Behaviour of Tylosin in Presence of UV Radiation

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ABSTRACT

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The occurrence of pharmaceuticals in the environment is a popular topic and is currently being heavily researched. Tylosin is a macrolide antibiotic used extensively in livestock farming and also in veterinary practices for other domestic animals. Tylosin is fatal to equestrian animals, however. In wastewater, tylosin has been measured to occur in the micrograms per liter range. In this experiment, the concentration is raised to 0.5mg/l to allow for easier detection. The rate of degradation should remain the same. Algae was to be used to degrade the tylosin, as it has been proven to work very well to remediate heavy metal laden waters. In the experiment, 0.5mg/L of tylosin was spiked into 4 beakers. Two of these beakers contained algae and two contained a phosphate buffer adjusted to a pH according to the algae's diurnal cycle pH. The beakers were then split into two groups with one algae and the corresponding pH for either the day or the night cycle. For each of the beakers, a replicate beaker was prepared to confirm results. This gave a total of 8 beakers, 4 beakers in each cycle. The beaker in the day cycle were placed under both UV and visible spectrum lights for 48 hours, and the night cycle beakers were placed in a light-tight box for 48 hours. When the samples were taken and processed using methanol extraction to separate the algae from the liquid and solid phase extraction to isolate the tylosin concentration, the algae sample results were not what was expected. The initial concentrations varied wildly and, in many cases, concentrations spiked in the middle of the experiment. The protocols were retested and confirmed to be correct and due to time constraints, a new experiment not containing algae was developed.

In this new experiment, phosphate buffers were used to simulate the pH in the algae diurnal cycle and left for 48 hours in the same configuration as the previous experiment. The same protocols were used to process the samples and analyze them. In this new experiment the initial concentrations were in the correct range of 0.5mg/L and the rest of the results were quite interesting. The tylosin degraded well in the experiment, suggesting that raising or lowering the pH levels destabilized the compound enough to degrade. The final concentrations bottomed at roughly 0.08mg/L. In an interesting note, the concentrations at 1 hour seemed to normalize to an average of about 0.24mg/L from initial concentrations ranging from 0.3to 0.9mg/L.
The errors in the results from the algae experiment were not possible to identify in the time allotted for the experiment. The protocols involved in the pre-treatment were checked for errors, but were found to be correct. The same protocols were used to treat the phosphate buffer experiment and the results showed slightly more rapid degradation from the day cycle with pH6 buffer, though degradation halted for most samples at 0.08m/L.

Contents

1 INTRODUCTION

The fate of pharmaceuticals in the environment is a subject of interest and as such is being extensively researched to find methods of remediation for these drugs. Pharmaceuticals are not limited to human use and are widely used in veterinary practice. Each pharmaceutical's biodegradation attributes are different, so it is imperative to research each pharmaceutical to find a protocol that works best. Some methods of degradation have a blanket protocol that works well with more than one or two different pharmaceuticals.

In this research, the veterinary pharmaceutical tylosin is studied and a protocol for degradation is tested. Tylosin is a "Macrolide antibiotic obtained from cultures of Streptomyces fradiae. The drug is effective against many microorganisms in animals but not in humans." (Pub-chem.com, 2014). Tyolsin is used as an anti-biotic and as a growth promoter in cattle, swine and chicken. The pharmaceutical is also used regularly in some domestic animals for chronic diarrhea and mycoplasmic infections. Tylosin can be administered via powdered form or an oral liquid, which is generally mixed into food, as an injectable dose. Tylosin is, however, not suitable for horses. Elanco, who is a global animal health company, warns:"do not administer to horses or other equines. Injection of tylosin in equines has been fatal" (Elanco, 2014). The drug gives them a strange case of fatal diarrhea, effectively dehydrating the horse until death occurs. Given this fact, it is clear that tylosin is not a viable anti-biotic for all animals.

The occurrence of tylosin in the environment has been quantified in varying fields by many researchers. An example of the concentrations found in swine wastewater and slurry in Mexico is taken from a recent study. In their report the researchers stated, "In grab samples of swine wastewater, the tylosin detected showed concentrations of 56, 72 and 8.6 μg L^{-1}, in breeding-gestation, nursery pigs, and grow/finishing area, respectively. In composite samples, the concentration of tylosin was 11.8 μg L^{-1} for the breeding-gestation area and 2.4 μg L^{-1} for the grow-finishing area. For slurry, the concentration of tylosin was 20.6 and 17.8 μg L^{-1}, for the breeding-gestation and grow-finishing area, respectively. (Garcia-Sanchez et al., 2013) It is clear to see that the concentrations that are occurring are quite small, in the microgram per liter range. The report goes on to say, however, that concentrations of tylosin were found in swine finishing areas where none of the swine were given tylosin. This transference could be related to the conditions swine are in and

it could be possible that the swine slurry could be ingested by other swine, who have not had tylosin administered.

Tylosin does degrade naturally in the environment, but because there are four factors of tylosin (tylosin A, B, C, and D) they degrade at different rates. In a research report by Dingfei Hu and Joel Coats states that "tylosin A is degraded with a half-life of 200 d in the light in water, and the total loss of tylosin A in the dark is 6% of the initial spiked amount during the experimental period. Tylosin C and D are relatively stable except in ultrapure water in the light. Slight increases of tylosin B after two months and formation of two photoreaction isomers of tylosin A were observed under exposure to light. (Hu and Coats, 2007)

1.1 Aim of the Experiment

The aim of this experiment is to determine the degradability of tylosin in a simulated algae pond. The purpose of the algae in the experiment is to take advantage of the diurnal cycle, during which the algae raises and lowers the pH of the algae pond. The change in pH occurs when, during sunlight hours, algae undergoes photosynthesis, producing oxygen and raising the pH levels. Conversely, at night, in the absence of sunlight, algae undergoes a process called dark respiration. Dark respiration refers to the consumption of O_2 and the production of CO_2 by algae.

2   Theoretical Background

To better understand the terms and ideas behind this experiment, it is important to define the expressions. In the following section are definitions of the terms and necessary knowledge to give a general idea behind this research experiment.

2.1 Definitions

Bioremediation has been defined as the "Remediate" means to solve a problem, and "bioremediate" means to use biological organisms to solve an environmental problem such as contaminated soil or groundwater" (Cornell University, 2009). Bioremediation is generally split into two methods, the first of which is to use the microbes that are present in the environment already that degrade pollutants by enhancing their growth, thus increasing their ability to degrade pollutants. The second is to introduce a certain group of microbes to address a particular environmental pollutant that the naturally occurring microbe population cannot handle or cannot remediate on its own. The method practiced in this experiment utilizes the algae that occurs naturally. Different algae species have different properties, but what this experiment is after is the ability to change the pH of the water naturally without the addition of chemicals. This is something that all algae species can do, though some are more effective than others. By giving the algae the nutrients it needs to flourish, the larger algae population can be more effective.

