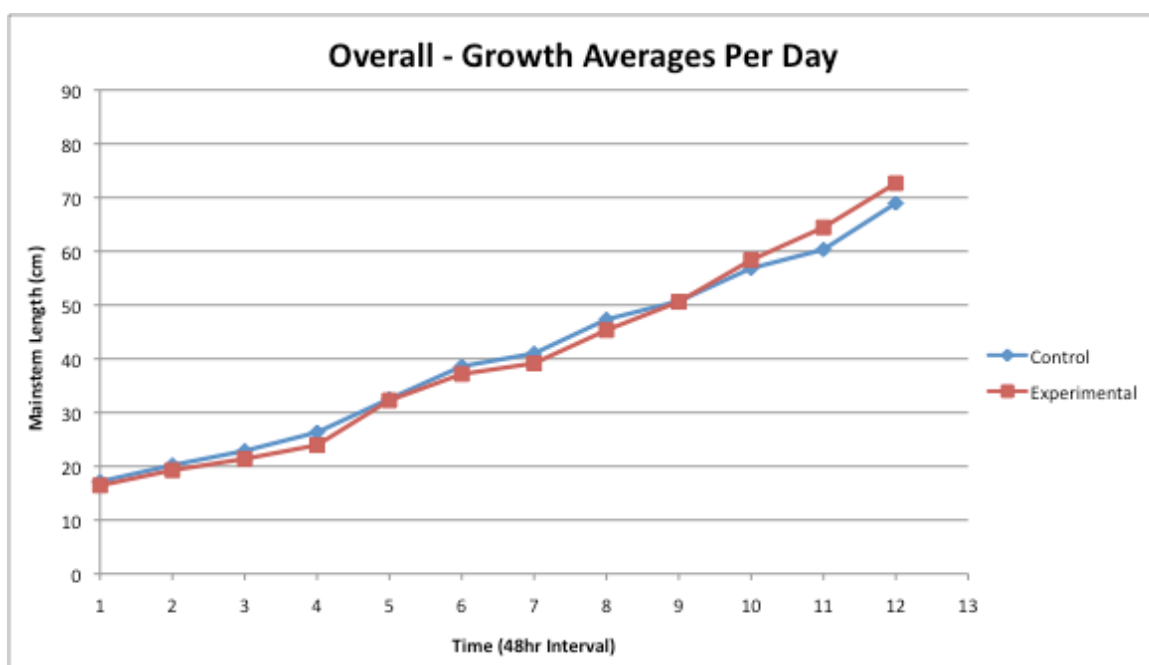
or not. In the case for our samples the F-tests return the result of equivalence. Once the F-Tests have been completed, the parameters to be used in our Students T-Tests have been defined. In the sample data used it can be seen that we shall conduct a two-tailed T-Test assuming equal variances.

With the data tested using the correct parameters for a two tailed students t-test with equal population size and equal variances the results given above show that this data is not statistically significant. Results that are statistically significant are unlikely to have occurred by chance. Because these outcomes are statistically insignificant, then the observed differences in plant outcomes may have occurred by chance. However, the data collected and compared in this experiment still demonstrate biological significance. Statistical significance takes into account only the mathematical relationship between data sets, but not the biological aspects of the experiment.

Biological significance is not something that can be calculated mathematically, however, it can be taken that the accelerated growth rates and increased fresh weights of a single group of plants are significant. Not only did this occur in a single trial; it was observed in all three trials. Although the degrees of growth acceleration and increases in weight vary, the significance arises because the same increased growth trend occurred for all three groups to measurable amounts.

### Graphical Results

The summary provided in Tables 5.1 and 5.2 only presents the final results of the plant growth. Graph 5.1 presents the plotted daily average growth of both the *Control* and *Experimental* groups. However, the difference between the two groups appearing on Table 5.1 may seem small, it is must be noted that the important values are the change in growth rates of each individual plant within the groups. A plot of the average growth per day eliminates the ability to present these values; as such the growth of each plant within the two groups is also presented below.



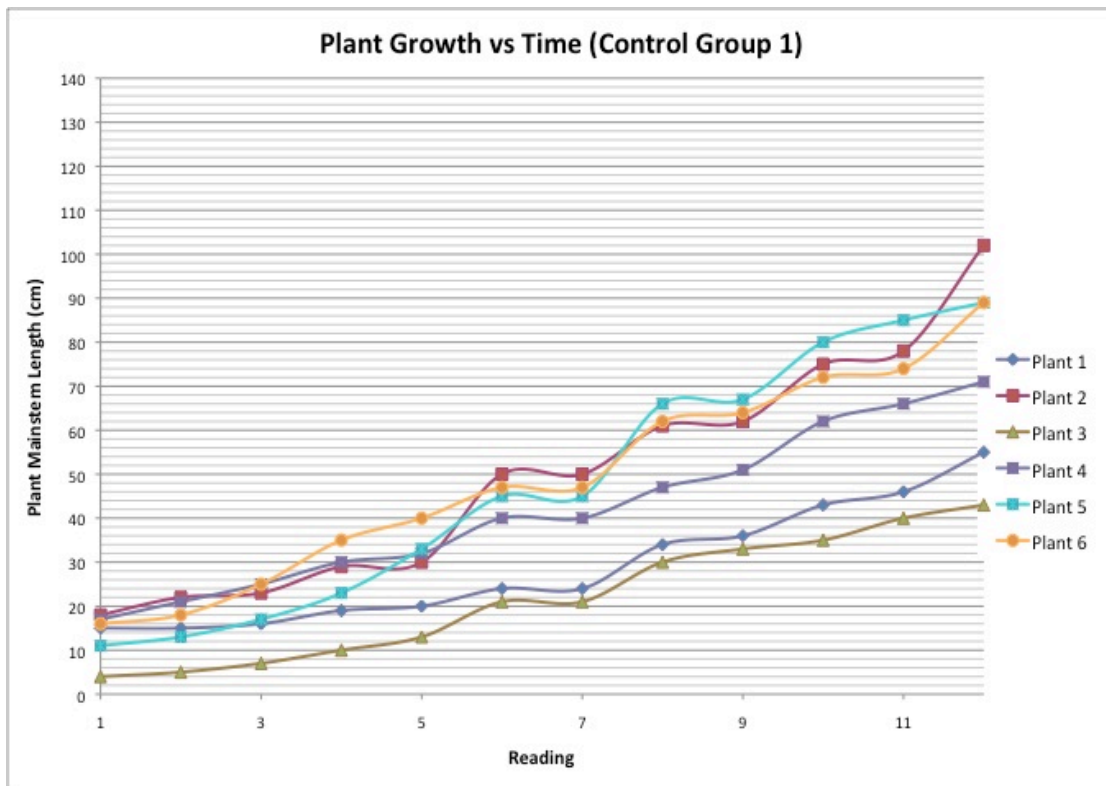
Graph 5.1 – Average Growth (*Control* and *Experimental*)

The following graphs show the growth pattern of each of the plants tested over time and present complete growth trends. All graphs possess the same layout, which contains the *Mainstem Length (cm)* on the vertical axis and the *Time (48hr Interval)* on the horizontal axis. Each of the coloured lines visible on the graph moving from left to right represent

