# The development of a method of estimating postmortem submersion interval (PMSI) using the colonization of artificial substrates by freshwater algae

By

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# Abstract

In terrestrial criminal investigations, entomological evidence is employed to determine the elapsed time from death until discovery or the postmortem interval (PMI). Certain insects will colonize a corpse in a predictable sequence as it decomposes, allowing for a PMI estimate to be made. For remains recovered in aquatic environments, no human flesh eating insects exist. Therefore, PMI estimates can not be made using standard terrestrial methods. A proposed alternative is to use the colonization and growth of epiphytic algae to determine the postmortem submersion interval (PMSI). Epiphytic algae are ubiquitous, present throughout the year and will colonize most submerged natural and artificial surfaces. A simple clamp apparatus was used to suspend two types of fabric and a human hair sample in two freshwater ponds of differing nutrient status. The two week trials commenced in June and continued through to October. Total diatoms were used as the indicator group on the cloth, while *Cocconeis pediculus* was used as an indicator species on the hair substrate. In the eutrophic pond, diatom colonization of these artificial substrates showed characteristic logistic growth while the mesotrophic pond showed slower growth over the two week submersion period. There was little seasonal effect on the rate of colonization in the mesotrophic pond; however, there was a temperature effect in the eutrophic pond between the June and October trials. This exploratory study examined the most effective method for removal and enumeration of the epiphytic algae, as well as the feasibility and accuracy of this method of estimating PMSI.

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I would also like to thank my committee members (Dr. Donna Young and Mrs. Nancy Loadman), the biology department technical staff and Dr. Moodie, for their assistance on this project. Finally, the Rossmere Golf and Country Club, as well as the Fort Whyte Center deserve acknowledgment for their generosity in sharing their much needed resources.

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# Introduction

## 1-1: A brief history

The field of forensic science has come along way since its reported beginnings in the 700s, when the Chinese used fingerprints to establish the identity of documents and clay sculptures (Rudin and Inman 2001). The term 'science' originally came from the Latin word meaning "to know", and the word is still sometimes used in this way; however many scientists today have come to refer to science in terms of the scientific method. Scientific method involves the collection, observation, and interpretation of data in a systematic, logical and objective fashion (Greenshields and Scheurman 2001). The word 'forensic' originates from the Latin word forensus, which means "of the forum." The forum was the general assembly where lawmaking, governmental debates and trials were held (Koppl 2005). Together these two terms evolved from their earlier definitions to refer to the application of the natural and physical sciences to matters of law. Forensic science is a broad field, encompassing all aspects of the scientific method in establishing the criminal guilt or innocence of a suspect in an objective manner (Saferstein 2004).

Many believe that Sir Arthur Conan Doyle had a significant influence on popularizing scientific crime detection methods and encouraging the imaginations of emerging forensic investigators. The character of Sherlock Holmes revolutionized crime solving techniques at a time when the "smoking gun" was necessary for confident convictions (Saferstein 2004). The simple deductive methods used by Holmes attracted a wide audience and stimulated interest in the scientific community (Synder 2004).

It may be just coincidence, but since Doyle's creation of Holmes there have been many scientific breakthroughs that have enabled considerable growth in the field of forensic science including: DNA fingerprinting; the physical examination of fibres, glass, paint, explosives, firearms, and soil; document examination; fingerprint analysis and the chemical analysis of drugs, bodily fluids, hairs and botanical materials (Saferstein 2004). Although these techniques have furthered the field of forensic science considerably, no problem has continued to eluded forensic scientists as much as the need to fix time of death to within the limits of probability (Jaffe 1983).

Similar to Holmes, present day murder mystery and crime solving shows such as 'CSI' and 'Law and Order' spend most of the episode carefully trying to determine cause of death and trying to find a suspect, while avoiding the question of time of death (Sachs 2001). In reality the challenge lies in determining the time of death. Cause of death is usually obvious to the investigator responding to the scene of the murder (Sachs 2001), as well, the techniques listed above provide a basis upon which to establish a suspect. However, simply establishing a prime suspect is insufficient for a guilty verdict, since in the modern criminal justice system a suspect is "innocent until proven guilty" (Sachs 2001). Time of death can be important in the determination of criminal guilt through the legal question of alibi and opportunity (Jaffe 1983). If the suspect is able to prove that he/she was at some other place when the fatal injury was inflicted then he/she has an alibi and his/her innocence is implied. Conversely, if the time of the fatal injury coincides with the time the suspect was known to be in the vicinity of the victim then the suspect had the opportunity to commit the crime.

**1-2: Determining the postmortem interval**

The determination of the postmortem interval (PMI) or the time elapsed from death until discovery of a corpse, has undergone rigorous scientific study. A classic trio of anatomical stopwatches; rigor mortis, algor mortis and livor mortis were first described in the third and fourth centuries and are still used to this day (Sachs 2001). Rigor mortis is the gradual muscle stiffening that manifests within the first 24 hours and disappears within 36 hours (Saferstein 2004). Algor mortis represents the slow cooling of the corpse as it equilibrates with ambient temperature (up to 24 hours) (Saferstein 2004). Livor mortis or lividity refers to the gradual settling and pooling of blood that begins the moment the heart stops pumping. The blood continues to settle up to 12 hours after death (Saferstein 2004).

These standard time pieces have proven unreliable due to deaths infinite variations such as; age, body size, heath, manner of death, ambient temperature, air movement, location, and victim's clothing (Sachs 2001). Although medical examiners are cautious to dismiss all variables, reasonable doubt still exists in these time of death estimations. Furthermore, two days after death, these three time pieces (already only marginally helpful) become useless (Sachs 2001). Other anatomical methods that were quickly dismissed due to their inadequacy when applied to actual murders included stomach contents identification, rise in potassium levels in the eye's jelly-like vitreous humor, and the waning ability of the cadaver's muscles to respond to mild electric shock (Sachs 2001).

It is self-evident that the longer the interval of time between death and examination of a body the wider the limits of probability (Jaffe 2001). That is why a researcher must be wary of relying on single observations to determine time of death and must also be aware of

the associated environmental influence (Jaffe 2001). In fact, the longer the PMI, the more likely that environmental evidence will furnish more reliable data upon which to estimate the time of death than will anatomical changes (Jaffe 2001).

In terrestrial environments, entomological evidence has now become a widely accepted tool in homicide investigations to determine the PMI (Wolff *et al.* 2001; Centeno *et al.* 2002). This is due to the fact that entomological evidence takes into account environmental variables and is not based on a single observation of the corpse's anatomy but on careful examinations of insect development on the corpse.

Invertebrates will colonize a corpse in a predictable sequence (Wolff *et al.* 2001; Centeno *et al.* 2002; Anderson 2002). This predictable sequence can be used to determine the time between death and discovery (Wolff *et al.* 2001; Hobischak and Anderson 2002). *Callipohoridae* or blow flies will colonize and lay eggs on the corpse immediately after death. These eggs hatch into maggots which continue to feed on the remains. Eventually the maggots will leave the body and pupate, forming a pupal case. The empty pupal case is discarded following metamorphosis and left behind as evidence that the cycle has taken place (Reiter and Grassberger 2002). The entire cycle takes a minimum elapsed time of 25-29 days (Anderson 2002).

The first successful use of entomological evidence occurred in the UK when two decaying bodies were discovered in a small ravine in Dumfriesshire, Scotland, in September 1935. Blow fly maggots were aged and provided a vital clue as to when the murders took place (Goff 2000). The use of entomological evidence did not continue to develop steadily from that point on. It wasn't until the 1980's that entomological evidence

gained acceptance in law enforcement (Goff 2000). Ground breaking research carried out by a group of researchers known as the 'Dirty Dozen' helped relate what was known about the reproductive and developmental cycles of insects to environmental conditions such as temperature and humidity. These two variables generally influence insect colonization and community composition (Sachs 2001). Such experiments have revealed that time of death estimates are approximations and are greatly influenced by a number of environmental variables. Also, these studies have shown that most variables can be accounted for by controlled experimentation (Goff 2000). The rigorous peer review process which this system has undergone has resulted in a valuable tool for forensic science and criminal investigations.

**1-3: Determining the postmortem submersion interval**

Presently, there is no method to determine time of death when bodies are recovered from water. This is known as the postmortem submersion interval (PMSI). Terrestrial invertebrates can not be used as indicators and aquatic invertebrates have not proven reliable (Casamatta and Verb 2000). Studies pertaining to corpses in aquatic habitats have focused primarily on terrestrial insects colonizing the corpse as they bloat and rise to the surface (Haskell *et al.* 1989). The time it takes for the body to bloat and rise to the surface is usually 11-13 days (Hobischak and Anderson 2002). Colonization by terrestrial insects can therefore only begin following this time period. If a body is recovered prior to the eleven day minimum a PMSI estimate can not be made.

Further attempts to estimate PMSI have focused on trying to find an equivalent invertebrate successional pattern in water as was observed on land. As Haskell *et al.* (1989)

point out, the primary problem in aquatic environments is that there are no purely sarcophagous or flesh eating aquatic insects, to compare with the common terrestrial indicator species, the blow fly. In 2002, Hobischak and Anderson tried to determine if non-flesh eating aquatic invertebrates would colonize pig carrion in a predictable fashion, however, it was quickly discovered that there were far too many variables that affected colonization.