Wastewater is separated into three categories: domestic sewage, industrial sewage, and storm sewage. "Domestic sewage carries used water from houses and apartments; it is also called sanitary sewage. Industrial sewage is used water from manufacturing or chemical processes. Storm sewage, or storm water, is runoff from precipitation that is collected in a system of pipes or open channels" (Encyclopedia Britannica, 2014). Tylosin is released into the environment mainly through organic fertilizer, or manure, which still contains traces of veterinary pharmaceuticals.

UV radiation is the "portion of the electromagnetic spectrum extending from the violet, or short-wavelength, end of the visible light range to the X-ray region. Ultraviolet (UV) radiation is undetectable by the human eye, although, when it falls on certain materials, it may cause them to fluoresce—i.e., emit electromagnetic radiation of lower energy, such as visible light" (Encyclopedia Britannica, 2014). UV radiation is typically located in the

wavelengths between 400nm – 10nm, and is separated into four classes: near (400nm-300nm), middle (300nm-200nm), far (200nm-100nm) and extreme (100nm-10nm). Furthermore, there are 3 conventional division of wavelengths according to absorption: "near (400–315 nm), which is absorbed relatively poorly by organisms; actinic (315–200 nm), which is absorbed most readily by organic matter and thus has the greatest effects on organisms; and vacuum (less than 200 nm), which is absorbed by most substances, including oxygen in the air (and below 100 nm nitrogen), and so is of little use in biological experimentation" (Encyclopedia Britannica, 2014). The UV lights used in this experiment were in the near category wavelengths.

Algae is defined as "a plant or plantlike organism of any of several phyla, divisions, or classes of chiefly aquatic usually chlorophyll-containing nonvascular organisms of polyphyletic origin that usually include the green, yellow-green, brown, and red algae in the eukaryotes and especially formerly the cyanobacteria in the prokaryotes" (Merriam-Webster, 2014) In the experiment, the algae that will be used is *Scenedesmus quadradica*. Algae have two cyclical processes, a day and a night cycle, referred to as a diurnal cycle. In the daytime, the algae consume CO_2 and produce and release O_2 and H_2O. This activity increases the pH slightly and can change the permeability of the pharmaceutical allowing for ionization, depending on the pH value needed for ionization. During the night, the algae reverses its respiration. This process, referred to as dark respiration, slightly lowers the pH in the water throughout the night and reaches the lowest pH in the morning before photosynthesis begins again. In this dark respiration cycle, O_2 is consumed and CO_2 and H_2O are released. As stated previously, this diurnal change in pH is the chief principal of bioremediation via algae blooms.

Liquid Chromatography- Mass Spectrometry analyzer, often shortened to the acronym LC-MS, is an array of different analytical machines that can detect varying levels of elements or compounds. In order to detect these element or compounds a protocol must be created and a standard of the element or compound must be prepared for the LC-MS to use as a template to identify and quantify concentrations of the desired substance.

The way LC-MS works can be broken into two separate, but linked portions. The first is High-Performance Liquid Chromatography (HPLC). This process is referred to by the Waters company as the most powerful approach. a sample is passed through a narrow column that contains a stationary phase. A mobile phase (solvent) is used to pass the

sample through the column and at different speeds according to the element or chemical contained in the sample.

According to Waters, a mass spectrometer "can measure the mass of a molecule only after it converts the molecule to a gas-phase ion. To do so, it imparts an electrical charge to molecules and converts the resultant flux of electrically charged ions into a proportional electrical current that a data system then reads. The data system converts the current to digital information, displaying it as a mass spectrum. (Waters, 2014). When HPLC and Mass Spectrometry (MS) work in tandem, the result is LC-MS. By splitting the elements of a sample up and then analyzing the amount of that element contained in the sample by comparing it to a standard solution, LC-MS can detect minute concentrations of many different elements or compounds as well as selectively analyze a single element or compound in a solution.

3 Protocols and Methods

To begin the experiment, protocols were drawn and tested to ensure accuracy. This includes: sampling, centrifugation, methanol extraction, ultra-sonifacation, vortex mixing, and solid phase extraction, followed by LC-MS. Each step of the sampling and pre-treatment phase was carefully planned and carried out exactly according to the protocol that was written. Each protocol was planned according to the need of this experiment and were recorded to be followed exactly during the sampling and pre-treatment process. The following paragraph are meant to explain the protocol as they were used.

3.1 Overview of Experiments

The original experiment was comprised of 4 1000ml beakers. Of the beakers, two contained 500ml algae water and 0.5mg/L tylosin, and two contained 500ml of pure water with a phosphate buffer and no algae, brought to a specific pH according to the algae samples diurnal pH, and 0.5mg/L tylosin. Each beaker was given 1 replicate to double check results and help eliminate outliers. This gave a total of 8 beakers in the experiment. The groups of beakers were placed in an incubator to remain at a constant temperature of 30°C for 48 hours. The beakers were split into two groups, a night cycle group and a day cycle group.

Table 1: Original Experiment Layout with Labelled Beakers and Replicates

NIGHT	DAY	NIGHT	DAY
Algae pH 6	Algae pH8	Phosphate pH6	Phosphate pH8
Algae pH6 Replicate	Algae pH 8 Replicate	Phosphate pH6 Replicate	Phosphate pH8 Replicate

 Each experiment group was done under the same conditions except for light. The night group of beakers, with 2 algae samples and 2 phosphate buffer samples, were placed in a light tight box below the group of day samples. The beakers were set in an EYELA multi shaker mixing tray, set to approximately 160 RPM. The incubator was outfitted with 4 visible spectrum 60w Phillips light bulbs and 2 20w Panasonic FL205 black light bulbs "that emit wavelengths of 300-400nm, peaking at 360nm (mainly UVA radiation)" (Danno, 1983). Samples were drawn at 0 hours, 1 hour, 2hours, 4 hours, 24 hours, and 48 hours. The phosphate buffer samples needed only SPE (Solid Phase Extraction), so any

phosphate buffer samples could be put immediately to the freezer to be treated after the experiment had run its course.

3.2 Sampling

The method of sampling used in the algae experiment involved using an auto-pipette with single use plastic tips attached. Each sample was drawn with a new, unused tip. The sample sizes were all 10ml, except in the case of the algae sample, where 5ml of pure water was used to flush the remaining algae particles from the pipette.

The algae samples needed to be processed right away to avoid possible further degradation of the tylosin. To this end, as soon as an algae sample was taken, pre-treatment was started at once and the following algae samples were queued. Four samples could be pre-treated at once, and since there were four beakers in the experiment that contained algae, each round of sampling was regarded as one sample to avoid confusion.