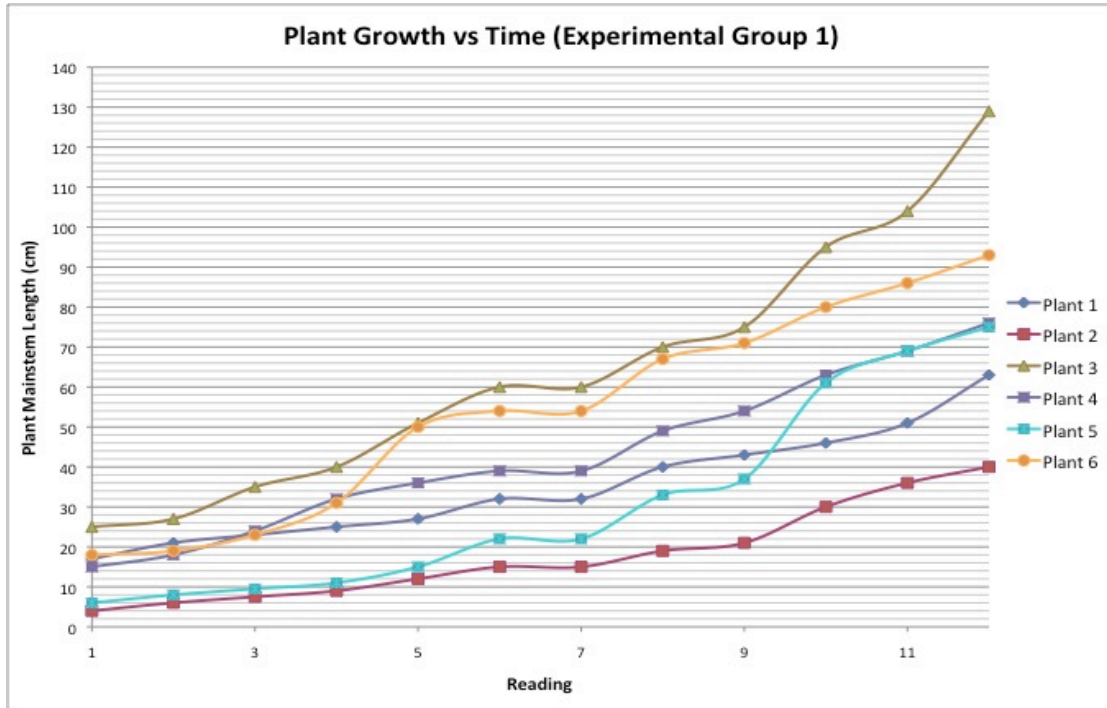


the growth of a plant. In order to record the data presented on the graphs below, measurements had to be taken on 48-hour intervals, labeled as ‘*Reading*’. Because the chosen plants are vines, the mainstem lengths were recorded and used as the primary marker for plant growth.

The following graphs depict the growth of each plant in both the *Control* group and *Experimental* group in Cycle 1.

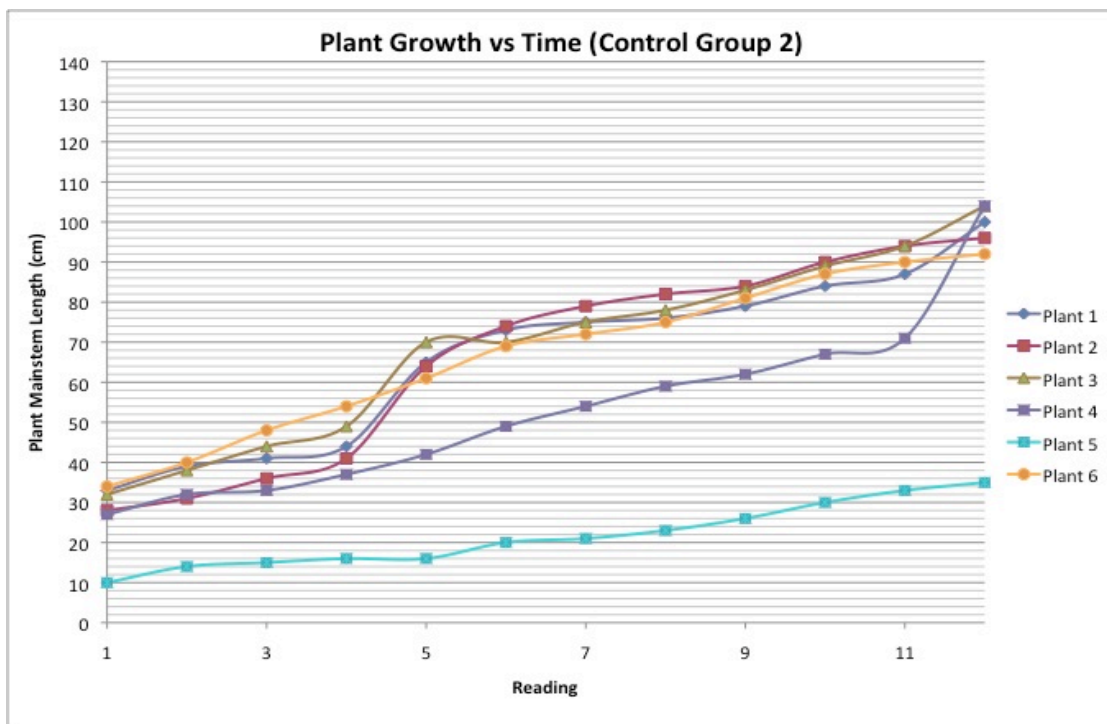


Graph 5.2 – Plant Growth Vs Time (Control Group) Cycle 1

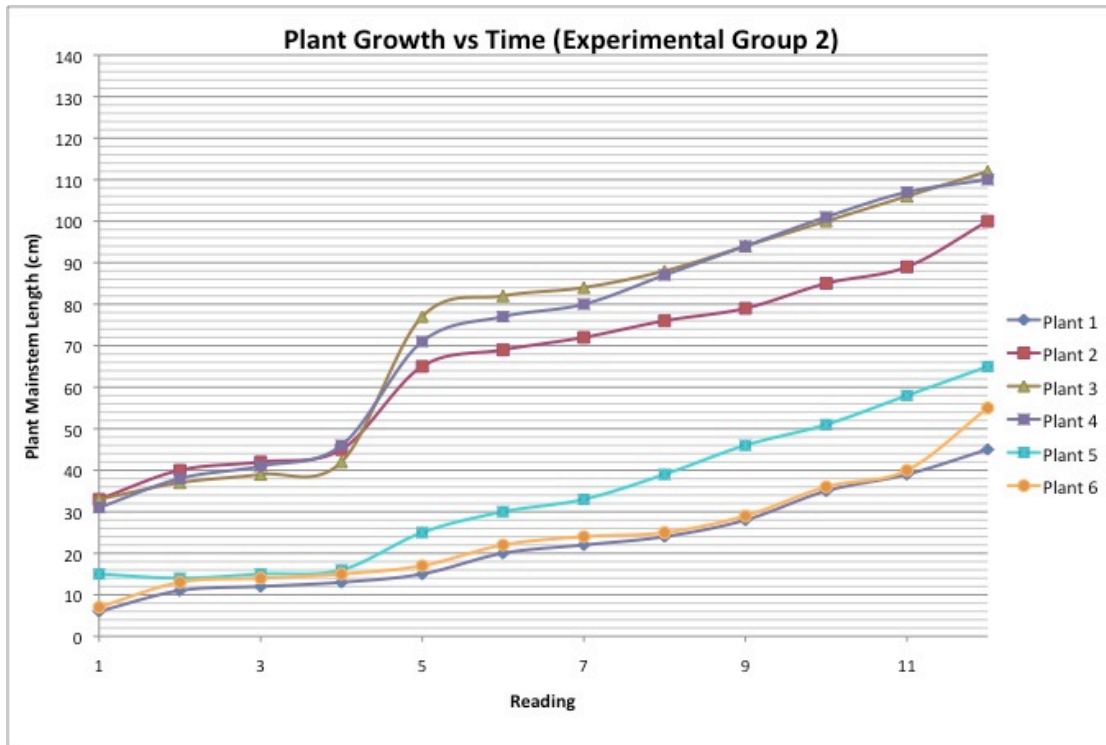


Graph 5.3 – Plant Growth Vs Time (Experimental Group) Cycle 2

The following graphs depict the growth of each plant in both the *Control* group and *Experimental* group in Cycle 2.