A direct estimation of PMSI following terrestrial models is currently not possible. A major constant component of all aquatic ecosystems is the algal community. Algae are ubiquitous, present throughout the year (Haefner *et al.* 2004) and epiphytic algae will colonize most submerged surfaces natural and artificial (Barbiero 2000). The use of community structure, succession and colonization of epiphytic algae has been suggested by two groups of scientists in PMSI estimates, Haefner *et al.* (2004) and Casamatta and Verb (2000). Their research was performed on pig carrion and rat carrion respectively. Both studies were preliminary tests which provided some evidence for the use of algal colonization as a useful PMSI clock. Casamatta and Verb (2000) pointed out that immature rat corpses do not directly approximate human skin and are therefore not a choice substrate. Haefner *et al.* (2004) chose pig carrion due to its close similarity to human skin. It was noted in their study that the presence or absence of clothing on the carrion affected algal growth. The carrion that were clothed showed less algal growth on the flesh than unclothed carrion. However, the study neglected to indicate whether the clothing itself had been colonized. In Canada, murder victims tend to be clothed therefore it is important to understand how clothing affects the algal colonization process.

**1-4: Objectives**

A possible alternative to using algal colonization on submerged carrion is to examine how algae will colonize substrates often associated with a submerged body such as hair and cloth. The goal of this research is to examine if the time of submergence of hair and cloth can be determined from the rate of colonization of epiphytic algae present in the water in which these substrates have been immersed. The experimental method assumes that the longer substrates such as hair and cloth are submerged the more algae will become attached and proliferate. The objectives of this study were to:

**1**. determine the pattern of species succession that occurs on the cloth over time,

**2.** examine whether population numbers increase over time,

**3.** identify certain indicator species or taxonomic groups that can be used as PMSI clocks, and,

**4.** determine the accuracy of this method of determining PMSI.

# Methods

**2-1: Study Sites**

The study was conducted from June to October 2005 in two ponds of different nutrient status in the city of Winnipeg, Manitoba (49° 54' N, 97° 14' W). The pond located at Rossmere Golf and Country Club will be referred to as a eutrophic (nutrient enriched and highly productive) pond because of its location in the center of a golf course, where chemical fertilizers high in nitrogen and phosphorus are readily leached into the pond (Wong *et al.* 1998). The Fort Whyte Center on the other hand, is a prairie preserve that is dedicated to sustaining the natural world. Therefore, the pond at the Fort Whyte Center is sustained at a moderately productive nutrient level and will be referred to as mesotrophic.

Open canopies at both sites permitted direct sunlight throughout the day to penetrate the pond water. The Rossmere pond was surrounded primarily by Kentucky blue-grass (*Poa pratensis* L.). The pond contained a variety of aquatic plants including cattails (*Typha latifolia* L.), water-milfoil (*Myriophyllum sibricum* Komarov), and pond weed (*Potamogeton pectinatus* L.). The Fort Whyte Center pond was surrounded by characteristic prairie species including Trembling aspen *(Populus tremuloides* Michx), Narrow-leaved willow (*Salix exigua* Nutt), and various grasses such as Big bluestem (*Andropogon gerardi* Vitman) and Little bluestem (*Schizachyrium scoparium ssp. scoparium* (Michx) Nash). The aquatic plants included various duckweed taxa such as *Lemna minor* L., *L. trisulca* L. and *Spirodela polyrhiza* (L.) Schleid and water-milfoil (*M.sibiricum*).

**2-2: Field collection:**

Sampling began on the 20th of June 2005 for twelve days at Rossmere Golf Course and 15 days at the Fort Whyte Center. Sample collections continued for the duration of the open water season with a total of eleven, two week trials completed. The final trial occurred October 11-23rd, 2005. Only the first (June) and last (October) trials were used for sample analysis procedures because of the marked temperature difference between the two months. The temperature range for the June trial was 22-25°C, while the temperature range for the October trial was 8-10°C.

Hair and cloth were used as substrates for periphyton growth. The hair substrate was provided by Colewaves Beauty College in Winnipeg, Manitoba. The cloth substrates consisted of newly purchased fabric (T-shirt cotton and nylon) and used clothing (denim). A simple clamp system made up of Lexan® and common hardware (Appendix I) was used to suspend the hair and cloth substrates in the two ponds at a depth of approximately 0.5 m. Cloth types consisted of cotton, a cotton derivative, denim and nylon. These fabrics were cut into 48, 12 cm long by 1 cm wide strips. The cloth was then suspended from the Lexan® clamps to create a fringe-like arrangement. Only two fabric types plus hair were used for each individual trial. The hair was arranged in small clumps of approximately 60 hairs and placed longitudinally on the same clamp apparatus as the cloth (Appendix I).

Daily samples of four strips of cloth and one, 60 hair clump were collected for 12 to 15 days. Once cut, the cloth strips and hair were placed in small vials containing Lugol's Iodine and returned to the lab for analysis.

Temperature readings were taken at the beginning, middle and end of each trial using the YSI Model 57 portable oxygen and temperature meter, to ensure that the temperature range did not shift drastically during the two weeks of submergence.

**2-3: Preliminary analysis**

A preliminary analysis was carried out prior to the commencement of the data analysis portion of the study to determine an effective method for the removal of the algae from the cloth samples. The use of a small motorized toothbrush was determined to be the most effective and least destructive method of algal cell removal. The other methods that were examined for cell removal from the cloth samples included sonication, vortexing and scraping.

**2-4a: Analysis of hair**

The hair was removed from the vial of preservative and the tip end of the hair as established, a one cm portion of hair was cut from the tip of eight hairs. Only hairs on the outer edges of the hair bundle were used. The cut portion of the hair was then examined at 400x magnification on a compound microscope. For each of the eight hairs, five randomly selected field views were counted. *Cocconies pediculus* Ehr. was determined as an indicator species for the hair. Therefore, the number of *C. pediculus* cells were counted for each of the five field views. The number of *C. pediculus* cells per mm of hair was determined by multiplying the average of the five field views by a conversion factor of 5.4, the diameter of the field at 400x magnification being 0.185mm. The eight hairs were then average and plotted.

**2-4b: Analysis of cloth**

The rate of diatom colonization on the cloth was estimated by determining the average total number of diatoms per square centimeter of cloth, for each day of an experimental run. For each day, five one square cm cloth samples were prepared as follows: each one $cm^2$ cloth sample was cut and brushed for one minute on one side using a small-motorized toothbrush into one of the wells of a large six chambered microplate. The toothbrush and cloth were rinsed with distilled water into the well of the microplate. The contents were then transferred into an Utermöhl settling chamber and allowed to settle for ten minutes. After ten minutes, the settling chambers were examined under an inverted microscope at 100x magnification. The total number of diatoms per square centimeter of cloth was estimated using ten randomly selected Whipple ocular micrometer grid counts. The ten counts were then averaged and multiplied by a conversion factor of 446.25 to convert to cells/$cm^2$. The average number of diatom cells per square centimeter of cloth was then calculated from these five samples.

**2-4c: Simulation test:**

To test the effectiveness of this proposed PMSI method, two case studies were initiated following the completion of the PMSI calibration curves. These simulation tests consisted of a blind run in which a sample of hair and cotton was obtained from one of the Rossmere June or October trials or the Fort Whyte Center June or October trials. These samples were analyzed and days of submergence were determined using the calibration curves.

**2-5: Statistical analysis**

Population growth curves showing standard error were created for both the hair and cloth substrates. In exploratory studies such as this one, it is important to determine the sample effort necessary to obtain a specified level of precision. Therefore, the sampling effort required to bring the sample means to within 5, 10 and 20% of the population mean, with a 95% confidence interval, was determined following methods described in Krebs (1999).

**2-6: Identification**

All algal identifications were made using Prescott (1982) and Vineyard (1979). However, only a general knowledge of algal taxonomy was required to identify diatoms as a group and to identify the indicator species *C.pediculus*.

# Results

### 3-1: Hair

*Cocconeis pediculus* Ehr. colonized the hair shaft more frequently than any other species of diatom (Table 1). However, colonization along the hair shaft varied from the free or tip end to the confined end next to the apparatus (Appendix II). Colonization also varied within the bundle. Figure 1 illustrates the colonization pattern of *C.pediculus* on the tip end of the hair substrate following immersion in two ponds of different nutrient status at two different temperature ranges. Data points could not be determined after day five for the Rossmere Golf Course October trial due to the increased colonization of the substrate by other groups of epiphytic algae and bacteria.

**Table 1**: Preliminary trial to determine the proportion of total diatoms, *C.pediculus* and other algal groups present on the hair in the Rossmere Golf Course pond for June 2005.

| Day | *C.pediculus* | Other diatoms | Total diatoms | Other algal groups |
|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 |
| 2 | 1 | 0 | 1 | 0 |
| 3 | 13 | 2 | 15 | 0 |
| 4 | 106 | 1 | 107 | 4 |
| 5 | 0 | 0 | 0 | 0 |
| 6 | 50 | 0 | 50 | 1 |
| 7 | 36 | 3 | 39 | 12 |
| 8 | 6 | 1 | 7 | 0 |
| 9 | 22 | 1 | 23 | 7 |
| 10 | 7 | 2 | 9 | 1 |
| **Total** | **241** | **10** | **251** | **26** |