3.3 Methanol Extraction

The methanol extraction protocol was used only with the algae samples and combines the centrifuge, ultra-sonifacation and vortex mixing. During this process the sample is centrifuged for 10 minutes and the liquid phase is removed into another test tube. 5ml of 1:1 KCl:Methanol is added to the solid phase and the sample is then vortexed for 3 minutes. After vortex mixing, the sample is placed in an ultra-sonic bath for 10 minutes and then centrifuged again for 10 minutes before separating the solid phase from the liquid. The process is repeated twice and the total volume of the algae sample after methanol extraction is 25ml.

3.3.1 Centrifugation

Centrifugation was used to separate the solid phase from the liquid phase. The centrifuge was set to operate for 10 minutes at 6000RPM. Centrifuge was used in addition to ultra-sonifacation and vortex mixing, to complete the methanol extraction process. The centrifuge was used 3 times for each sample in total.

3.3.2 Vortex Mixing

The vortex mixer is a small machine with a rubberized, pressure sensitive head that, when pressed down upon begins to rapidly oscillate, creating a vortex with the liquid inside the test tube. The Vortex mixer is used twice for 3 minutes each time. This ensured that the algae is well mixed in the methanol solution and allows the tylosin to break free from adsorbtion by the algae. The vortex mixer does not have specific RPM settings and instead has stepped speed settings. The speed setting used in this experiment was step 6 of 10 steps.

3.3.3 Ultra-sonifacation

The final step, before returning to the centrifuge is ultra-sonifacation. In this protocol the algae samples, now well mixed with the methanol solution, are put into an ultrasonic bath. This phase last 10 minutes and allows any remaining adsorbed tylosin to break away from the algae so it can be extracted.

3.3.4 Evaporation

After methanol extraction is complete, the methanol in the sample must be removed. If there is any methanol remaining in the sample during the sample loading process in the solid phase extraction protocol, the methanol will cause the some of the sample to leach through the cartridge and will not be recoverable. The easiest way to accomplish this is by evaporation. At this stage the sample contains 10ml of methanol that must be removed. Methanol evaporates at a lower temperature than water and a simple hot water bath, set at a temperature of 35°C, with a suction pump was used to evaporate and remove the methanol from the remaining sample. The evaporation time was 5 minutes, at which time the suction was turned off and the pressure released. The flask was then carefully removed as the pressure in the bottle returned to normal. If the flask is removed to quickly, there is a risk of the sample being forced out of the flask by a sudden influx of air.

3.4 Solid Phase Extraction for Algae Sample

After methanol extraction was completed, Solid Phase Extraction (SPE) needed to be performed in order to isolate the Tyolsin concentration remaining in the sample. SPE will also remove any fine particles that might clog the LC-MS. In the protocol for the algae samples, Waters SPE C-18, 6cm^3 cartridge with 200mg of sorbent material with a 30μm particle size were used. The C-18 cartridge resembles a syringe with no plunger inside,

with absorbent packing in the tip. According to Waters, the protocol used in this experiment is referred to as Normal-Phase Chromatography. This is defined briefly as "a mode that is classically used to separate neutral organic compounds whose chemical nature ranges from hydrophobic to moderately polar. (Waters, 2014) The protocol that was outline by the water company was used to perform the SPE procedure below.

In this protocol, after methanol extraction was completed, the algae samples were diluted to 100ml in pure water adjusted to pH3 using HCl. To avoid confusion of samples, the cartridges and samples were given a number in black for an original sample and in red for replicate sample. The samples were also given a duplicate label with any information pertaining to that sample to be placed on the finished sample vial at the end of SPE. Once sample loading began, this was the only way to tell the samples apart from one another.

The SPE suction manifold was prepared by checking that the suction valve was secure and blocking any ports in the lid that would not be in used to ensure proper suction. Up to 6, C-18 cartridges were then inserted tip-first into the ports securely. The cartridges needed to be primed before loading the 100ml samples. To do this 5ml of HCP grade methanol was added to each cartridge. After priming, it was vital to ensure that no methanol remained in the cartridge, as this would cause the sample to fail to be cached in the sorbent material. To this end, 10ml of pH3 water by HCL was added, 5ml at a time. As the pH3 water flushed the cartridge, a 60ml syringe was inserted into the top of the cartridge with a small adapter. When the meniscus of the pH3 water had roughly one quarter left to travel before reaching the sorbent material in the cartridge, the samples were poured into the 60ml syringes. This process is called sample loading, as the sample drains through the sorbent material, the tylosin will be cached, or will be "loaded" into the material. During sample loading a suction pump is used and the pressure inside the SPE manifold was regulated a 0.2 PSI. It is important to note that during this stage of SPE, priming, washing and sample loading, it is vital that the sorbent material is not allowed to dry.

After the sample has been loaded, 5ml of pure water was flushed through the cartridge to wash out any contaminants. This is done to ensure that on the tylosin remained cached in the sorbent material, so when methanol was added on the tylosin will be obtained. After washing with pure water, the pressure valve is closed and the suction was brought to maximum at 0.5psi. This is left for 10 minutes to allow the cartridge to dry out. Once this is finished, the methanol and water mixture caught in a dish in the bottom of the SPE

manifold is disposed of properly and a test tube rack is placed inside the manifold. The test tube rack is designed to line up with the ports on the manifold cover. 10ml glass vials are place in the slots that correspond to the ports in use. Next the ports must be primed with methanol. To do this, one by one, the cartridges were taken out and, over a collection dish, methanol was passed through the port. Once all the ports had been primed and the cartridges had been returned to their original position the elution stage could take its course.

Elution is the defined by the Merriam-Webster Dictionary as "the removal of (adsorbed material) from an adsorbent by means of a solvent" (Merriam-Webster, 2014). In this experiment methanol is used as the solvent to elute the adsorbed material (tylosin) from the sorbent material. To do this 10ml of methanol was added to the C-18 cartridge and a 10ml syringe with an adapter tip that fit firmly into the top of the cartridge was used to slowly force the methanol into the dried sorbent material. When the first drop appeared from the bottom of the port, the syringe was removed and the methanol was left to elute the tylosin from the cartridge and the next cartridge was prepared for the same process.

Once the elution was finished and all the methanol had passed through the C-18 cartridge, the 10ml syringe with the adapter tip was used again. In this use the syringe was used to force air into the cartridge and push any remaining methanol out of the cartridge and into the glass sample vial. Following the final removal of methanol from the sorbent material in the C-18 cartridge, the cartridge was disposed of. At this point the vials were all checked to ensure the 10ml of methanol remained in the vial and were corrected by either adding methanol to the sample or evaporating a portion of the methanol out of the sample. If evaporation at this stage was needed, a small test tube evaporator was used. This evaporator was simply a series of tubes into which the vial was placed and the tube was heated to 35°C. Above each vial receptacle was a thin piece of metal tubing through which nitrogen gas was allowed to flow. This process allowed precise control over the rate of evaporation by allowing the user to control the gas flow.