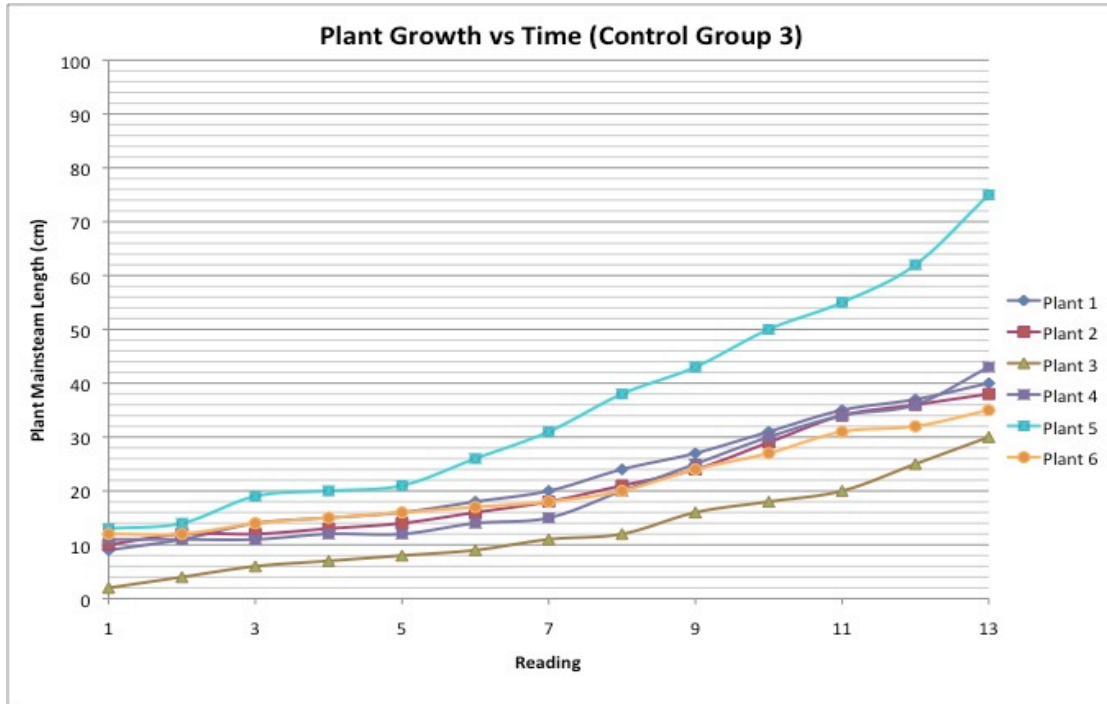


Graph 5.4 – Plant Growth Vs Time (Control Group) Cycle 2

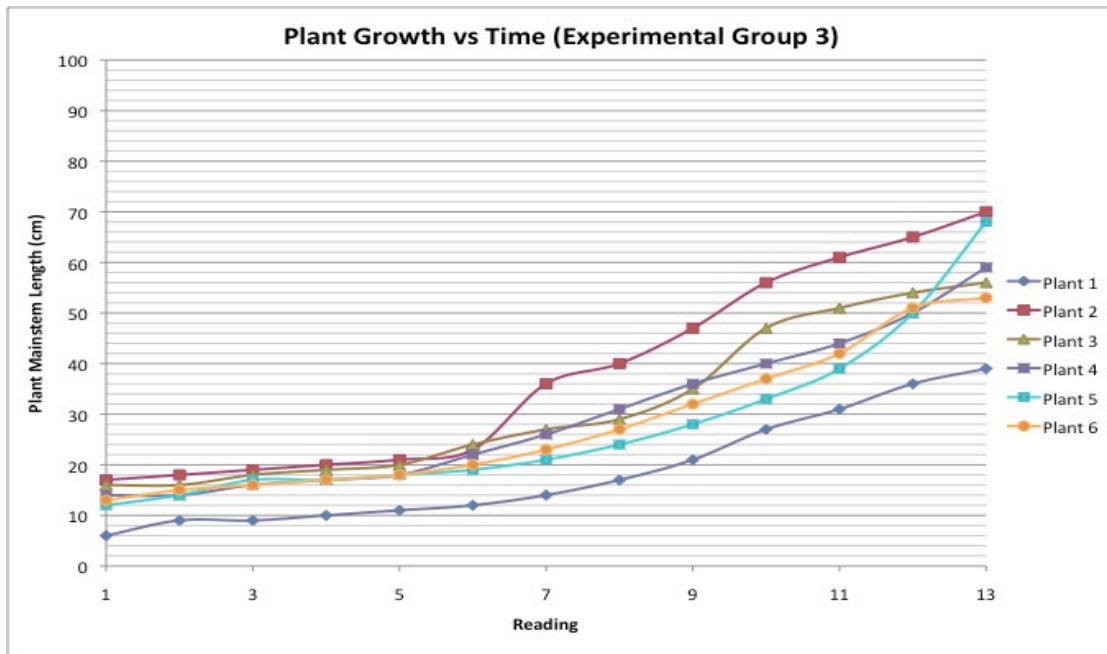


Graph 5.5 – Plant Growth Vs Time (Experimental Group) Cycle 2

The following graphs depict the growth of each plant in both the *Control* group and the *Experimental* group in Cycle 3.



Graph 5.6– Plant Growth Vs Time (Control Group) Cycle 3



Graph 5.7 – Plant Growth Vs Time (Experimental Group) Cycle 3

From the graphs representing daily plant growth presented above the trend is clearly visible. These plotted points do not play as important of a role as the change between

individual plants. However, they do display that the *Experimental* groups produce, on average, larger plants.

#### Results and Analysis of Water Uptake Data

The data collected regarding water uptake has been analyzed and the results have been organized into Table 5.4. This Table shows the rate of growth of each plant throughout the entire cycle, which is represented as column 1, '*Overall Ave. Water Depth*'. The following column 2 displays the calculated average water depth for each plant prior to the addition of SWCNTs solution, which is shown as "*Water Depth Ave. Before CNTs*". Column 3 is the '*Water Depth Ave. After CNTs*' and finally column 4 represents the difference between the average water depths recorded for before the addition of CNTs and after the addition of CNTs. This column is presented as the "*Difference in Depth (Before & After)*" and measures the change in the water depths averages within a group, in the data sets labeled "*Control*" and "*Experimental*" for all cycles. It should be noted that the *Control* groups in each cycle were never given SWCNTs, yet the columns use that event as a temporal marker.

The values listed under the *Control* and *Experimental* sections are compared in the third section of data listed as "*Percent Difference (%)*". The percent difference shows that during the first 14 days where all plants (in both the *Control* and *Experimental* groups) grow in only the Milli-Q water and nutrient solution, the growth rates are relatively close. This data is shown in the first two data sections (*Control* and *Experimental*), in the first two columns.

The data discussed in the previous section shows that these groups begin to grow at an accelerated rate, with this increased growth rate Table 5.4 shows the amount of water taken up by the plants also increases. With this accelerated rate we can see that the change of water uptake in the *Experimental* groups is far larger than that in the *Control* groups. During the growth of the plants the key data regarding water uptake is the change in uptake from before the addition of SWCNT solution to after the addition of the SWCNT solution. This is the change between column 2 and column 3, with the results shown in column 4 on Table 5.4. This data eliminates the variable size of the plants, which can affect the results. For example, larger plants will naturally uptake more water, which changes the calculated average water uptake. If we look at the changes between the addition of the SWCNT solution it compares data within the group and leaves only a picture of whether the new solution increases uptake, decreases uptake, or if it remained the same.

When analyzing the data it becomes clear that the addition of the SWCNT solution causes the plants to accelerate in growth, and uptake larger amounts of water.

<b>Water Usage Summary</b>				
<i>Cycle</i>	<i>Water Depth Ave. Overall</i>	<i>Water Depth Ave. Before CNTs</i>	<i>Water Depth Ave. After CNTs</i>	<i>Differences In Depth (Before&amp;After)</i>
<b>Control</b>				
<i>Cycle 1</i>	6.265	6.575	5.646	0.929
<i>Cycle 2</i>	6.563	6.760	6.167	0.594
<i>Cycle 3</i>	6.622	6.787	6.250	0.537
<b>Experimental</b>				
<i>Cycle 1</i>	6.167	6.250	6.000	0.250
<i>Cycle 2</i>	6.563	7.115	5.458	1.656
<i>Cycle 3</i>	6.571	6.889	5.854	1.035
<b>Percent Difference</b>				
<i>Cycle 1</i>	0.588	2.243	3.377	30.676
<i>Cycle 2</i>	0.000	5.105	12.186	94.444
<i>Cycle 3</i>	0.777	1.490	6.540	63.328
<b>Students Two Tail T-Test</b>				
<i>Cycle 1</i>	0.897	0.683	0.550	0.437
<i>Cycle 2</i>	1.000	0.485	0.027	0.060
<i>Cycle 3</i>	0.520	0.227	0.066	0.043

Table 5.4 – Water Usage Summary

The standard deviation for the *Water Depth Ave. Overall* category encompasses both phases of growth, before and after the SWCNT solution is added. This will greatly affect the standard deviation, as well as the rest of the statistical models. It was previously seen that the *Experimental* group undergoes a period of accelerated growth, which is accompanied by increased water uptake, and thus the data cannot be compared between both groups because the results produced become skewed. Once standard deviation is calculated, the variances for each data set are found.