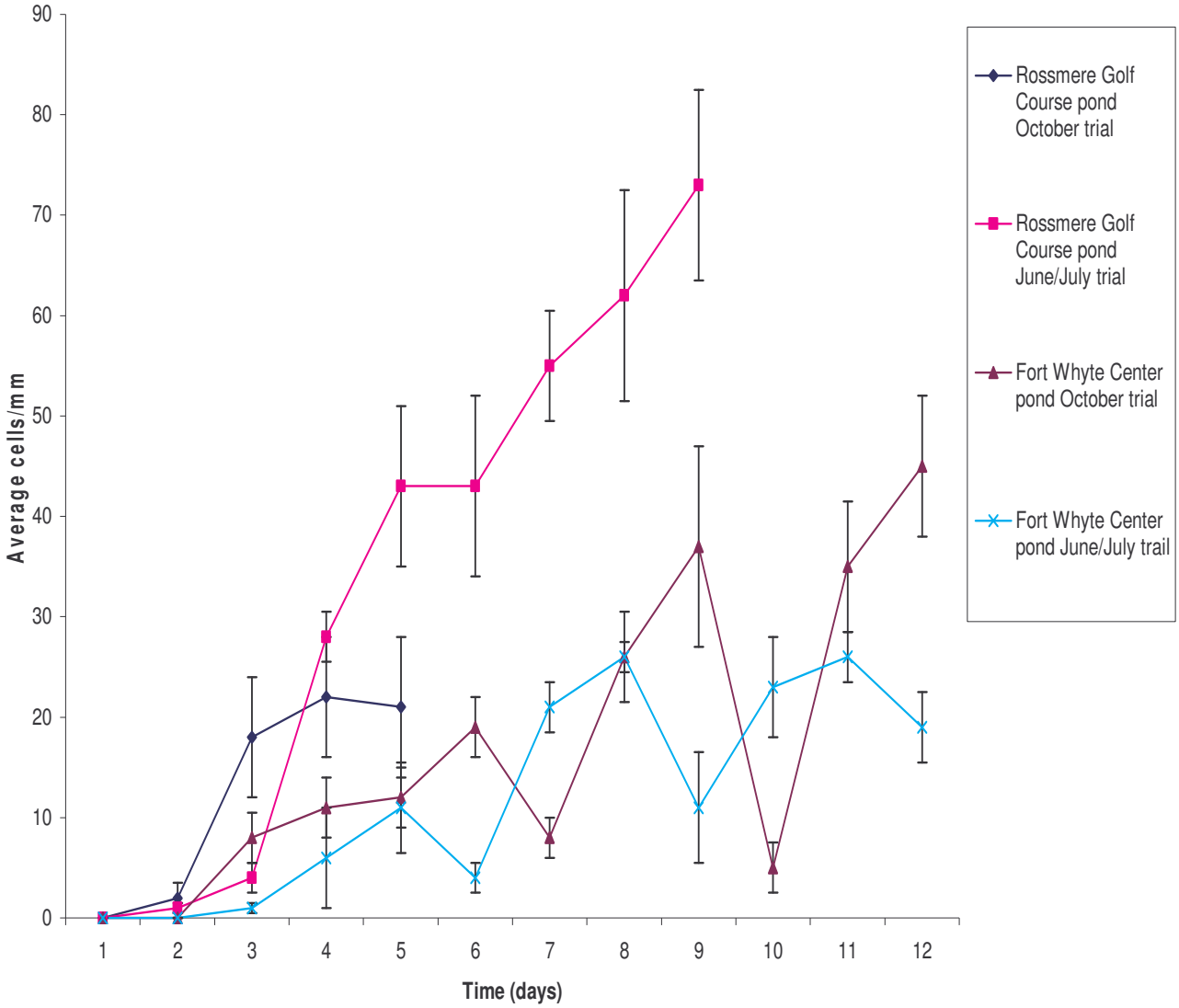


**Figure 1**: Hair calibration curve for PMSI estimates using standard error. Hair was submerged in the Rossmere Golf Course eutrophic pond and the Fort Whyte Center mesotrophic pond during June and October of 2005. Data points for the Rossmere October trial could not be determined past day five and for the June trial past day ten due to overgrowth by eubacteria.

**3-2: Cloth**

Cotton was the only fabric that was submerged at both temperature ranges in both ponds (Figure 2). Nylon was submerged in the June trial (Figure 3), while denim was submerged in the October trial (Figure 4). Figures 3 and 4 only show the colonization of nylon and denim substrates in the eutrophic Rossmere pond. The colonization of nylon and denim in the Fort Whyte Center pond is shown in Appendix III.

The different substrates all appeared to show an increase in population numbers over the course of submergence. Algal colonization on the cotton substrate appeared to have a typical S-shaped pattern of growth, not unlike growth that follows a logistic model. The scale of Figure 2 displays cotton colonization in the Fort Whyte Center pond as linear however, when scaled up, the growth curve does seem to be more S-shaped (Figure 3 in Appendix III).

In all instances cell density on the cotton substrate was greater than the nylon or denim substrates. Algal cells also appeared to colonize the cotton cloth substrate slightly sooner than the nylon or denim (Figures 3 & 4 and Figures 1 & 2 in Appendix III). There appeared to be no overall difference in colonization between the natural and synthetic fibers. Both cloth types showed an increase in algal cell density over time. However on the nylon, after day eight algal cell densities decreased drastically (Figure 3).

**3-3: Environmental Variables**

**3-3a: Temperature**

In the Rossmere Golf Course pond a difference in colonization related to temperature was seen for both the hair and cloth substrates (Figures 1 and 2). The June

trial demonstrated a higher cell density compared to the October trial on both substrates. Temperature variation did not appear to have as marked an effect on colonization in the Fort Whyte Center pond.

**3-3b: Nutrient Status of the water body**

Cell density was much higher in the Rossmere Golf Course eutrophic pond. In the June cotton trial, the Rossmere Golf Course pond had a cell density six times that of the Fort Whyte Center mesotrophic pond (Figure 2). In the October trial, cell density was three times higher in the Rossmere Golf Course pond compared to the pond at the Fort Whyte Center. The hair substrate showed a similar trend with the June cell density values three times higher in the eutrophic pond than in the mesotrophic pond (Figure 1).

**3-4: Simulation test**

The simulation test showed that for the Rossmere June trial (case 1), submergence could be determined using the algal PMSI methods (Table 2). The Fort Whyte Center October trial (case 2) underestimated submergence by three days.

**3-5: Statistics**

Tables 3 and 4 illustrate the sampling effort required to receive an accuracy of 5%, 10% and 20% of the mean, using a 95% confidence interval. Appendix IV presents results for the entire trials.

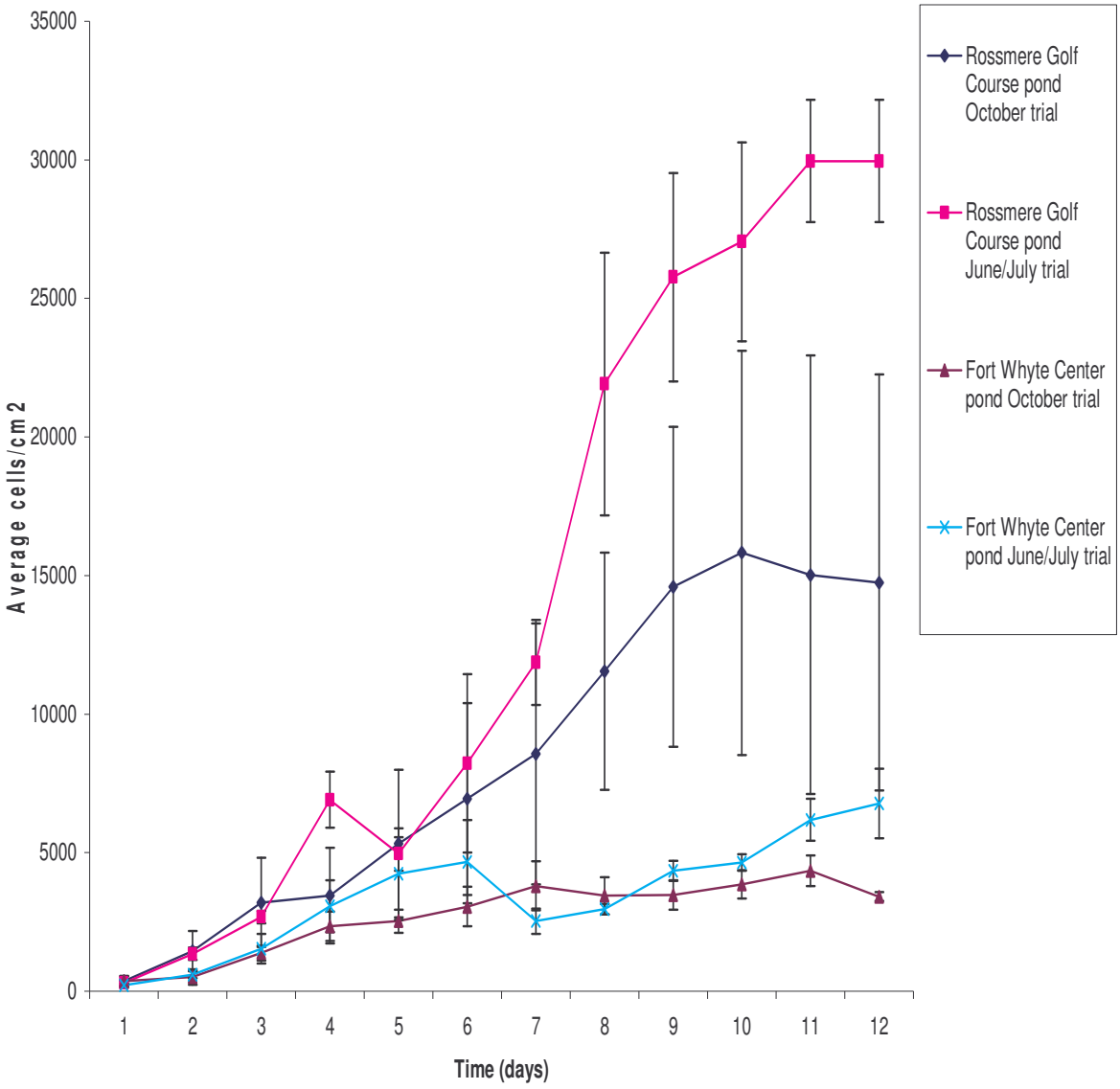


**Figure 2**: Cotton calibration curve for PMSI estimates using standard error. The cotton was submerged in the Rossmere Golf Course eutrophic pond and the Fort Whyte Center mesotrophic pond during June and October of 2005.