Following SPE, the samples could be frozen and analysed at a later time within 3 weeks. The samples at this stage were now ready to be analysed in the Liquid Chromatography Mass Spectrometry anaylzer.

3.4.1 Solid Phase Extraction for Phosphate Buffer Samples

The protocol for the phosphate buffers that were frozen after sampling is similar to the protocol for the algae samples. It is not necessary to perform methanol extraction or any evaporation at this stage for the phosphate buffer samples. In this protocol, only SPE is needed and a small cartridge is used. This protocol calls for a $3cm^3$, C-18 cartridge with 60mg of sorbent material. With this smaller cartridge, a smaller amount of methanol and pH3 water are also needed.

The preparation is very much the same as the algae sample's SPE protocol. The phosphate buffer samples were all diluted up to 100ml with pH3 water by HCl. Up to 6, $3cm^3$, C-18 cartridges were loaded into the suction manifold tip-first. In this protocol 3ml of methanol was used to prime the sorbent material. After the sorbent material was primed, and just before the meniscus reached the sorbent material, 6ml of pH3 water was added to each cartridge, 3ml at a time to ensure no methanol remained. Once the last 3ml of pH3 water was added the 60ml sample loading syringes with their adapter tips were fitted firmly in place on top of each cartridge. When the pH3 water was nearly drained from the cartridge, the samples were added to the 60ml syringe and allowed to drip. At this stage the suction pumped was turned on and the pressure was regulated at 0.1psi until the samples were loaded to the sorbent material. Once finished, 3ml of pure water was used to wash the contaminants from the sorbent material.

At this point the cartridges needed to be dried for 10 minutes at maximum psi (approximately 0.5psi). Once the drying stage is completed, the sample can be frozen again or they can be eluted immediately. In this experiment the samples were eluted before being frozen. Elution in this protocol was prepared the same way it was for the algae samples. The test tube rack was placed inside the suction manifold and 10ml glass vials were placed into the appropriate positions. Each port was then primed with a small amount of methanol and the cartridges were repositioned. Elution was performed by adding 6ml of methanol to the cartridges and forcing enough air into the cartridge with the 10ml syringe equipped with the adapter tip to allow the first drop to appear in the vial. Once elution was completed, the same 10ml syringe was used to push air into the cartridge and force out any remaining methanol. At this stage the volumes of the vials were examined to ensure that there were 6ml of methanol. If there were any vials that had to little methanol, the volume was increased to the appropriate 6ml. If any vials had more than 6ml, the evaporation apparatus was used to evaporate enough methanol to confirm 6ml remained

in the vial. The phosphate sample were now ready to be analysed by LC-MS or frozen to be analysed at a later time.

3.4.2 High Pressure Liquid Chromatography-Mass Spectrometry Analysis

The LC-MS protocol started with an existing protocol to detect tylosin in small concentrations and was optimized to fit the experiment, requiring nearly half the retention time in the column. The existing protocol required 18 minutes of retention time in the column to analyze the elements of the sample, whereas the optimized protocol required only 10 minutes, effectively doubling the amount of samples being analyzed. Each sample volume is only 6ml, however this is more than sufficient for the injection into the column as the injection volume is between 1 an 2ml. The samples are passed through a Sunfire C-18 column with a particle size of 3.5µm containing a stationary phase of 1.6% Formic acid in Acetonitrile for 10 minutes. The mobile phase of 3% Formic acid in a 1000ml solution on 10mM of Ammonium Formate was used to pass the sample through the column.

The LC-MS procedure is automated after the initial setup with the software, and loading the samples to the autoloader tray. The setup procedure will be different, with different with different software or machines. In this experiment the software Empower was used.

4 Results

Each set results from the LC-MS came in groups of 3, corresponding to the number of injections tested from each sample. The 3 results were averaged together, unless there was an obvious outlier, in which case the outlier was excluded from the average. Once all of the results were averaged for the duration of the experiment, the averages for those sample are put into a scatter graph in Microsoft Excel.

There were 3 experiments in total. The first was a simple test experiment to determine possible time frames for sampling and experiment length. The second experiment was with *Scenedesmus* algae degrading tylosin and phosphate buffer degrading tylosin under UV radiation. The third experiment involved only a phosphate buffer degrading tylosin under UV radiation. The following section will reveal the results from these experiments.

4.1 Test Experiment

Before the experiment began, a test run was performed using only pure water at a pH of 7.3 and 0.5mg/L tylosin. This test was run for 24 hours and was done to estimate how long to run the main experiments. The samples collected were analysed against a standard solution, which can be seen in Figure 2. The results of this showed both an error and the data needed for a rough estimate of the time required for the experiment. As seen in the graph, Figure 1, the initial concentration is incorrect. The initial concentration should have been 0.5mg/L, and here it is approximately one order below that concentration at about 0.065mg/L. This lead to a double checking of the protocol for the stock solution and the error was identified as a miscalculation of dilutions. The stock solution was re-made correctly and checked via LC-MS for errors.
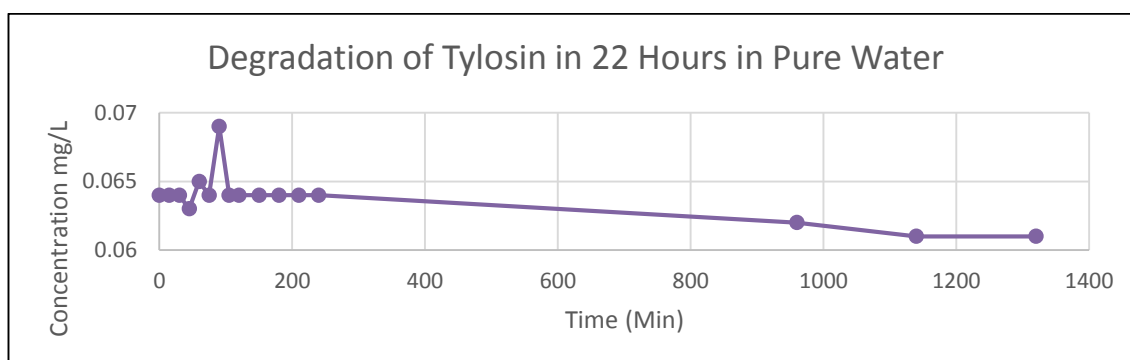