The variances are compared, and for the samples used in calculation, it has been concluded that the variances can be taken as equal through the use of a standard F-Test. The F-Test conducted takes into account the variances and produces a result that allows

them to be regarded as equivalent or not. In the case for our samples the F-tests return the result of equivalence. Once the F-Tests have been completed, the parameters to be used in our Students T-Tests have been defined. In the sample data used it can be seen that we shall conduct a two-tailed T-Test assuming equal variances.

With the data tested using the correct parameters for a two tailed students t-test with equal population size and equal variances the results given above show that this data is not statistically significant. Results that are statistically significant are unlikely to have occurred by chance. However, some comparisons in this section do return results that are statistically significant, and some that are very close to being significant. Data presented in Cycle 2, under the column '*Water Depth Ave. After CNTs*' and Cycle 3 comparing the '*Difference In Depths (Before & After)*', return significant results: the differences are not due to chance alone. However, this was not the case for the entire comparison set, thus must be discounted. It does suggest that if more then 3 cycles had been completed the chances of more of the comparisons returning significant results increases.

However, as with the observed growth between the plants, the data collected and compared in this experiment show biological significance. The increased water uptake observed in all three trials. Although the degrees of water uptake vary, the significance arises from the fact that the same uptake trend occurred for all three groups to measurable amounts.



### Extended Statistical Analysis

The previous statistical analyses using the Students t-Tests would only test if there were significant differences between the *Control* and *Experimental* groups within a cycle. However, the division into cycles, which were a practical, experimental necessity, could create uncertainty in that the cycles themselves could introduce an unknown variable. An analysis of variance (ANOVA) can determine if such variables have had statistically significant effects. In regards to this experiment, an ANOVA test will determine if the number given to plant (Plant 1-6), the cycle the plant was grown in (Cycle 1-3), or the group the plant was assigned to (*Control* or *Experimental*) had any significant effect on the outcome of the experiment. To determine these effects a number of ANOVA tests had to be conducted, the following will describe the ANOVA test which was conducted, the data used in its calculation, and the results.

### Check for Normality

The previous Student t-Tests and analysis assumed normality. To check for normality, both the Kurtosis and Skewness numbers of our data (*Difference in Growth Rates (Before & After)*) have been calculated. These numbers, compared to the critical values verify that our data used can be considered statistically normal. Table 5.5 shows the calculated values.

	<b>Control</b>	<b>Experimental</b>
<b>Kurtosis</b>	0.84	-1.07
<b>Skewness</b>	1.12694759	0.719522432
<b>Critical (+/-)</b>	1.15470054	1.154700538

Table 5.5 – Normality Test

*Before and After SWCNT Solution, Phase and Cycle*

The factors examined in these Two-Factor ANOVA tests are the Cycle, and the ‘*Before*’ and ‘*After*’ phases in both the *Experimental* and *Control* groups. In order to conduct these tests the 6 plants in the *Experimental* and *Control* groups have to be taken as two sets of replicates. Doing this will determine if there was a significant change between the ‘*Before*’ and ‘*After*’ phases in each cycle. Two ANOVA tests have to be conducted, first for the *Control* groups in each of the 3 Cycles, and the second for the *Experimental*.

<b>ANOVA Two Factor With Replication</b>						
<b>Control</b>						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Sample	83.21	1	83.21	33.51	2.5187E-06	4.17
Columns	38.25	2	19.13	7.70	0.0019969	3.32
Interaction	102.31	2	51.16	20.60	2.3401E-06	3.32
Within	74.49	30	2.48			
Total	298.27	35				
<b>Experimental</b>						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Sample	173.84	1	173.84	95.64	7.6661E-11	4.17
Columns	91.60	2	45.80	25.20	3.7866E-07	3.32
Interaction	154.82	2	77.41	42.59	1.7233E-09	3.32
Within	54.53	30	1.82			
Total	474.80	35				

Table 5.6 – Two-Factor ANOVA for Phase and Cycle

Table 5.6 presents the results obtained through these two ANOVA tests. It can be seen that for both the *Experimental* and *Control* data in all 3 Cycles the results remain the same. The F-Statistic obtained is greater than that of the F-Critical. When looking at our factors involved, Phase (*Before* and *After*) and Cycle, this result states that both have a significant effect on the experimental results.

When looking at the Phase results, it states the plants growth rates *Before* and *After* are significantly different within the two groups. Interestingly, the control group also shows

that there are significant effects due to phase and cycle. However, the effect of phase in the control group appears to be noticeably less based on the generated F-Statistics. This suggests that the addition of SWCNT solution in the experimental could have some effect. However, these results also state that the cycle a plant was grown in also has a significant effect on plant growth rate.

A Single Factor ANOVA using the ‘*Difference in Growth Rates (Before & After)*’ the addition of the SWCNT solution will verify that the effect of the cycle is indeed significant. The F-Statistic being greater than the F-Critical states they cannot be considered as equal.

<b>ANOVA Single Factor</b>						
<b>Control</b>						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	199.86	2	99.93	26.31	1.2454E-05	3.68
Within Groups	56.98	15	3.80			
Total	256.84	17				
<b>Experimental</b>						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	309.57	2	154.78	53.72	1.4502E-07	3.68
Within Groups	43.22	15	2.88			
Total	352.79	17				

Table 5.7 – Single-Factor ANOVA for Cycle

Table 5.7 shows the same results as Table 5.6 for the analysis of the cycle a plant was grown in. The F-Statistic is greater than that of the F-Critical, therefore the effects of the cycles are significant.

However, when looking at the calculated data presented from the Single-Factor ANOVA in Table 5.8 it can be seen that the ‘Variance’ and the ‘Averages’ for both Cycle 1 and 2

remain similar. Running the Single-Factor ANOVA with only Cycle 1 and Cycle 2 in the data set returned an F-Statistic less than that of the F-Critical. These new results show that Cycle 1 and Cycle 2 can be said to be equal and thus not affect the plant growth. Cycle 3's data appears to be too different and thus it cannot be said to be the same. This difference in data is due to Cycle 3 containing younger plants (approx. 1 week younger), thus these plants development proceeded at a rate different to that of the other two cycles. As a result Cycle 3, although important within itself, is judged to be too different from Cycles 1 and 2 to permit a combined statistical analysis of all three cycles. Instead, the Two-factor ANOVA will be re-run using only Cycles 1 and 2 to avoid the uncertain variability that Cycle 3 appears to introduce.

<b>Control</b>				
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Cycle 1	6	6.07	1.01	0.75
Cycle 2	6	2.94	0.49	3.06
Cycle 3	6	46.83	7.81	7.59
<b>Experimental</b>				
<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
Cycle 1	6	12.69	2.12	2.63
Cycle 2	6	5.12	0.85	1.71
Cycle 3	6	61.28	10.21	4.30

Table 5.8 – Single-Factor ANOVA for Cycle, calculated data

With this conclusion conducting another Two-Factor ANOVA test for the effect of Cycle 1 and 2, and Phase on our results produces the following results.

<b>ANOVA Two Factor With Replication</b>						
<b>Control</b>						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Sample	2.60	1	2.60	2.02	0.17	4.35
Columns	0.02	1	0.02	0.02	0.89	4.35
Interaction	0.17	1	0.17	0.13	0.72	4.35
Within	25.73	20	1.29			
Total	28.53	23				
<b>Experimental</b>						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Sample	13.24	1	13.24	9.71	0.01	4.35
Columns	1.01	1	1.01	0.74	0.40	4.35
Interaction	2.40	1	2.40	1.76	0.20	4.35
Within	27.29	20	1.36			
Total	43.94	23				

Table 5.9 – Two-Factor ANOVA for Cycle 1,2 and Phase

The results in Table 5.9 show that the effect of phase is no longer significant in the *Control* groups, but is significant in the *Experimental* group. Also, it shows that the interaction between the Phase and Cycle is not significant.

#### *The Numbering of Plants Within Each Cycle*

A Two-Factor ANOVA without replication is used to determine if the random numbering of the 6 plants contained in each group (*Experimental* and *Control*), within each cycle plays a significant role in the experimental results.