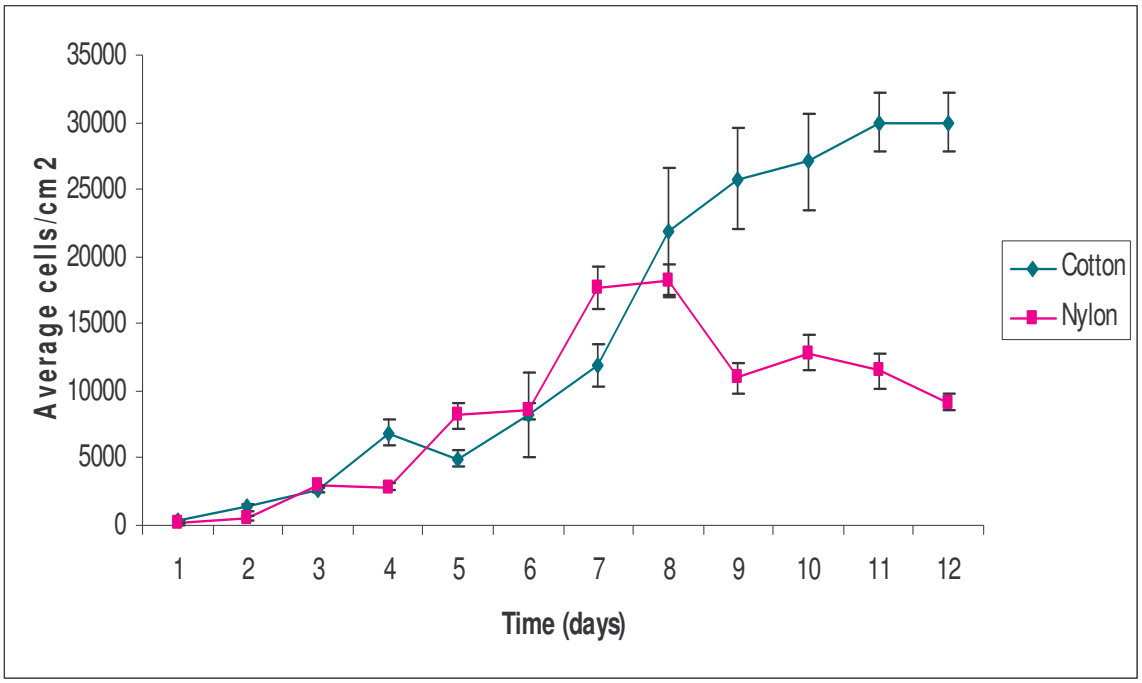


**Figure 3:** Algal colonization on cotton and nylon for a Rossmere Golf Course eutrophic pond, June 2005. Bars showing standard error of the mean.

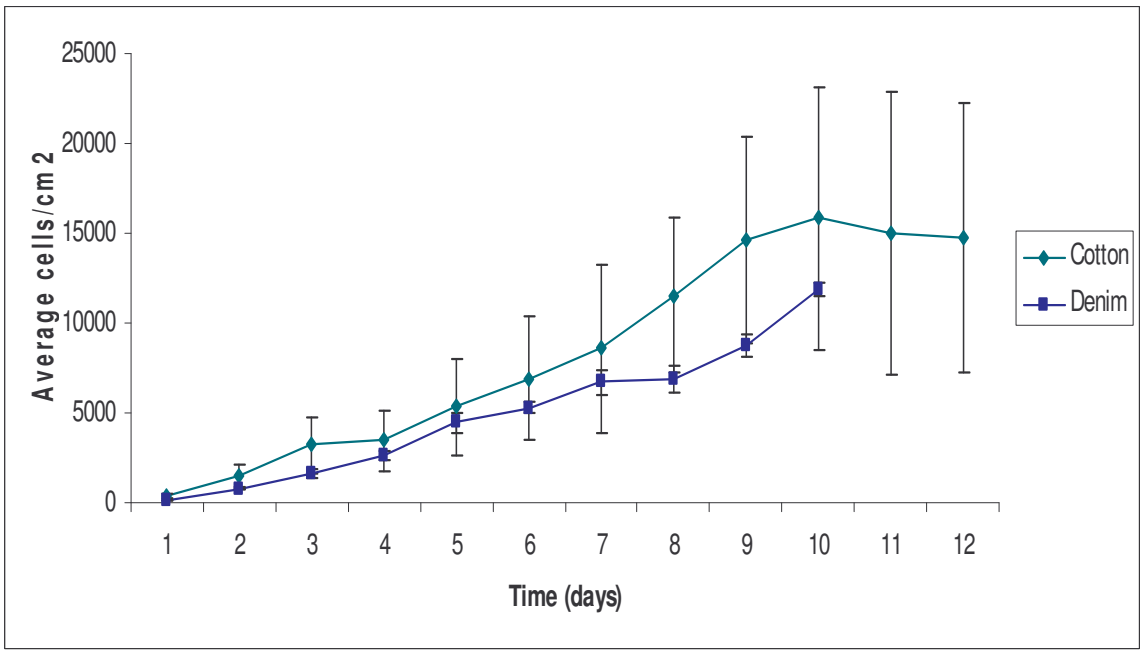


**Figure 4:** Algal colonization on cotton and denim for a Rossmere Golf Course eutrophic pond, October 2005. Bars showing standard error of the mean.

**Table 2**: Simulation test to determine the applicability of the algal PMSI method in determining time of submergence. Unknown samples were obtained from one of the four trials and an estimation of PMSI was made based on cell density and calibration curves for that pond. The estimation was then compared to the actual day of submergence.

| Experimental Run | Cell density on the cotton (cells/cm$^2$) | Cell density on the hair (cells/mm) | Estimated PMSI (days) | Actual PMSI (days) |
|---|---|---|---|---|
| June/July Rossmere Golf Course (Case 1) | 9255 | 17 | 4 | 4 |
| October Fort Whyte Center (Case 2) | 1062 | 8 | 3 | 6 |

**Table 3**: Sample size estimations for days 1, 6, and 12 for the Rossmere Golf Course October Cotton trial. All sample estimations used a confidence interval of 95%. Expanded table found in Appendix IV.

| Day | Average cells/cm$^2$ | Standard deviation | Sample estimate for 5% of the mean | Sample estimate for 10% of the mean | Sample estimate for 20% of the mean |
|---|---|---|---|---|---|
| 1 | 366 | 618.66 | 45734682 | 11433670 | 2858418 |
| 6 | 6935 | 526.69 | 92293 | 23073 | 5768 |
| 12 | 15021 | 2306.21 | 377167 | 94292 | 23573 |

**Table 4**: Sample size estimations for days 1, 6, and 12 for the Rossmere Golf Course October Hair trial. All sample estimations used a confidence interval of 95%. Expanded table found in Appendix IV.

| Day | Average cells/cm$^2$ | Standard deviation | Sample estimate for 5% of the mean | Sample estimate for 10% of the mean | Sample estimate for 20% of the mean |
|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 |
| 6 | 4 | 3 | 9671111 | 2417778 | 604444 |
| 12 | 19 | 7 | 2082874 | 520718 | 130180 |

# Discussion

**4-1a -Determination of indicator group**

*Q: Is there a particular group or species that can be used as an indicator group in PMSI estimates?*

It was determined through several preliminary sample analyses that a group of epiphytic algae from the Class Bacillariophyceae could be used as a potential indicator group in PMSI estimations. This group is commonly known as the diatoms. Diatoms are non-motile, photosynthesizing algae that have a siliceous skeleton or frustule. They are also found in almost every aquatic environment (Moser *et al.* 1996).

Diatoms preserve well due to their siliceous skeleton (Moser *et al.* 1996). Also, they are usually only between 20-200 microns in size and are therefore not visible to the naked eye (Peabody 1999). For these two reasons, they have been implemented as a valuable tool in forensic science for decades. Diatoms have been used to determine cause of death (Peabody 1977) and to place suspects at crime scenes (Siver *et al.* 1994). If a body is recovered from water and diatoms are present in the bone marrow or other enclosed tissue it can be determined that the victim died by drowning (Peabody 1977; Matsumoto and Fukui 1993). Furthermore, a suspect can be linked to a particular pond by the comparison of pond diatoms, with diatoms present on the suspects clothing (Siver *et al.* 1994). No other algae is known to be resistant enough to persist on clothing (Peabody 1999).

A preliminary hair trial for the pond at Rossmere Golf Course (June) showed that diatoms colonized the hair 9.7 times more than any other group (Table 1). Antoine and Benson-Evans (1986) reported similar findings in their study of periphyton development on artificial substrates; diatoms contributed 80-100% of the total algal counts.

In addition, diatoms colonized the substrates within 1-2 days of submergence whereas, the other groups tended to colonize the substrates later. Hoagland *et al.* (1982) described a comparable colonization sequence to that encountered on both the hair and cloth substrates. Colonization began with a fine organic coating of bacteria, followed immediately by low profile diatoms, then by an upperstory of long-stalked and large rosette diatoms and finally by filamentous green algae.

**4-1b -Epiphytic algal removal**

*Q: What is the most efficient method of cell removal from the cloth substrates?*

A pilot study was initiated in the summer of 2005 to determine the best removal technique for the epiphytic algae from the cloth. Sonication, vortexing, and physical scraping were all attempted. It was determined that sonication and vortexing were too destructive to algal cells making identification under the microscope impossible. The sonication and vortexing processes broke algal cell walls and diatom frustules.