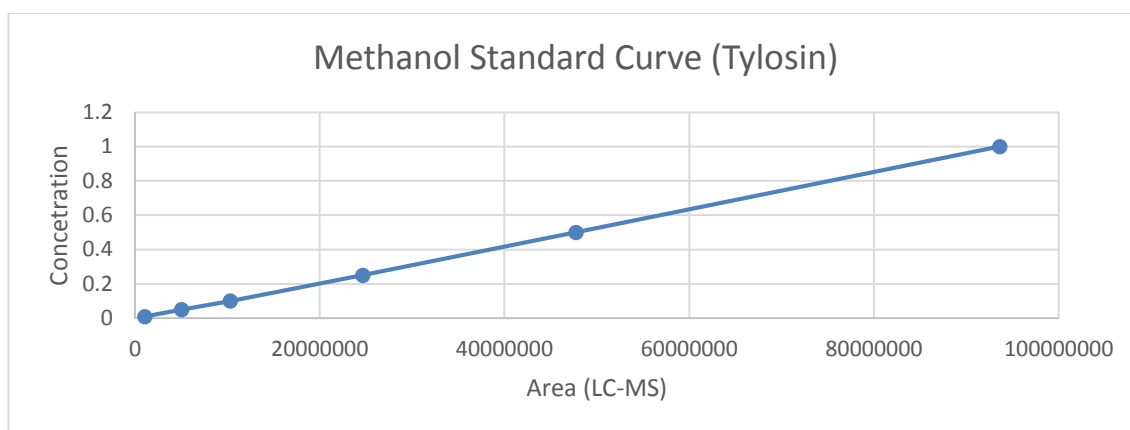Figure 1: Pre-test Experiment Results

Figure 2: Methanol Standard Curve for Concentrations from 0.01mg/L to 1mg/L

The graph also shows that no major degradation occurred in pure water at pH7.3 in 22 hours. This is because tylosin is most stable at pH7 and is not easily degraded. This was expected, but the graph suggests that an experiment run for 48 hours at higher or lower than pH7 will show degradation in the allotted time.

4.2 Algae, Phosphate Buffer and UV Radiation Results

The results of the algae and buffer solution experiment were unexpected. In the experiment the algae samples underwent rather extensive pre-treatment before finally being analysed. It can be seen in figures 3-6 that the degradation curve is not what is expected and doesn't appear logical. The initial concentrations seem to be incorrect, even after preparing a new stock solution that was tested and confirmed to be correct.
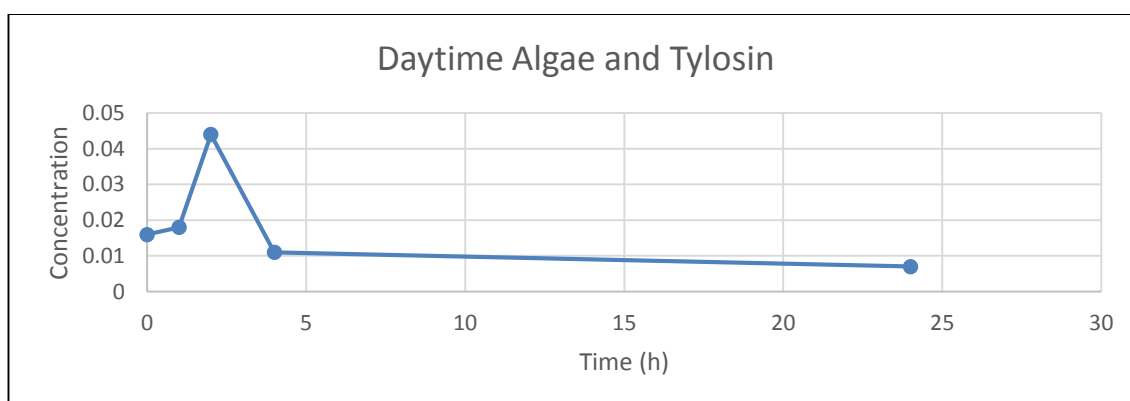

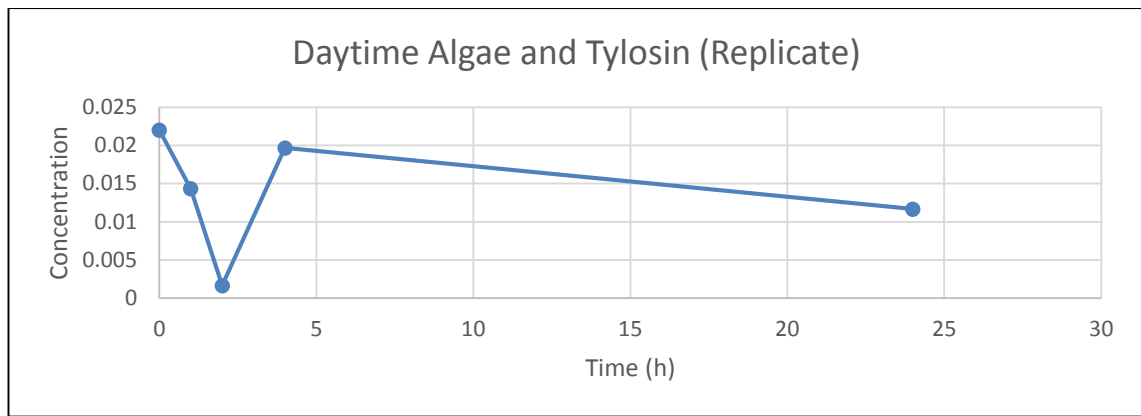Figure 3: Day results from original algae experiment pH8

Figure 4: Day results from original algae experiment pH8 (Replicate)
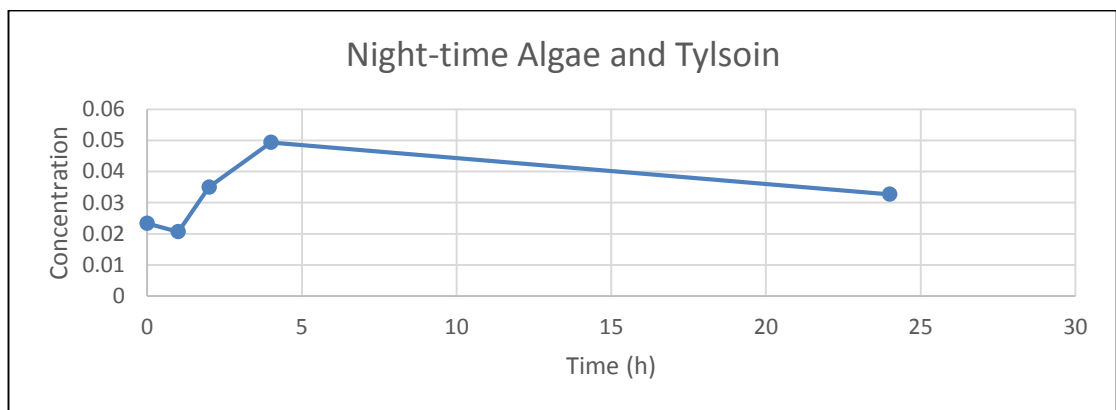