<b>ANOVA Two Factor Without Replication</b>						
<b>Control</b>						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Rows	19.92	5	3.98	1.07	0.43	3.33
Columns	199.86	2	99.93	26.97	9.3635E-05	4.10
Error	37.06	10	3.71			
Total	256.84	17				
<b>Experimental</b>						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Rows	10.76	5	2.15	0.66	0.66	3.33
Columns	309.57	2	154.78	47.68	7.7048E-06	4.10
Error	32.47	10	3.25			
Total	352.79	17				

Table 5.10 – Two-Factor ANOVA for Plant Number

The results obtained in Table 5.10 present an F-Statistic that is less than that of the F-Critical, which states that the number given to each plant with its *Control* or *Experimental* group does not significantly affect the results of the experiment.

#### *Comparison of Plant Growth Data to Water Usage Data*

To verify the results obtained through each of the ANOVA tests conducted above the same tests were performed on the Water Usage data. The results of these tests will not be presented in this document but will be contained in Appendix B, as they show the same conclusions already discussed. The *Before* and *After* phases play a statistically significant role, however the Plant Number within a group (*Experimental* and *Control*) and the cycle a plant was grown in, do not have a statistically significant effect on the experiment.

#### *Total Organic Carbon (TOC) Monitoring*

Throughout each cycle the Total Organic Carbon (TOC) content of the plants growth solutions were monitored. During each cycle, a sample of the growth solution was taken

from all of the odd numbered plants and the TOC content results recorded. When the SWCNT solution was added, a sample was then taken from every plant in the *Experimental* group. Along with the samples taken from growing plants, samples were also taken from a *Control* solution in which no plant was grown, thus providing a baseline in which any increased or decreased TOC content can be attributed to the plant or the SWCNT solution when applicable. The monitoring of the TOC was used to determine the purity of the growth solution, as well as track the rate at which the SWCNT solution settled. The base readings determined for Milli-Q water and the nutrient solution are as follows:

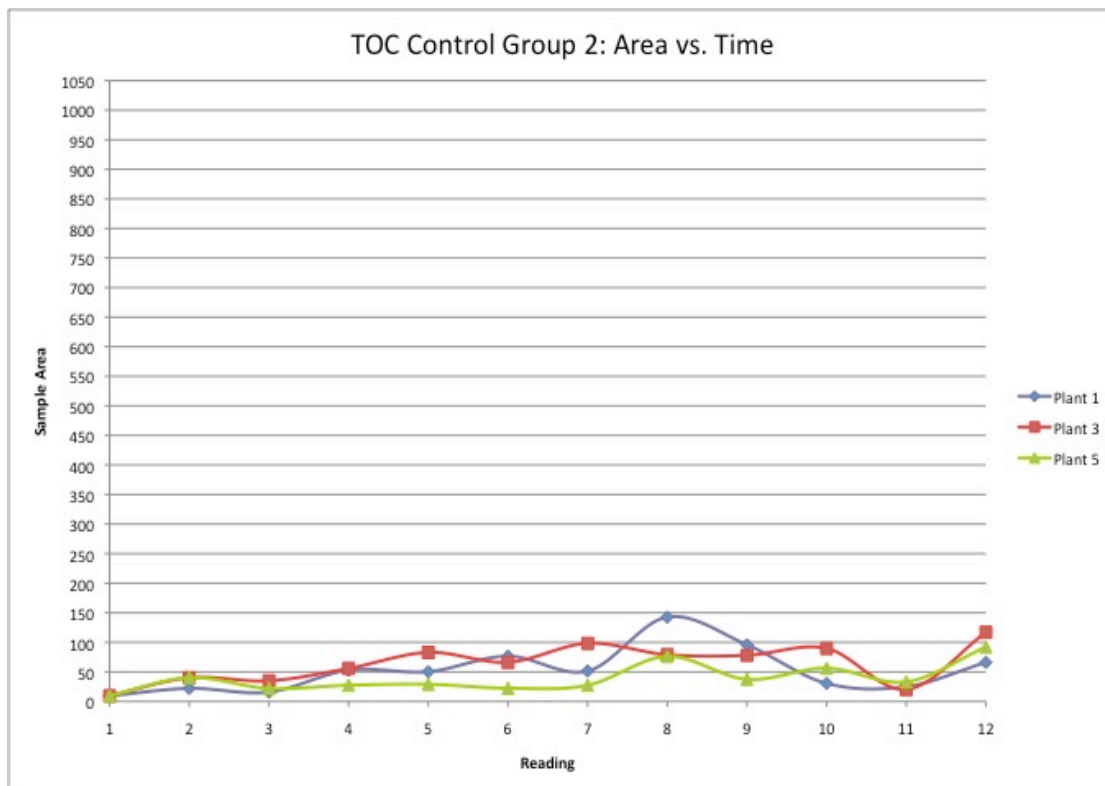
$$\text{Sample Area} = 9.559, \text{ Sample Concentration (mg/L)} = 1.798$$

These readings are used to determine whether the TOC content has increased or decreased.

#### *TOC Content Graphical Analysis*

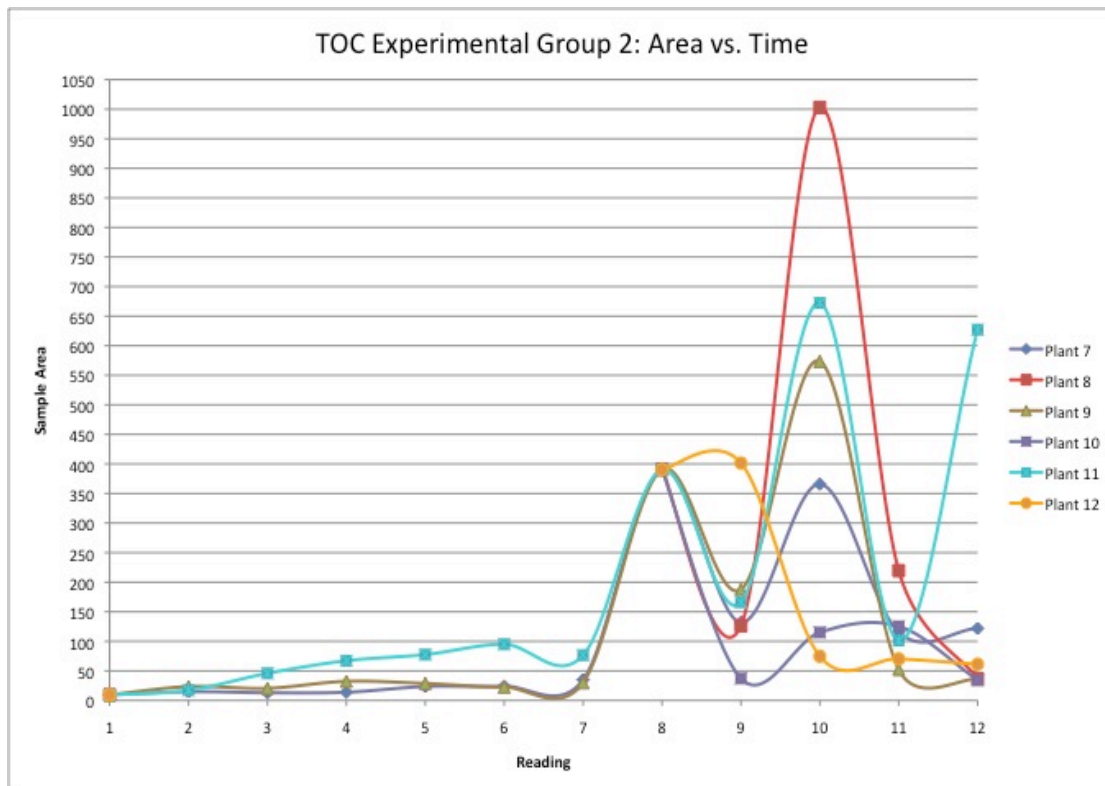
The following graphs display the increase and decrease of each cycle's sample area, and sample concentration. It can be clearly seen that each *Control* group shows an increase in both area and concentration throughout each cycle. In regards to the peaks found on the *Control* graphs, they can be attributed to decaying organic matter that has made its way into the solution, such as leaves or bugs, that may have fallen. Also in some cases these peaks correspond with the addition of nutrients to the plants. These cases are clear because the peak is not as dramatic as those found in the *Experimental* groups, do not occur with all of the plants in that group, and often decrease quickly back to levels consistent with the rest of the group.