Physical scraping using a scalpel did not remove a large proportion of the algal cells present. Many small epiphytic diatoms wedged themselves between the interwoven fibers of the cloth and could not be released by scraping alone. Examination of the piece of cloth under the microscope after scraping revealed a large number of diatoms still in place. The most effective method thus far proved to be the toothbrush removal procedure outlined in the methods section.

**4-2: Hair**

Hair is unlike most other types of biological evidence in that it is remarkably stable to most environmental conditions and will not degrade easily (Jackson and Jackson 2004).

In water, hair does not slough off until the active stage of carrion decomposition which occurs between 42-105 days post submergence (Anderson and Hobischak 2004). Hair is considered to be a desirable substrate for PMSI estimates because of its resiliency and persistence on the corpse.

**4-2a- Hair strand Anatomy**

The cortex, cuticle and medulla are the main layers that comprise the hair shaft (Appendix V) (Saferstein 2004). The cuticle is what makes the hair resistant to chemical decomposition and allows the hair to retain its structural features over long periods of time (Saferstein 2004). The cortex is contained within the cuticle. It consists of cells with keratin (protein) which contain the pigment granules that impart color to the hair (Langford *et al.* 2005). Finally, the medulla is an air-filled channel in the core of the hair (Langford *et al.* 2005). The presence and appearance of the medulla varies from individual to individual and even among hairs of a given individual (Saferstein 2004). The medulla can be classified as being continuous, interrupted, fragmented, or absent (Appendix V).

**4-2b- Algal colonization along the hair shaft**

*Q: Is there an individual species that colonizes the hair more frequently than any other?*

*Cocconeis pediculus* cells colonized the hair shaft 96% more than other diatom species in the Rossmere Golf Course pond in June 2005 (Table 1). *Cocconeis* species are considered early colonizers and are superior competitors (Oemke and Burton 1986). C.*pediculus* is a small, ubiquitous, epiphytic diatom (Appendix VI), whose frustules are broadly ovoid-elliptic in valve view (Prescott 1982).

*Cocconeis pediculus* is epiphytic on filamentous algae and aquatic plant roots and at times flourishes so well on these substrates that it forms a complete coating over the host surface (Prescott 1982). It is also frequently encountered on stock microscope slides of filamentous green algae (personal observation). The similarity in morphology between filamentous algae and a hair strand is evident and it comes as no surprise that *C. pediculus* is the main colonizer of the hair shaft. Also, it is not surprising that the hair shaft experiences a similar phenomenon as Prescott (1982) pointed out on the plant roots, in that the hair shaft becomes completely covered by *C. pediculus* after several days of submergence.

*Q: What affect does the bundling of hair have on colonization?*

During the analysis portion it was discovered that for the same day certain hair shafts were completely un-colonized while others were heavily colonized. Initially, it was believed to be a factor of the medulla. Hairs that did not have a medulla or had fragmented medullas appeared to be more heavily populated than hairs that had continuous medullas. However, following a thorough investigation into this phenomenon this was discovered not to be the case. All hairs used for analysis were darkly pigmented hairs, but those that possessed a medulla were even darker due to the thick central core. Therefore, diatoms colonizing the surfaces of these hairs were less visible due to the dark coloration of the hair shaft. Hairs lacking a medulla were more transparent and diatoms could be seen easily using less light. Consequently, microscopic techniques required greater lighting when hairs possessed a medulla. With increased light hidden diatoms became apparent.

Continued investigation into which hairs were colonized and which were not revealed that the bundling of hair samples in the apparatus affected colonization. The hairs in the center of the clump appeared to be un-populated while the hairs on the outer edges of the clump were heavily populated. It is therefore believed that when the hair is submerged the outer hairs will be more available to colonizing epiphytic algae than the inner hairs. As the outer hairs become more populated, algal cells will begin to colonize the inner area of the bundle. This bundling effect appeared to occur on all the hairs tested.

The manner in which the hair samples were arranged in the apparatus is not responsible for this effect. Hair naturally forms small bundles in water. Therefore, hair recovered from a submerged corpse would experience the same 'bundle effect' as seen on the apparatus.

*Q: Why is the tip of the hair shaft colonized more frequently?*

*Cocconeis pediculus* will colonize the tip of the hair strand more frequently than the center (Appendix II). The central portion of the hair is closer to the apparatus. Using the same logic as the bundle effect, hairs are more closely gathered near the apparatus and are therefore less available to colonizing algae. The hair tips are less confined and thus more available for colonization.

An even greater amount of variation, due to the bundle effect, was experienced when the entire hair strand was examined (Appendix II). The hair strands on the outer edge of the hair bundles were more heavily colonized on all portions of the hair stand and produced a greater overall cell density than hairs on the interior of the bundle. Hairs in

the interior of the bundles were only colonized on the tips. Therefore, cell density counts for the entire hair strand relied solely on the tip counts.

**4-3: Cloth**

**4-3a: Cloth characteristics**

Cloth is a textile fiber used to make garments and clothing. Individual fibers have unique characteristics and patterns (Saferstein 2004). There are two types of fibers, natural and synthetic (Langford *et al.* 2005). Natural fibers are derived from animal or plant sources. These include wool, silk, cotton or hemp (Saferstein 2004). Synthetic fibers are man-made polymer based fibers, such as polyester and nylon (Langford *et al.* 2005). Both synthetic (nylon) and natural (cotton) fiber types were examined for algal colonization. The fibers that compose cotton are flat and twisted while the nylon fiber is a smooth clear rod (Appendix VII). High quality cotton has long, strong fibers knit into a plain tight weave that is resistant to abrasion. Denim which is derived from cotton is constructed in a twill (diagonal) weave with indigo and white 100% cotton yarns. Denim is a durable fabric however it frays and loses its dyes easily (Bendure and Pfeiffer 1972). Both denim and white T-shirt cotton were examined as colonizing substrates because of their differences in texture.

**4-3b: Cloth as a colonizing substrate**

*Q: Is there variation in algal colonization on the different cloth substrates?*

On June 26$^{th}$ and 27$^{th}$ 2005, the Rossmere Golf Course pond experienced a high degree of precipitation (Appendix VIII). This high degree of precipitation corresponded well with the substantial decrease in algal cell density on the nylon cloth substrate for day

nine of the Rossmere June trial (Figure 3). The cotton cloth substrate did not experience this same decrease in algal cell density. It is therefore presumed to be a factor of the smooth nature of the nylon fiber. The untwisted synthetic nylon fiber creates an even tighter knit than the cotton fabric, which may have prevented cell adhesion to the nylon fabric. Therefore, nylon appears to be more susceptible to environmental factors such as precipitation and current.

The denim cloth substrate frayed easily during algal removal and the dye used in the fabric obscured cell counts. Dyed cotton fibers were released during the algal cell removal technique. These small fibers disguised the presence of diatoms during visual enumerations. This may have led to the lower cell density throughout the run for the denim curves compared to the cotton curves (Figure 4 and Appendix VI). There were very few fibers released when removing diatom cells from the cotton which made enumerations easier. This may have been due to the tight long fiber weave used in cotton t-shirts over the easily frayed twilled yarn weave used in the denim garments (Bendure and Pfeiffer 1972).

Although, the denim frayed easily and visual cell counts were obscured, it appeared that the denim substrate produced a more precise estimate of PMSI than the cotton (Figure 4 and Appendix VI). The variation experienced between samples during the analysis of the denim substrate was less than that experienced on the cotton substrates.

*Q: Are there other techniques rather than visual enumeration for the cloth?*

The cloth substrates experienced a high degree of colonization by other groups of algae during the later portion of the trials. In the Rossmere Golf Course October trial the last two data points for the denim substrate could not be determined because of the

increase in algal growth by filamentous green algae, protozoans and an abundance of eubacteria (Figure 4). In an effort to account for all species present on the fabric substrate regardless of whether it was a diatom or not, chlorophyll determinations and optical density tests were attempted. However, both methods proved to be unsuccessful as algal PMSI estimating techniques.

Determining the chlorophyll content of the samples was not possible due to high concentrations of Lugol's Iodine preservative and dyes from the denim which interfered with the chlorophyll spectrum (Figure 1 in Appendix IX). The spectra for both the test sample (denim, Lugol's and algae) and the reference sample (denim and Lugol's Iodine) did not continue past 500nm. Figure 2 in Appendix IX depicts a normal chlorophyll absorbance spectrum in which peaks occur at 460nm and 680nm. The normal algal chlorophyll peaks were therefore masked by the Lugol's Iodine and the fabric dye in the test sample.

Future studies should attempt daily chlorophyll determinations prior to the addition of preservatives. Haefner *et al* (2004) used chlorophyll-a concentrations to determine the PMSI of pig carrion and found that the chlorophyll concentrations were strongly correlated with time since submersion. The use of chlorophyll concentrations in determining PMSI was not initiated early on in this study because it was believed that quantitative visual enumeration would garner the most information. Also, samples required preservation because they could not be examined immediately upon collection.

The optical density test was attempted following the discovery that samples appeared to have a bacterial fuzz growing on them that would not be accounted for using

the diatom enumeration technique. It was determined from a heavily colonized day six cotton sample, that a wavelength of 580nm would be used for the optical density measurements. The slight increase that can be seen from days one through twelve is off-set by the sporadically large peaks and troughs experienced throughout the run (Figure 3 in Appendix IX). The corresponding diatom visual enumeration graph (Figure 3 in Appendix III) did not show a similar trend. Consequently, it was concluded that these inconsistencies were too great and the optical density method was rejected for this project.