Figure 5: Night results from original algae experiment pH6
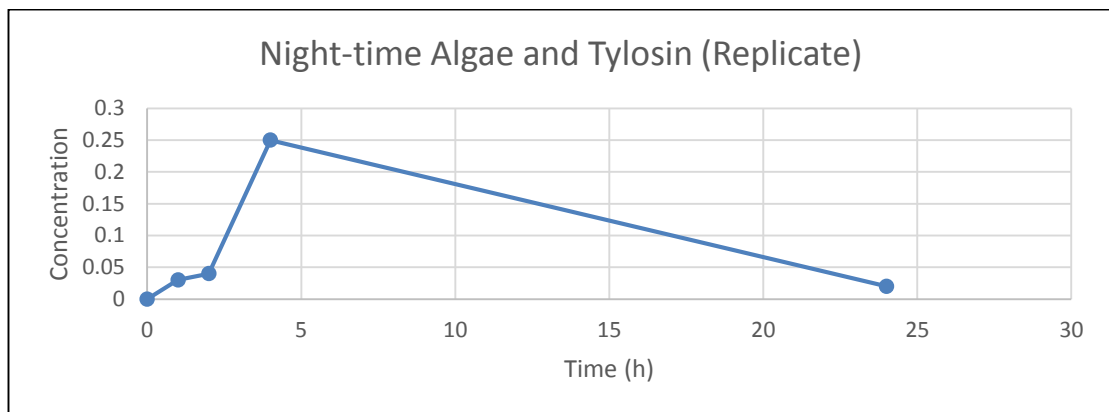

Figure 6: Night results from original algae experiment pH6 (Replicate)

The erroneous results and pre-treatment protocols were re-examined by the professors and peers to find any error in the protocol that could have led to the results obtained from this experiment. Any possible mistakes in the protocols were tested separately and each one was proven to be correct. It is unknown at this stage in the experiment what could have caused such concentrations.

4.3 Phosphate Buffer and UV Radiation Results

In the experiment containing only phosphate buffers, the results were much more in line with what was anticipated. The experiment ran without any issues and, because there is no algae in this experiment, the pre-treatment was minimal. The results of this experiment can be seen in figures 7-14. In the results for this experiment, it can been seen that the initial concentrations are much closer to the desired 0.5mg/L, with a standard deviation of approximately 0.3mg/L. The concentrations are measured against the methanol standard solution seen in Figure 2.
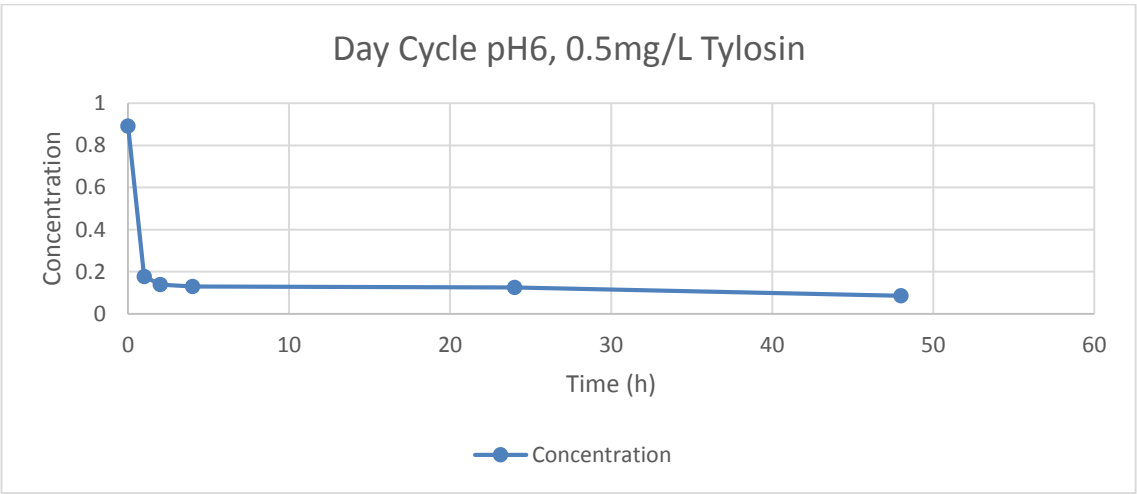

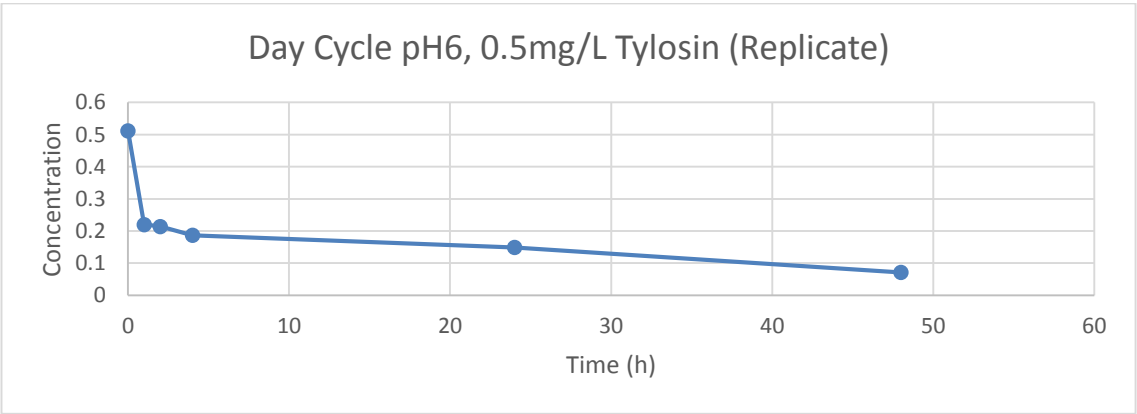Figure 7: Results from 2nd experiment with phosphate buffer at pH6, day cycle