As an example the graphs for Cycle 2 are displayed below; the remainder are provided in Appendix B.

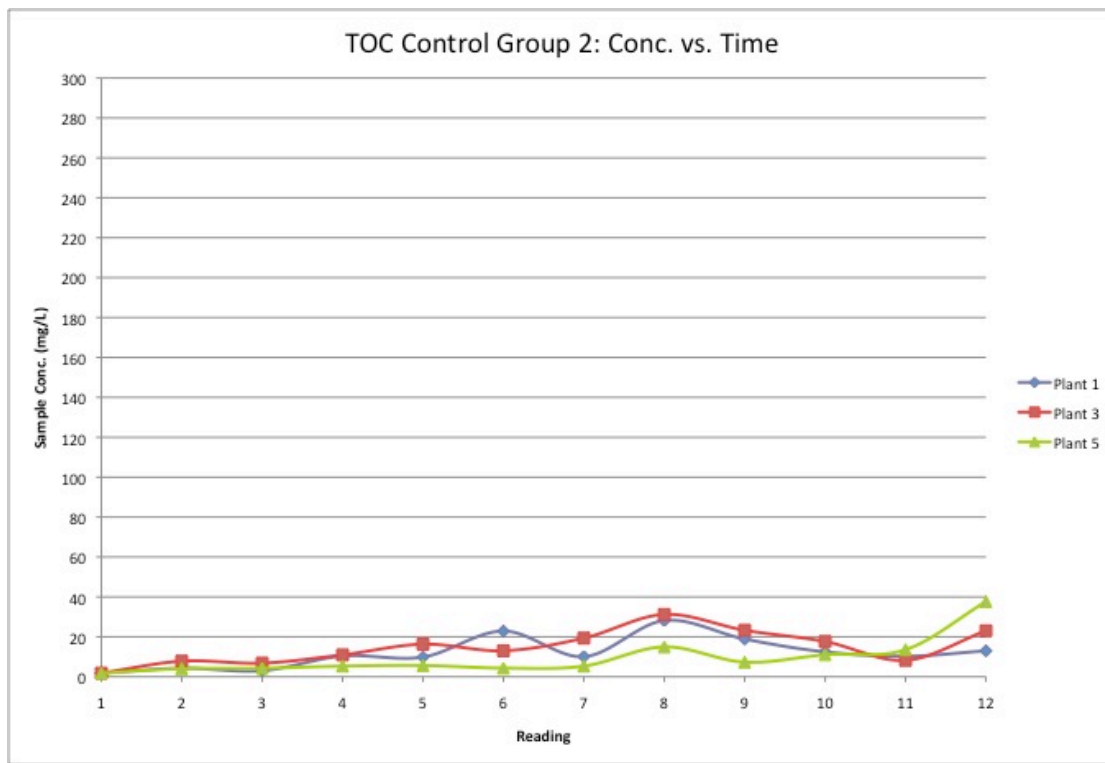


Graph 5.8 – TOC Area vs Time (Control Group) Cycle 2

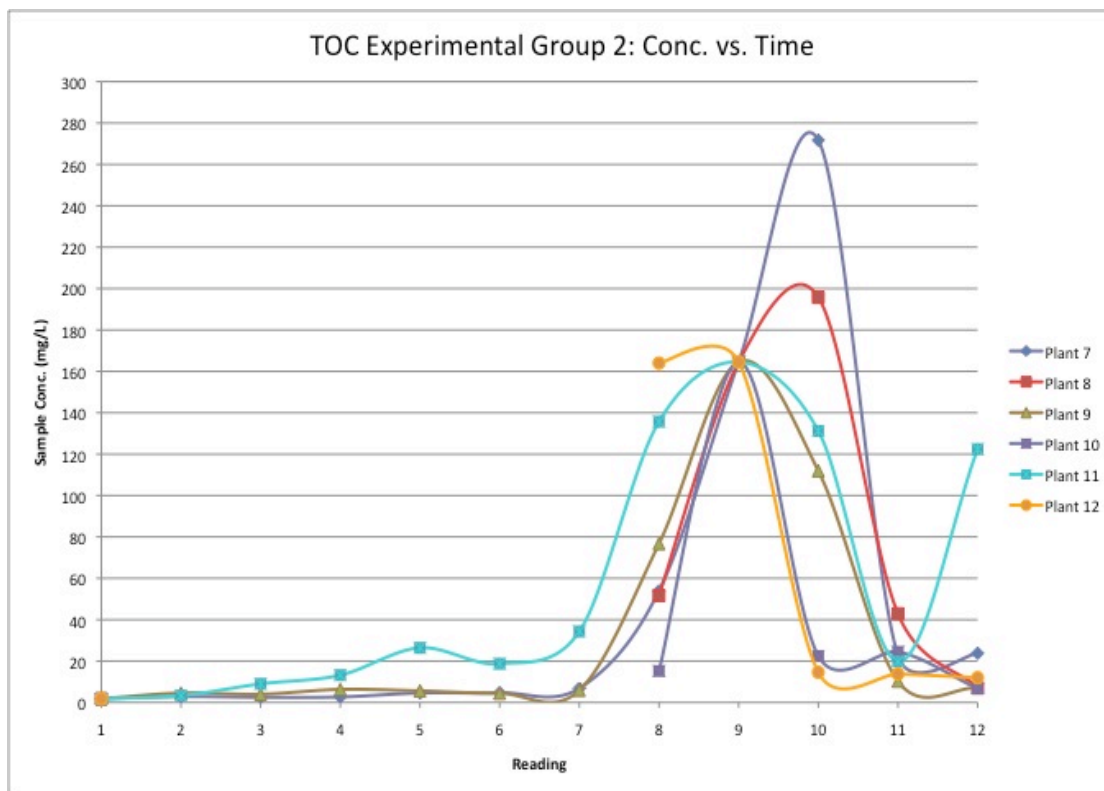




Graph 5.9 – TOC Area vs Time (Experimental Group) Cycle 2



Graph 5.10 – TOC Conc. Vs Time (Control Group) Cycle 2



Graph 5.11 – TOC Conc. Vs Time (Experimental Group) Cycle 2

The graphs displayed can be compared showing the clear change that occurs when the SWCNT solution is added. This shows the water has become contaminated. In each of the *Experimental* groups, this event is marked by the significant peak; in the *Control* groups no change is apparent because there was no addition of SWCNTs. Throughout each cycle it becomes apparent that the SWCNTs are settling as time progresses, the sample areas and concentrations decreasing over time demonstrate this settling. It can also be seen that within the *Experimental* groups the readings tend to waver; they seem to decrease, then increase, then decrease finally, as opposed to decreasing in a strict linear fashion. This observation likely is because of the varying settling rates and the movement inside of the jar cause by the air bubbler. Each jar holding the suspensions has an air supply hose that

is connected to an aquarium bubbler that supplies air to the plants roots and prevents the water from stagnating. This addition of air cause constant movement in the solutions, which can change the concentrations of the samples at any given time. However, it can be seen that even with the wavering results the overall trend is a decrease in area, and concentration in the *Experimental* groups.

## CHAPTER VI

### ASSESSING CARBON NANOTUBE INTERACTION WITH PLANTS

Through the data presented in Chapter 5, the addition of a SWCNT solution to developing plants increases growth rate and water uptake based on biological observations. However, during examinations of the plants after the completion of each cycle there has been no conclusive evidence of CNTs being taken up into the plants themselves. It appears that the CNTs only interact with the root structure of the plants that they come into direct contact with. In Figure 6.1, the roots contacting the SWCNT solution blacken; where as the roots that did not contact the SWCNT solution remain white. Figure 6.2 shows the all white roots of the *Control* groups, which had no SWCNT solution added. These results strongly suggest that the CNTs did not translocate through the root system, but that the SWCNT remain in the affected root system. The remainder of the plant - leaves, flowers or fruits - are unlikely to contain CNTs. , in turn this will not affect any wildlife as studies have shown during mammalian.

After each cycle the plants in the *Experimental* group had their roots systems washed in an attempt to remove the CNTs, yet on all attempts the roots remained stained. *Figure 6.2* shows the results after extensive washing.