**4-4: Environmental Variables**

**4-4a -Temperature**

*Q: Does water temperature affect colonization?*

Temperature did not appear to be an influential factor in algal colonization in the Fort Whyte pond. However, in the Rossmere Golf Course pond a temperature effect was experienced between the June trial and the October trial on both the hair and cloth substrates (Figures 1 and 2). Colonization was slow in the fall (8-10°C) trial but very rapid during the summer (22-25°C) trial. By the end of the June trial on the cotton substrate algal cell density was twice that of the October run. Hoagland *et al.* (1982) describe this seasonal trend; however they also describe a shift in species dominance due to seasonality that was not looked for in this study.

The Fort Whyte Center pond trials did not appear to show any variation due to temperature on either substrate. On both the hair and the cotton substrates the algal growth curves remained similar regardless of water temperature.

**4-4b -Nutrient status**

*Q: What affect does the nutrient status of the pond have on algal colonization?*

Nutrient status of the ponds appeared to have a considerable effect on diatom species abundance and algal cell density. Both nitrogen and phosphorus concentrations directly affect diatom colonization and reproduction (Stevenson and Yangdong 1999). Cell density on the cotton substrate was six times higher in the eutrophic pond than the mesotrophic pond (Figure 2). Similarly, diatom cell density on the hair substrate was three times higher in the eutrophic pond.

The shape of the growth curves were also affected by the different nutrient levels. The logistic growth curve in the eutrophic pond hit a plateau around day ten. In the mesotrophic pond, the curve remained in the exponential stage of growth until the end of the trial. The growth experienced in the mesotrophic pond will not continue to increase exponentially, factors such as interspecific substrate competition and resource competition eventually cause the population to a plateau (Hoagland *et al.* 1982). Due to increased cell density in the eutrophic pond, the diatoms experienced resource limitation (space) sooner than the mesotrophic pond and therefore showed a characteristic S-shaped logistic curve.

**4-4c-Other environmental variables**

*Q: Are there other environmental variables that may affect colonization?*

This study focused primarily on the effects of temperature and nutrient level variation on colonization. However, during the course of the study additional variables such as precipitation, water current, and grazer impacts became increasingly evident. Although this study was not designed to examine these variables, a brief mention of their

observed effects is of value. An extensive examination into these variables is needed if this method of algal PMSI estimation is to become widely adopted.

Oemke and Burton (1986) describe the impact of water current on diatom communities. They explain that spring flooding serves to reset the algal community to its earlier successional stages. Even small unnoticed floods can have a profound effect by resetting diatom colonization. The nylon cloth substrate shows an example of this phenomenon (Figure 3). The small summer flood that occurred the last week of June 2005 (Appendix VIII) reduced diatom numbers on the nylon substrate. The Fort Whyte Center nylon trial did not show a significant decrease in algal cell density after the high rainfall (Figure 1 in Appendix III). This might be explained by the placement of the apparatus in a sheltered region of the pond. The Fort Whyte Center apparatus was attached to a floating dock whereas; the Rossmere Golf Course apparatus was attached to a bridge. The bridge was not in contact with the water and the apparatus was therefore subjected to the impact of current changes in the pond. Current changes in the Fort Whyte Center pond were not as influential on diatom colonization because the apparatus was sheltered by the dock.

It is also important to note that current velocity has been known to affect diatom immigration rates by controlling the rate that algae are introduced to the substrate (Stevenson 1983). Colonization may therefore be restricted in waters that experience a high degree of wave action or are readily susceptible to flooding. Understanding the influence of water current is important in order to properly construct useful PMSI calibration curves.

In the Fort Whyte Center pond for both the June and October trials, the hair substrate experienced periodic algal cell density decreases. These decreases can not presently be explained with certainty. However, a possible reason for this trend may be that C.*pediculus* colonization is being influenced by algal grazers. Epiphytic algal cell density and biomass is readily influenced by grazers such as herbivorous snails (Rosemond 1993). Snails were observed on the apparatus on day six for the June Fort Whyte trial. In Figure 1 day six is marked by a sharp decrease in algal cell density on the hair substrate. It would be of value to investigate this phenomenon further by creating controls in which the grazers are restricted access to the apparatus.

**4-5: Application to Forensic Science**

**4-5-a: Simulation test**

Case 1: samples of an unknown date were obtained from the Rossmere Golf Course pond from the June trial. Using the PMSI estimation method it was determined that these hair and cloth samples had been submerged for four days. This was determined by overlapping estimates from both the hair and cotton. Using the cotton, a submergence of four or six days was estimated. Using the hair, a submergence of three or four days was estimated. Overlapping both estimates yielded a PMSI of four days. Case 1 proved that the method can work.

Case 2: samples of an unknown date were obtained from the Fort Whyte Center pond from the October trial. In Case 2 an underestimate of the PMSI was made using the algal PMSI estimation methods. The sample represented a submersion time of 6 days, the algal cell count from the hair and cloth were low and placed the PMSI at a much shorter

time interval of 3 days. It may be that hair and cloth samples used for the case study were no longer useful after construction of the calibration curves because all colonized samples were already enumerated. It may also be that the number of hair and cloth counts performed on each of the test samples was too small. Appendix X illustrates the analysis and determination of each simulated case in more detail.

**4-5-b: Considerations for use in Forensic Science**

*Q: What sample size is appropriate?*

The sample sizes used for this study were too small in comparison to those determined in the sampling effort exercise and variation between samples was too great for acceptance as a rigorous method in forensic science.

The high variation seen between samples can be attributed to inaccuracies and inconsistencies during sampling analysis. During the analysis of the cloth it was difficult to produce cloth samples that were perfectly cut to one $cm^2$. Each sample of cloth had a slightly different surface area. This certainly would have led to a large variation between samples. Also, the apparatus used a fringe-like arrangement to hang the substrates. This allowed cloth to be colonized on both sides of the fabric. During sample analysis it was decided that cloth associated with actual submerged remains would only be colonized on one side, therefore only one side of the cloth was brushed for diatoms. However, during the brushing procedure it is possible that in some instances diatoms were dislodged from the opposing side of the cloth. This also may have lead to variation between cell counts. In addition, the algal cell removal technique is a possible source of variation. A motorized toothbrush was chosen over a standard toothbrush to minimize variation in brushing from

one sample to the next. However, variation in the removal of diatoms may have been produced due to differences in pressure applied during brushing.

Variation experienced between hair samples was discussed in Section 4.2. Hair sample variation was attributed to: where on the hair strand visual counts were made (the cut end or the tip end), the placement of the hair strand in the bundle and finally, the pigmentation of the hair strand. Future research should examine possible ways to minimize the variation on both the hair and the cloth substrates.

*Q: How effective is this method of PMSI estimation?*

The simulation test provided evidence for and against the use of this method of PMSI estimation. A margin of error of three days is far too great given the legal burden of proof. However, decreasing the variation between samples and increasing sample sizes may provide the rigor needed for use of this method in forensic science.

**4-5-c: Legal Considerations for Acceptance of New Forensic Methods in Court**

As with any new methodology, technology or standard, wide spread acceptance by the scientific community is required. Similarly, in the courts it is important to ensure quality assurance of the testing procedures, validation of the procedures and validation of those conducting the procedures (Miller-Coyle 2005). There are several factors outlined by Miller-Coyle (2005) that the U.S. Supreme Court addresses when considering the admissibility of a new forensic method in court including:

-whether the theory or technique in question can be or has been tested,

-whether it has been subjected to peer review and publication,

-what its known error rate is,

-whether standards exist to control and maintain its operation, and finally,

-whether it has attracted widespread acceptance within a relevant scientific community.

These factors are fairly standard in the scientific process and can also be applied to admissibility in Canadian courts. Further criteria would include an examination of the prestige and background of the expert, whether the results can be interpreted objectively and finally whether the experts can explain the data in a manner that the fact finder can draw conclusions from (Koppl 2005). As with many criminal cases, once these elemental features are established, challenges can then be made on a case-by-case basis, based on issues of contamination, laboratory procedures and reliability of the scientist who performed the actual testing (Koppl 2005).

This proposed method of estimating PMSI is far from that stage, however early considerations of the rigor and scrutiny required of a new forensic test will only aid in it's development. Like any new scientific method, standards will need to be met through intensive research, inquiries and validation (Miller-Coyle 2005). It is critical to the algal PMSI method to ensure that the methodology and the statistical tests are sound during its inception to guarantee later acceptance.

# Recommendations

The following section is a list of recommendations to help further the field of 'forensic phycology' by suggesting ways to minimize variation, inconsistencies and inaccuracies in both sampling procedures and analysis.

**1. Alteration of the sampling apparatus:**

In order to minimize variation caused by the colonization on both surfaces of the cloth an alteration of the apparatus is suggested. A very minor change that may decrease variation would be to fold the cloth and place it into the apparatus so that the folded edge is exposed. Folding the cloth prevents some colonization on the inner surface of the cloth, while allowing the apparatus to remain simple in its design.

A more complex solution would be to create a cylindrical apparatus and surround the cylinder with fabric. This will prevent colonization on both sides of the fabric. The fabric would only be able to surround the cylinder loosely in order to allow for sample collection.

The existing apparatus does not take into account the addition of nutrients from a decomposing corpse which may increase algal colonization on the surfaces. In an attempt to keep the apparatus simple, it is suggested that bacon strips or fat be incorporated into the clamp system, perhaps layering the bacon between the strips of Lexan®. Future research should eventually incorporate decomposing carrion into the methods.

In order to determine the effect of algal grazers on colonization, it is suggested that a control apparatus be created. The control apparatus should be contained within a fine grid cage in order to allow algal colonization but prevent the entry of snails and other herbivores.

**2. Alteration of the sampling procedure:**

Sampling procedures could also be improved. Strips should not be cut into the fabric prior to submersion. Rather, one cm wide strips should be cut the day of sample collection. An indication of which side is the colonizing surface would also be needed.