Figure 8: Results from 2nd experiment with phosphate buffer at pH6, day cycle (Replicate)
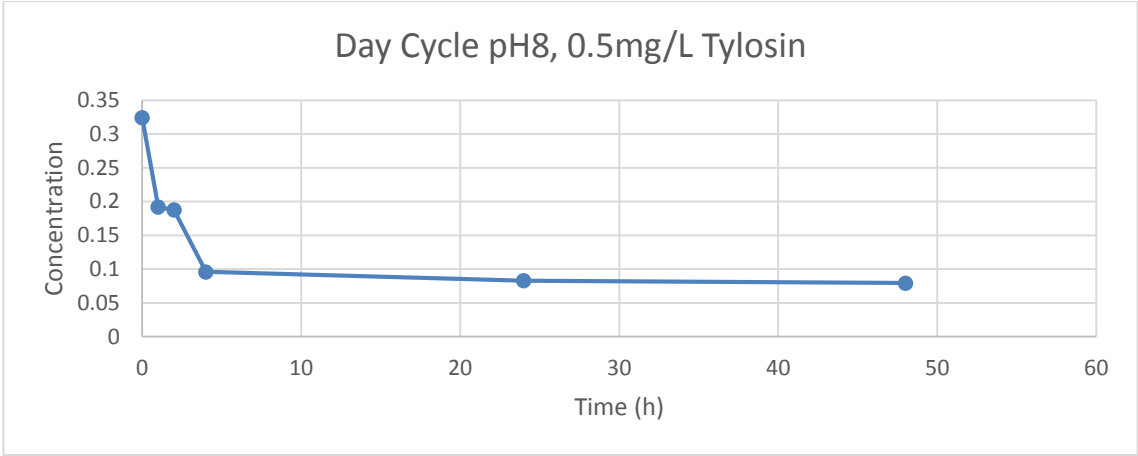
Figure 9: Results from 2nd experiment with phosphate buffer at pH8, day cycle
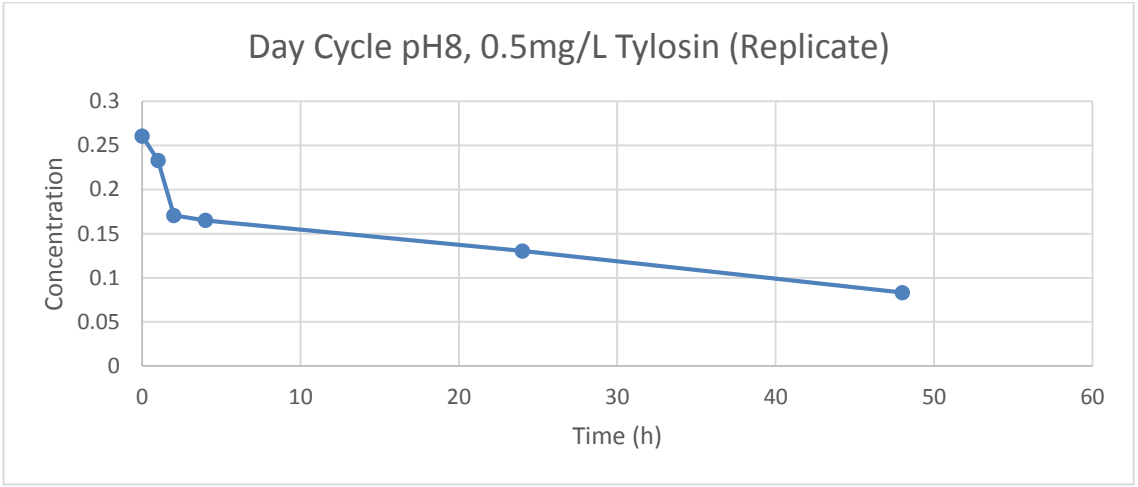

Figure 10: Results from 2nd experiment with phosphate buffer at pH8, day cycle (Replicate)
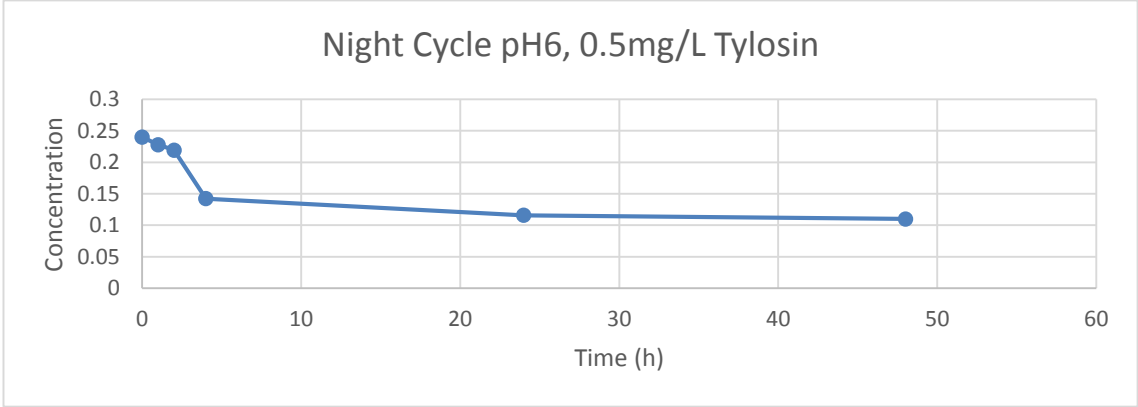

Figure 11: Results from 2nd experiment with phosphate buffer at pH6, night cycle
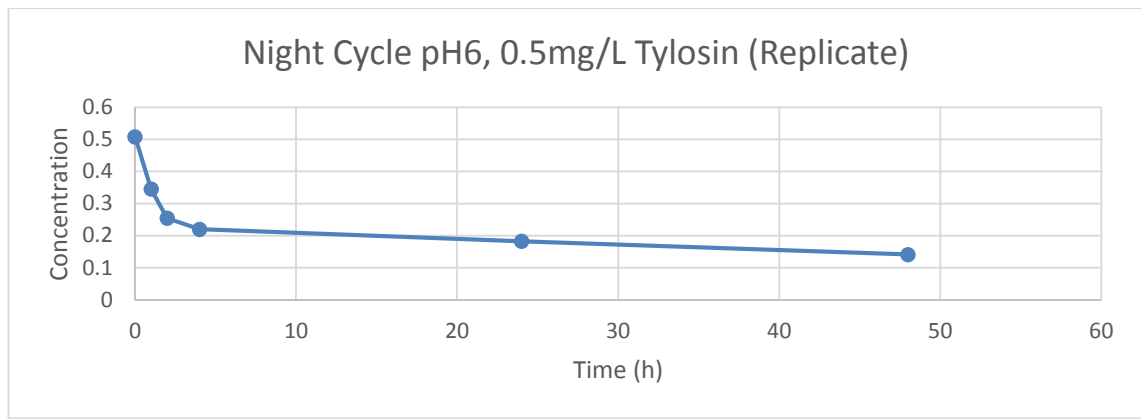
Figure 12: Results from 2nd experiment with phosphate buffer at pH6, night cycle (Replicate)

Figure 13: Results from 2nd experiment with phosphate buffer at pH8, night cycle

Figure 14: Results from 2nd experiment with phosphate buffer at pH8, night cycle (Replicate)

It can be concluded that the experiment was successful in degrading tylosin from the data acquired. Even with differing starting concentrations the final concentrations are similar, with an average of 0.08mg/L with a standard deviation of approximately 0.02mg/L. These final concentrations are of interest because each of the samples ceased degradation at

roughly the same concentration. This could have been due to time restrictions in the experiment. If the experiment had been allowed to run for 72 hours, the final concentration could possibly have been lower. Though it is possible that unless the pH was changed to lower or higher values, respective of the starting value, the final concentrations of tylosin could have become stable again at those concentrations at the pH 6 and pH 8.