Figure 6.1 – Plant Roots after contact with SWCNT solution



Figure 6.2 - Control Plant Roots



Figure 6.3 – Experimental Plants root system after washing

The black stain that remains on the root systems suggests the roots are actually absorbing the CNTs through the pores but not translocating these foreign objects through the plant. This may explain the increased water uptake and growth as well. When the SWCNT solution is introduced into the system, the CNTs begin interacting with the nutrients already present. CNTs have been shown to possess excellent sorptive properties thus they are very capable of adhering to the nutrients they contact, much like a sponge. When these CNTs are then taken into the root system the plant can drain the CNTs of the additional nutrients held by the CNTs, leaving the CNTs lodged in the pore. These remaining CNTs then act to help the plant uptake more water as they can act much like a siphon hose would, allowing more water to enter the roots system. These hypothesized

mechanisms for increased nutrient and water uptake are the possible reasons for increased growth rates.

The CNTs that interact with the nutrients and are not taken into the root system begin to settle out. This settling removes those nutrients from the solution and prevents the roots from absorbing them. The sorptive properties of the CNTs can be used to aid the plants in the early time periods after SWCNT addition, but over time, will remove nutrients from the solution.

In the case of this occurring in a closed system where no more nutrients are to be added the short term result shows the increased development of the plants. However if the time span of this experiment was to be extended, it is possible that the CNTs would remove enough nutrients through settling to ultimately hinder the development of the plants. In the real world application of CNTs as a remediation method the CNTs would be present in the ground water where the plants would interact with them for only a short period before they begin to be washed out. Given that any ground water system will generally be recharging and thus have continual water movement, it is possible that plants would not suffer the negative effects of the nutrients settling out. The CNTs would be able to adhere to the contaminants present as well nutrients, and would be collected or dispersed quickly enough that the long term negative effects affecting a closed system would be of little or no concern. Additional testing of these hypotheses on: 1) long term, closed environment CNT presence; and 2) continually water recharging open (natural) systems and CNTs

would confirm if SWCNT pose any actual challenges. Based on the testing in this research, the use of SWCNT could be a feasible remediation approach.

## CHAPTER VII

### CONCLUSION

The data analyzed through the course of this experiment demonstrate that CNTs appear to aid plant development in the short term. Based on the previously performed statistical analyses the addition of the SWCNT solution appeared to increase the growth rate and water usage of the plants in the *Experimental* groups. These were not entirely expected and to some degree, counter to what was possibly expected given the prior research into the generally negative effects of CNTs on organisms, and the variety of conclusions reached by previous studies. Long-term effects can only be theorized at this point.

Both cases of nanomaterial interaction with developing plants referred to in the literature review give similar results in that a positive outcome for growth was found. In the case presented involving the germination of tomato seeds, the author concludes that the CNTs penetration of the seed coat allows the seeds to absorb more water at an earlier stage in development. This same feat is demonstrated in this research where the root system is penetrated by the CNTs, thus allowing the plant to absorb more water. In a nutrient rich environment these CNTs further likely carry the nutrients they absorb with them into the plant for further aid. However, drawbacks of adding CNTs to a system are also present. It was stated that over time the CNTs settle out, this settling would remove large amounts of nutrients from the water supply feeding the plants. If no new nutrients are added to the



system this will likely begin to impact plant development. This decline in development would follow the initial boost in growth. Once again, this would only affect the isolated or closed system.

When used in both water or soil remediation, in the field, it appears as though the CNTs will successfully adhere to the targeted contaminate (pending proper functionalization) and be either removed or dispersed following their application. CNTs that would go on to interact with vegetation would not seem to be taken up into the plants, and would not pose the threat of depleting the environment of nutrients. In a real world setting these CNTs would be continuously moving while nutrients would be added over time. Based on the research, the largest concern for use in remediation techniques would be the interaction with animal organisms rather than plants. However, it has been demonstrated that the functionalization of the nanomaterial can change its interaction with cells, and it would then have to be left to case-by-case trials in order to determine if a particular functionalized product used for remediation poses any threats to the environment. Long-term benefits or drawbacks of SWCNTs have yet to be tested. Furthermore, when conducting future tests it would be important to account for varying developmental rates of plants to avoid unforeseen analytical outcomes as demonstrated with the three cycles in this research. As it stands, unaltered SWCNTs, when suspended in an aquatic environment, seem to enhance the growth of plant life in the short term.

## APPENDICES

### APPENDIX A

#### PREVIOUS METHODOLOGY AND APPROACH

The use of cell suspensions will shed light on how individual plant cells interact with carbon nanotubes and the events that unfold will be observed through the use of fluorescently labeled SWCNTs interacting with the plant cells directly. This fluorescence of effected should be observed in the first stage of testing, and possibly predict which plants will be more vulnerable to the nanomaterial when its introduced for uptake during growth in an aqueous medium, as such will be the case in Stage II. The use of Gallic Acid (GA) throughout Stage II of this experiment is based on recent studies conducted at the Helsinki University of Technology, by Dr. E. Salonen and colleagues. These studies revealed that the interaction of fullerenes with natural phenolic acids (such as GA) induces rapid cell contraction, resulting in death (Salonen et al. 2008). The results of this study showed GA molecules rapidly adsorbed onto the surface of fullerenes through hydrogen bonding and enhanced through Van der Waals attractions. The self-assembly then translocated across the cell membranes to enter the nuclear membranes and the consequent contraction of the membranes (Salonen et al. 2008).

From these findings it is fair to conclude that the self-assembly of CNTs and GA will provide a similar effect when in direct contact with plant cells and after uptake, thus causing cell constriction and death, aiding in the determination of whether or not the CNTs have been absorbed by the plants during the second stage of testing. Stage II of testing will introduce a CNT solution and a GA solution to plants being grown in an aqueous medium. The control plants will be grown in three different solutions. The first

control set will be a simple nutrient solution. The second control set will be grown in a nutrient solution with the addition of GA and the third control set will be grown in a nutrient solution with the addition of a CNT solution.

The test set of plants will begin growth in a nutrient solution where the CNT and GA solution will be added together. Plants will be allowed to grow in the nutrient solution for roughly seven days after transplantation into the final system, when a CNT solution will be added followed by a GA solution after seven more days. Plants will then grow for the last seven days in the final mixture.

### Expected Results

Results of this experiment may come in many forms, as it may very well be possible that the nanomaterial in question is completely ignored by the plant during normal nutrient uptake. Expected results would be conclusive evidence, from one of the experimental stages: cell suspensions, or plant uptake. Conclusive evidence obtained through cell suspensions (Stage I) comes in the form of the observed fluorescent illumination when the suspended cells are viewed under a fluorescence microscope. Conclusive evidence during plant uptake (Stage II) may be obtained through observed alteration of the plant/root physiology or termination of the host plant. Alteration of the cell structure, or cell death will prove that the small size of the nanomaterials can penetrate the cell walls, either forcibly or through cellular uptake, and cause harm to the organelles. Finally, alteration of the plant/root physiology will show that the CNTs are contacting the roots in a manner similar to the plants natural nutrient uptake procedure. However, because of

reasons yet to be determined the attempted absorption of the nanomaterial may cause damage to the plant/roots either resulting in termination or impairment of the normal functioning. Through the control plants we can verify normal plant/root physiology to determine the damages that occur from the CNT solution. Also, we can verify whether the CNT absorption directly terminated the plant or if it was taken up and the addition of GA terminated the plant. This will allow us to determine which plants do not absorb the CNTs, which plants absorb the CNTs and survive and which plants are killed by the CNT solution. Beyond this method there has been no procedure developed to quantify the amount of CNT absorbed by each plant, thus this experiment is developed to simply state whether or not the absorption of CNT has occurred.

#### TESTING PROTOCOL

The method used for completion of this experiment will be broken into two stages at which observations throughout must be made and a final analysis will be required before moving to the following stage. The plants that will be cultured into cell suspensions in Stage I and grown in the aqueous medium in Stage II are as follows:

##### *Pumpkin (Cucurbita maxima)*

The protocols presented are needed to complete the experiment whereas protocols required for media, solution, or laboratory preparations are presented in the appendix. Each protocol has been adapted from 'Plant Cell Culture' by D.E Evans, J.O.D Coleman and A Kearns for use with this experiment and its requirements.