Variation may still arise during the sample analysis of the cloth because the cloth pieces may not be perfectly calibrated to one $cm^2$. A possible solution may be to use a fabric punch, similar to a paper hole punch but for fabric. An appropriate size is needed in order to allow for removal of cells following the motorized toothbrush technique ie: no smaller than a half a $cm^2$.

For the hair it is suggested that a number of small clumps of hair rather than just one clump be recovered from the corpse and preserved separately. An indication of whether the tip end or the cut end is being analyzed is necessary; as well as an indication as to where the hair was located in the bundle.

**3. Modification in sample analysis methods:**

It is suggested that the chlorophyll determination method be reexamined, without preservative. If the chlorophyll method proves valuable in PMSI estimations it does still have disadvantages. In the chlorophyll method, samples can not be reanalyzed at a later date to validate results. Also, samples would need to be analyzed immediately upon collection in order not to over estimate the number of colonizing cells. However, it would still be beneficial to reexamine the method.

Procedures outlined in Siver *et al.* (1994) can be attempted to minimize the variation caused by the presence of other algal groups on the cloth substrate. The brushed samples

can be transferred to a beaker containing sulfuric acid and potassium dichromate, in order to remove all organic matter and complicating cloth fibers. This would allow for easy enumeration of the diatom frustules.

The only suggestion to minimize variation caused during the brushing technique would be to increase the sample size. Altogether, larger sample sizes are needed for both the hair and cloth substrates.

**4. Introduction of an algal PMSI database:**

*Environmental variables:*

It is important to account for all environmental variables that may affect diatom colonization. Such variables include: temperature, water current, nutrient status of the water body, light levels, pH, and other stressors such as pollutants and toxins.

Each basin, pond, reservoir, stream bed or well, will have different environmental characteristics that will influence algal colonization. Therefore, it is suggested that a database of all water body types be created. This would entail placing an apparatus in each water body type and creating calibration curves.

Similar to the entomological methods outlined by Goff (2000), a case by case investigation will be required once the method is adopted. That is, for each case, an apparatus will need to be placed in the water body in which the corpse was recovered in order to create a calibration curve for that particular water body. It is imagined that over time a large database encompassing all water bodies within a province will be created similar to the CODIS database for DNA.

*Substrate variables:*

It is also proposed that a database encompassing all substrate variables be constructed. A thorough investigation into how diatom colonization differs on the different hair types is essential. It has already been suggested that visual enumerations on the hair substrate are more difficult on darker pigmented hairs. The solution was to increase the lighting used during microscopic examination of the hair. However, it is possible that when lightly pigmented hairs are examined the cell density may still increase, not because lighter hairs provide better substrates but because visual enumeration is made easier. It is important to construct calibration curves for each hair pigment (chemically treated or not), in each body location.

In addition to the hair, cloth calibration curves need to be constructed for different fabric types. This study only investigated three cloth substrates. It is important to understand how colonization is affected by other fabric types including: leather, polyester, hemp, and wool.

# Conclusions

1. Algal population numbers do appear to increase on both the hair and cloth substrates over time.

2. Diatoms appear to be a very useful taxonomic group for postmortem submersion interval estimates.

3. Species succession follows a very characteristic pattern, similar to what has been described in the literature beginning with a thin mat of bacteria, followed by low profile diatoms, an upperstory of long-stalked and large rosette diatoms and finally by filamentous green algae.

4. Variation in colonization appears to occur along the hair shaft and within the hair bundle due to the availability of the hair for colonization.

5. Denim appears to yield a more precise PMSI estimate than cotton, as there was less variation in the individual samples, however there was greater cell density and earlier colonization on the cotton cloth substrate.

6. Nutrient limitation appears to be the primary factor limiting algal growth, with temperature affecting the rate of colonization only in nutrient rich waters.

7. Other environmental variables such as precipitation, water current and herbivorous grazing seemed to effect diatom colonization and cell density. Precipitation and water current effects were only observed on the nylon cloth substrate, whereas, possible grazer effects were only observed on the hair substrate. Further investigation into these variables is needed.

8. Sample sizes used in this preliminary study proved to be too small and variation proved to be too great for adoption of the method in forensic science.

9. Further research is recommended in order to minimize inaccuracies, inconsistencies and variation in sample analysis and procedures.

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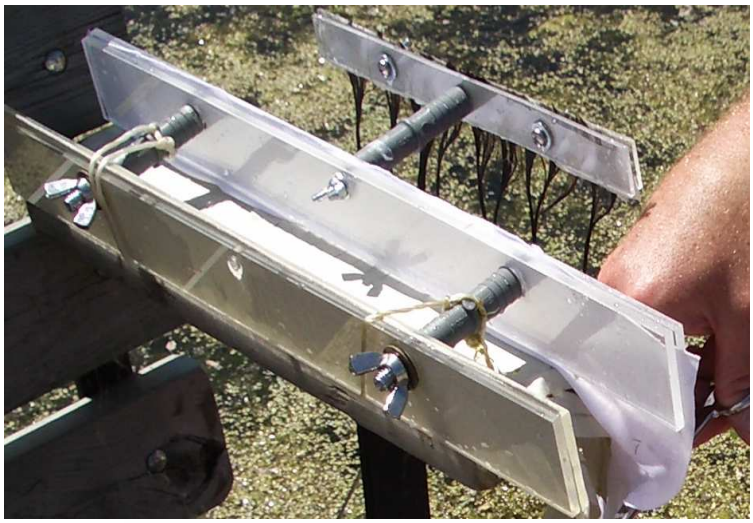
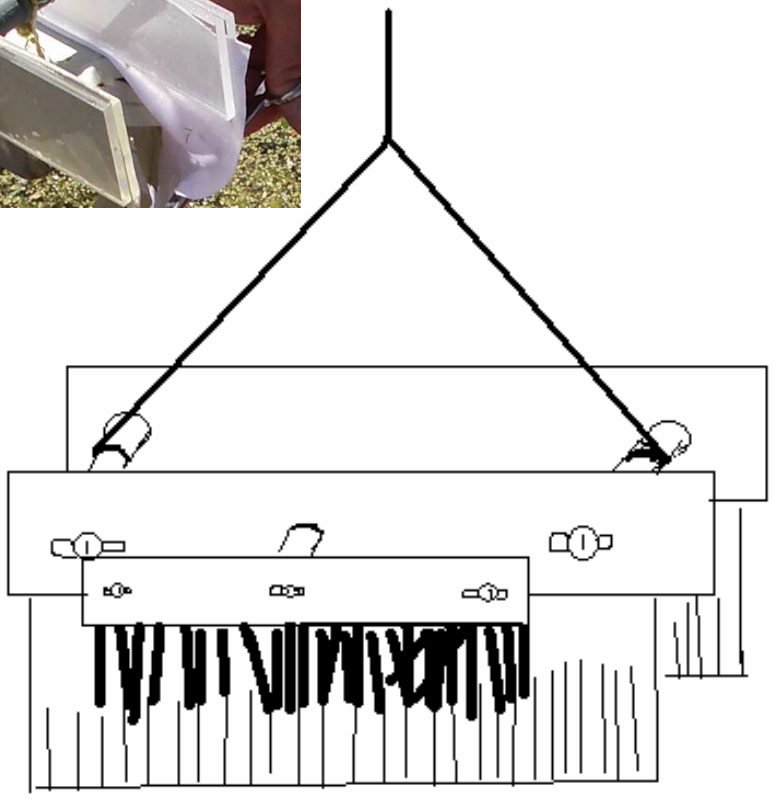
# Appendix I

## Sampling apparatus:

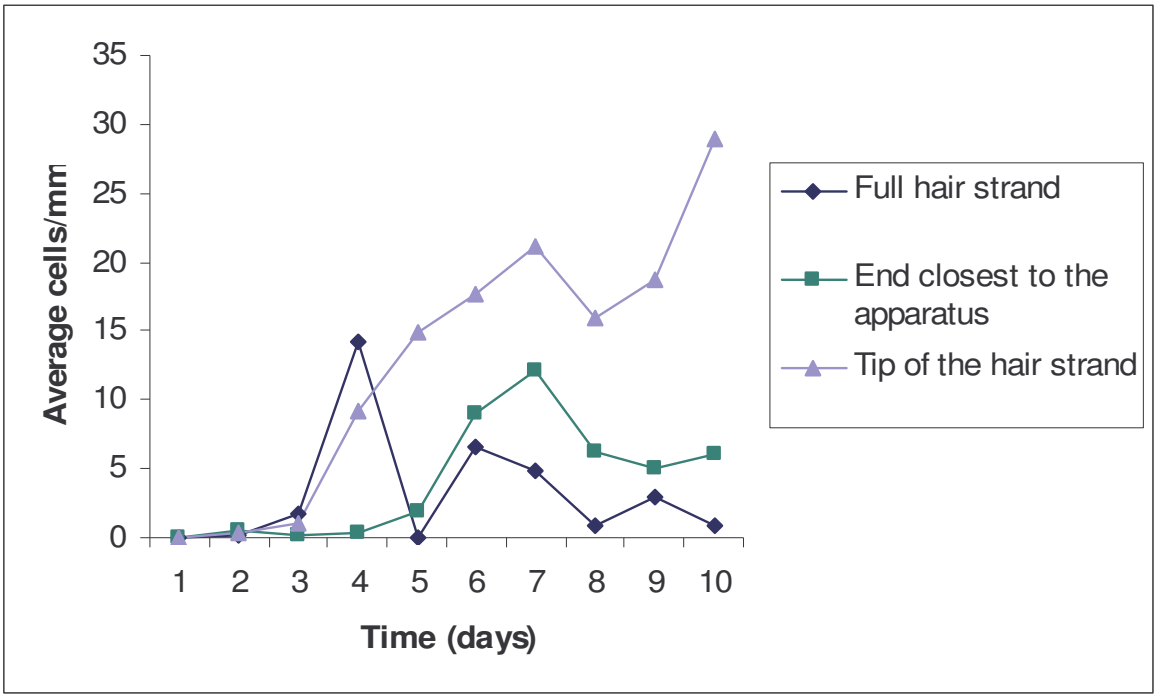




Apparatus used to suspend hair and cloth substrates in water. Above is a photograph of the apparatus used to suspend cotton, nylon and hair in the Fort Whyte Center pond in June 2005. Below is a schematic diagram of the apparatus.