5   DISCUSSION

The original experiment with algae was intended to be the only experiment performed, along with the test experiment and methanol standard curve analysis. The experiment containing only phosphate buffers and tylosin was constructed under time constraints and a need for accurate results for the degradation of tylosin. Given more time, the cause for errors in the initial algae experiment could have been determined and correct.

5.1   Algae Results Errors

In the algae experiment, any portion of the pre-treatment protocol that was suspected of interference with the results was tested on its own. Each section of the protocols for sampling, methanol extraction and SPE were taken into account as much as possible due to time constraints in the experiment.

5.1.1 Pure Water Addition

Initially it was thought that the 5ml of pure water used to wash the sampling pipettes was diluting the sample. This would have explained the low initial concentrations retrieved from the samples, however, was quickly discounted because, in the end, the sample is run through the SPE protocol which only caches the tylosin. Adding 5ml of pure water at the time of sampling would have no effect on the concentration of tylosin that was in the sample to begin with.

5.1.2 Evaporation of methanol

After the methanol extraction protocol, the methanol that was added to the sample to remove the tylosin from the algae particles needed to be evaporated. The volume of methanol in the sample at this stage is 10ml of methanol of the total 25ml of liquid in the sample. In the protocol the 5 minutes allowed for evaporation should have been enough to evaporate all of the methanol in the sample. If any methanol remained in the sample after this stage, the SPE protocol would be compromised, because the methanol would allow the tylosin to be flushed through the absorbent material in the cartridge rather than caching.

To check that no methanol was being left in the sample, a false sample was prepared with 10ml of methanol in 5ml of pure water. This false sample was attached to the evaporator in a bulb flask and allowed the standard 5 minutes to evaporate in a water bath set to the

standard 35°C. When the time was up, not only had the methanol evaporated, but also approximately 1ml of pure water had evaporated from the flask as well. This proved that there could not have been any amount of methanol in the sample after the evaporation stage was complete and that the protocol set was, in fact, correct.

5.1.3 Possible SPE errors

The last possible protocol that could contain errors was the SPE protocol. This was unlikely as the protocol is nearly the same for any compound being cached in the C-18 cartridges. The protocol required testing regardless of this fact. To test the SPE protocol, the method of confirming recovery rate of the SPE protocol was called into service. In this instance, the false sample was a sample taken from stock solution made to a concentration of 0.5mg/L of tylosin in pure water. The false sample was then run through the protocol exactly as the algae samples had been before. After the sample was eluted and ready to analyze, a 6ml sample was run through the LC-MS. The results can be seen in appendix 4. The recovery rate showed a very good result. Given this information, the SPE protocol was ruled out as the cause for error. The recovery rate results also ruled out any errors in preparing the stock solution as the same protocol was used in the experiment in the beginning.

5.2   New experiment

The primary factor behind creating a new experiment, without algae involved at all, was time. There was a significant amount of work to do to discover what the problem could have been in the protocols. The buffer solution in the algae experiment had an erroneous initial concentration, but the rest of the results appeared to be more or less correct. Creating a new experiment containing only the phosphate buffer with tylosin in pure water seemed to be the next logical step.

The protocols in place in this new experiment were, again, the very same protocols used in all of the experiments. The results that were taken from this new experiment had small deviations, but overall were reliable in the fact that the initial concentrations were only a standard deviation of approximately 0.03mg/L.

5.3   What the results show

It is known that tylosin is stable in water at a pH of 7 and does not degrade well. The results of this experiment show that tylosin is easily degraded at least one order from its

initial concentration when the pH is at higher or lower than pH7, thus destabilizing the compound enough to allow degradation. The results also show that although the initial concentrations were slightly different, the degradation had a final concentration of roughly 0.08mg/L. This can be better seen in Table 1.

Table 2: Tylosin Degradation under UV Radiation in Presence of Phosphate Buffer at Respective pHs

Time (h)	Day pH6 mg/l	Day pH6 Rep mg/l	Day pH8 mg/l	Day pH8 Rep mg/l	Night pH6 mg/l	Night pH6 Rep mg/l	Night pH8 mg/l	Night pH8 Rep mg/l
0h	0.90	0.51	0.32	0.26	0.24	0.50	0.79	0.58
1h	0.18	0.22	0.19	0.23	0.23	0.35	0.26	0.27
2h	0.14	0.21	0.19	0.17	0.22	0.25	0.20	0.20
4h	0.13	0.19	0.10	0.16	0.14	0.22	0.19	0.20
24h	0.13	0.15	0.08	0.13	0.12	0.18	0.18	0.18
48h	0.08	0.07	0.08	0.08	0.11	0.14	0.08	0.17

The reason for this halt in degradation could be as simple as a time issue or could be related to a second order degradation. It is a standing question whether or not the tylosin would degrade further if the experiment had 72 hours instead of 48 hours.

The results in the table also show that, on average, the UV radiation in the day cycle degraded the tylosin slightly faster than the night cycle, which had no UV radiation. In addition to this there are the different pH levels. It seems that the samples at pH 6 degraded very slightly better that pH8 samples in the day cycles, however in the night cycles pH8 degraded slightly better. It is also interesting to note that, given the rather large discrepancy of initial concentrations, the levels seems to have normalized to some extent to right around an average of 24mg/L at 1 hour into the experiment. This is certainly interesting, especially when coupled with final concentration results. It is curious that the concentrations seem to normalize and degrade roughly around the same rate, though they started at different concentrations.

The UV radiation used in the experiment was enough to degrade even this large concentration of 0.5mg/L of tylosin, though in wastewater the concentrations of tylosin are much smaller, in the microgram per liter range. It is unknown if the methods used in this experiment can be transplanted to a real-world application where the concentrations are quite small. This question requires further experimentation and fact finding to answer. Also the use of algae, which was the original focus of this research, is an interesting and proven bioremediator of heavy metal laden water. Dr. Seema Dwivedi stated that "it is also possible to use mainly algae to clean waste water because many of the pollutant sources in waste water are also food sources for algae. (Dwivedi, 2012). It seems logical that algae could degrade pharmaceuticals as well as heavy metals. A few research experiments concerning algae and pharmaceuticals have shown promise and gave rise to this experiment, though under such time restraints, the errors in the results could not be identified and corrected, and so in the end the experiment had to be modified to the current experiment with no algae.

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