### Stage I

Stage I consists of the preparation of the various seeds used in the regeneration of callus cultures which are then broken down into individual cell suspensions. Once adequately prepared, the seeds are germinated and through the protocols presented in previous sections are turned into cell suspensions, which are then ready to be incubated with a prepared solution containing SWCNTs labeled noncovalently with fluorescein isothiocyanate (FITC). The control set will show the effect of pure incubation, while the labeled CNT solution will determine the role played by the addition of CNT. The growth of each suspension will be observed and when the incubation period is complete the cells from each suspension will be analyzed to determine how the CNT solution has affected cell viability, growth, and structure.

### Stage I Materials

In order to follow the protocol presented in the next section it is necessary to have the following materials, media and solutions prepared or on hand ready to be prepared when necessary.

#### Sterile

- Autoclave
- Conical flask (250ml), sealed with aluminum foil
- Petri Dish
- Tea strainer, or muslin squares, autoclaved in foil packets
- Sterile containers for sterilizing plant material
- Sterile Pipette

- Sterile germination vessels
- Spatula
- Scalpel
- Small dissecting scissors
- Forceps
- Non-Sterile
- Binocular Dissecting microscope
- Gas permeable tape
- Growth Cabinet (22°C , continuous white light or 16hr light 8hr dark)
- Incubator
- Orbital shaking water bath
- Magnetic Stirrer and bar
- pH meter
- Fluorescein isothiocyanate (FITC)
- Fluorescences Microscope
- Single walled Carbon Nanotubes

### Stage I Protocol

Stage I consists of seed preparation and germination of seedlings in a laboratory environment, using commercially purchased media to ensure uniformity, as well as the growth of callus cultures and cell suspensions for use with the labeled CNT mixture. The following protocol will be used to prepare the seeds and germinate the seedlings for callus production.

**Protocol 1.1 – Preparation of Plant Material and Germination of Seedlings** (Evans et al. 2003)

1. Place selected seeds in a tea strainer, make a muslin bag to hold them and place directly into a 250ml conical flask
2. Submerge the seeds in absolute alcohol for a few seconds (3-5s). Pipette off alcohol and discard.
3. Immerse the seeds in hypochlorite with a few drops of surfactant (ie. Tween 80) for 15-20 minutes (the duration of the immersion can be varied – less for more sensitive seeds and longer if infection is a problem.)
4. Rinse the seeds 3x5 minutes with 250ml of sterile distilled water. If seeds float to the surface, either pipette off liquid or try a low-speed centrifugation step at 1000g for 5 minutes.
5. Working in aseptic conditions, place the seeds in a sterile Petri dish, then transfer them to germination medium in either a Universal bottle or other suitable sterile germination vessel. (Germination medium: MS medium with 2% sucrose, 0.8% Agar. Sterilized by autoclaving.)
6. The seedlings can then be germinated in a suitable incubator. (Temperature and lighting will depend on species, typically requiring 16hr light, 8 hr dark.) These seedlings, after germination can be used to provide sterile material from which callus can be regenerated.

Once the seedlings have been germinated adequately to allow sufficient root and stem material to develop (time of germination varies with plant species) they are ready to be dissected for regeneration of callus cultures. The following protocol will be used to

produce the callus cultures needed to yield the cell suspensions used with the CNT solution.

**Protocol 1.2 – Generation of Callus from a Dicot Root** (Evans et al. 2003)

(Working in a sterile laminar flow hood)

1. Transfer seedlings to the sterile surface of a Petri dish lid.
2. Excise the roots and section them into roughly 1mm lengths.
3. Carefully transfer the cut pieces to the surface of the callus induction medium using a sterile spatula. (Induction medium: Gamborgs B5 basal medium (sigma G5768) with 2% glucose, 0.8% Agar, ~pH 5.7)
4. Seal the Petri dishes around the edge with a piece of gas-permeable tape and place in an incubator at 22 degrees C with continuous white light or 16hr light, 8hr dark.
5. Monitor over 2-3 weeks. Growth of callus will 3-5mm over this period.
6. Callus may be subcultured by removing it and placing onto fresh medium periodically (every 4-6 weeks). It may be subdivided using a sterile scalpel to generate more individual calli.

For production of cell suspensions, agitation of friable callus cultures is required. This agitation will allow newly divided cells to separate and become free floating cells in the suspension medium. Once the cell suspensions have been produced they can be subcultured with the addition of the labelled CNT solution. After the incubation periods have been met each of the subcultured suspensions must be thoroughly examined and compared to determine the effects of the added supplements.



**Protocol 1.3 – Initiating Cell Suspension** (Evans et al. 2003)

1. Thoroughly sterilize the laminar flow cabinet with 70% alcohol. Leave running for 20 minutes prior to use.
2. Spray foil wrapped tools with 70% alcohol and leave dry in the running cabinet.
3. Before bring plates into the cabinet wipe all surfaces with paper soaked in 70% w/v alcohol.
4. Spray flasks of medium with 70% alcohol and allow to dry.
5. Unwrap sterile tools and Petri dishes of callus.
6. Using forceps and scalpel move callus to sterile Petri dish and break apart into small pieces. Ideally the tissue should be approximately 2-3mm in diameter. Close Petri dish. (This step is required to create 2 cell suspensions from a single callus culture for each callus culture used.)
7. Resterilize the tools and allow cooling before use.
8. Remove foil cap from fresh flask of medium.
9. Using forceps, pick up callus and transfer into flasks while avoiding the sides of the neck.
10. Seal necks of flasks with a fresh foil cap.
11. Move flasks to orbital shaking water bath. Monitor growth of material by eye. Discard contaminated flasks. Within a one to two weeks it should be possible to observe an increase in the number of free cells in the medium, which have broken away from the callus.

12. After 7-10 days, the cultures should be passaged, whether or not a large increase in mass has occurred. Allow the material to settle to the bottom of the flasks. Pipette off the liquid medium and replace with fresh medium.
13. Then pipette a volume of cells (ie. 10ml into a 100ml) from the old flasks into new flasks filled with fresh medium. (This step must be repeated to create the adequate number of new cell suspensions from a single passaged suspension.)
14. The new cell suspensions will be labeled suspensions 1-18 for tracking and identification purposes.
15. Suspensions 1-6 should be placed on the orbital shaking water bath for 7-10 days, and should be incubated without change, as Control Set 1.
16. Suspensions 7-12 should be placed on the orbital shaking water bath for 7-10 days and should be incubated with the addition of 1ml of pure FITC solution for every 5ml of suspension, after 5 days, leaving 2-5 days for incubation. This will be known as Control Set 2.
17. Suspensions 13-18 will be placed on the orbital shaking water bath for 7-10 days and should be incubated with the addition of 1ml of the labeled CNT solution for every 5ml of suspension, after 5 days leaving 2-5 days for incubation. This will be known as Test Set 1.
18. Repeat steps for each species to be tested and change the numbering accordingly to maintain organization of records.

With the completion of protocol 1.3 and analysis of the various cell suspensions through fluorescence microscopy, Stage I is complete.

## SUMMARY OF PROJECT COMPLETION

Preliminary testing for Stage I yielded successful results through the non-covalently labeling of the SWCNTs using both FITC and Gallic Acid. Comparing the luminescence of the two labels under a fluorescence microscope it was seen that FITC labels provided a noticeably brighter result.

Once confirming that it was possible to produce SWCNTs that would fluoresce, pumpkin seeds were germinated to produce roots for the generation of callus cultures. After germination the plants were harvested and specimens were exercised for placement in an agar induction medium. The initial fourteen-day passage produced results that allowed the calli to be re-sectioned for the production of more calli.

Roughly five days into the second passage of the callus cultures a power outage occurred which resulted in the incubator being reset to its default temperature and humidity settings. These default settings were not discovered for three days at which time the calli had been damaged beyond repair. In order to continue with this stage of testing a new group of plant seeds would have to be prepared, germinated, sterilized, roots sectioned, and calli would then have to be induced. Stage I would then be set back a minimum of five weeks if no further incidents arose. At this point it was decided to cease testing in Stage I and move directly to the following of the new procedure contained in this document.

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## VITA AUCTORIS

Matthew Ray was born in 1985 in Windsor, Ontario. He graduated from St. Josephs Catholic High School and went on to the University of Windsor where he obtained a B.A.Sc. [Honours] in Civil Engineering in 2007. Following this he moved into Environmental Engineering and obtained his Masters of Applied Science from the University of Windsor in 2010.