# Appendix II

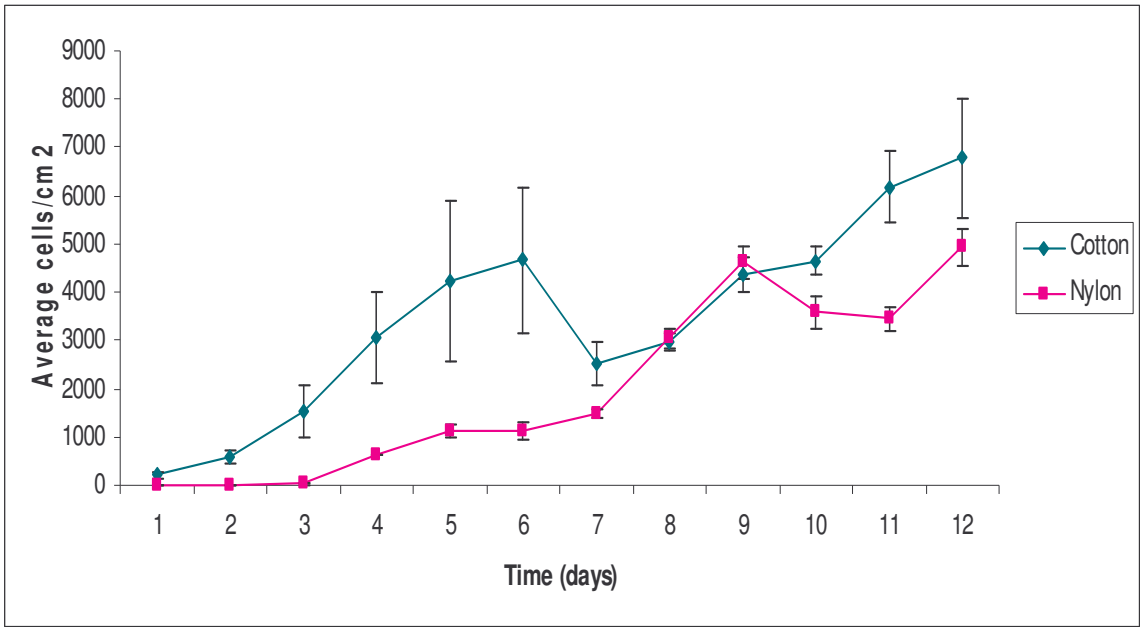
Hair strand variation:



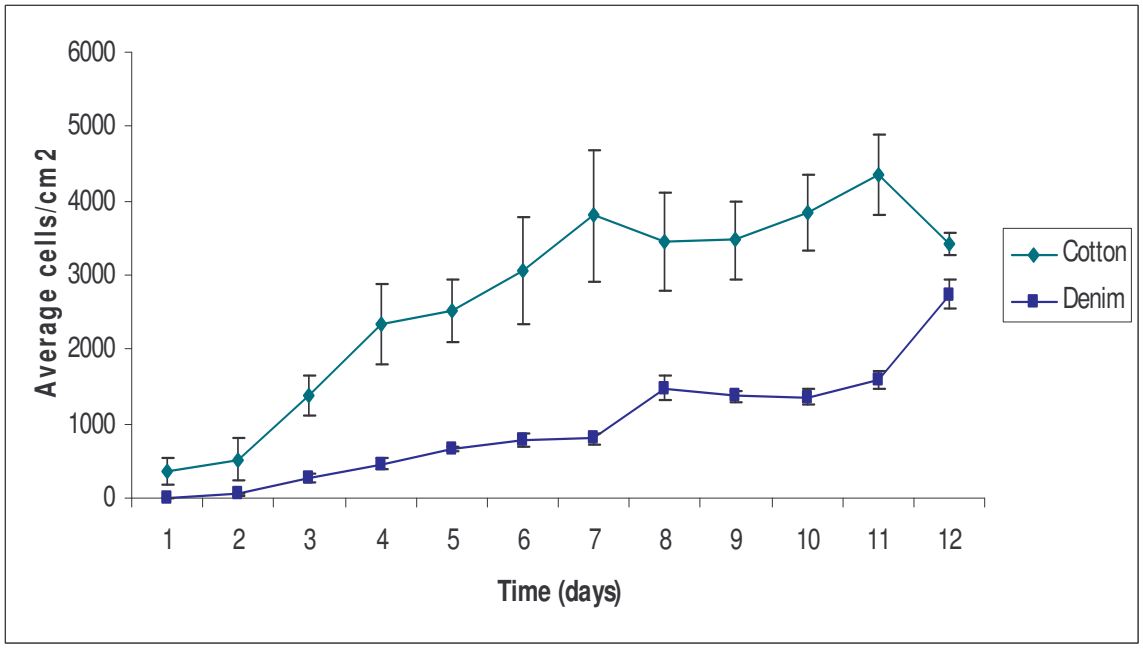

Three graphs comparing algal colonization and variation experienced on a full hair length, the cut portion (closest to the apparatus) and the tip of the hair shaft.

# Appendix III

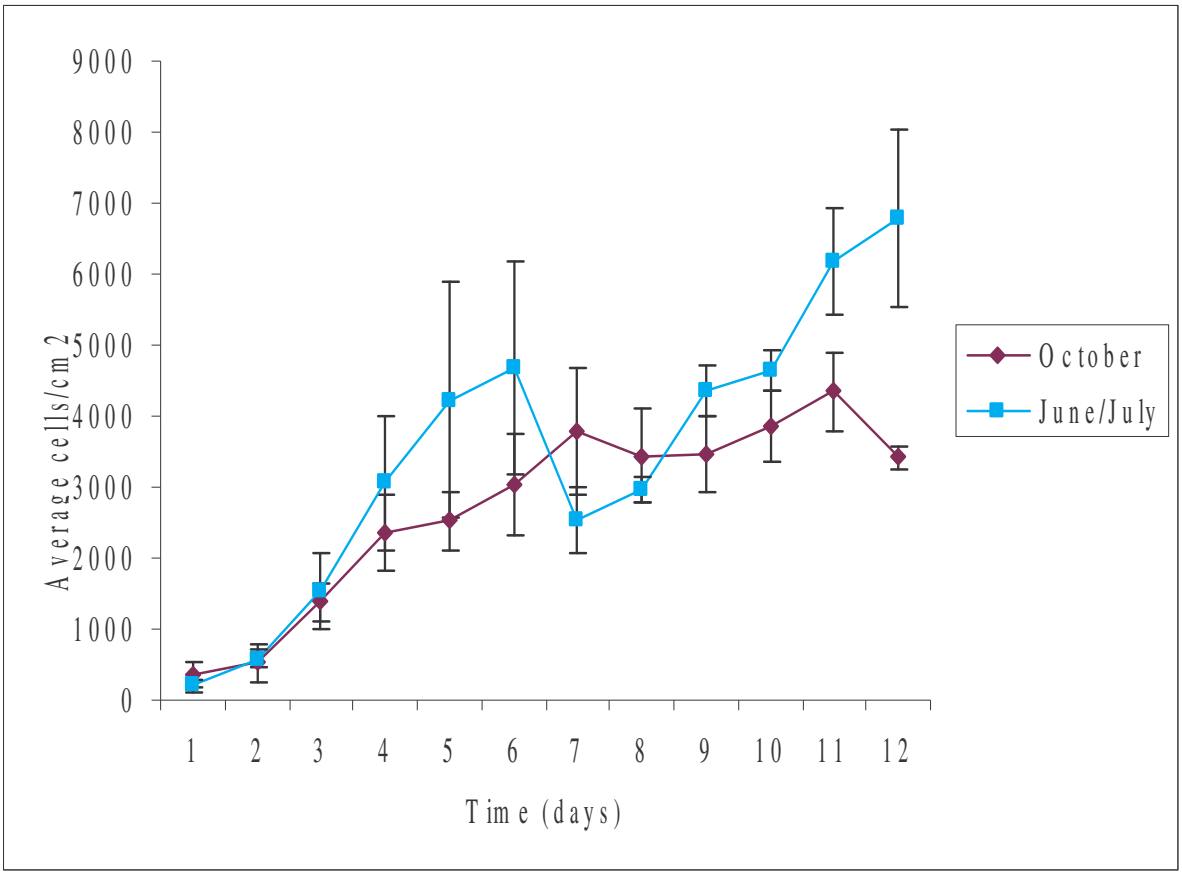
## Colonization of cloth in the Fort Whyte Center pond:




**Appendix III; Figure 1:** Algal colonization on cotton and nylon for the Fort Whyte Center mesotrophic pond, June,2005. Bars showing standard error of the mean.




**Appendix III; Figure 2:** Algal colonization on cotton and denim for the Fort Whyte Center mesotrophic pond, October 2005. Bars showing standard error of the mean.




**Appendix III; Figure 3**: Scaled down version of figure 3 in text showing colonization on cotton for the Fort Whyte Center pond in June and October, 2005. The scale has been enlarged to demonstrate the logistic growth pattern in the Fort Whyte Center pond. Bars showing standard error of the mean.

# Appendix IV

## Sample size estimations for cloth and hair:

**Appendix IV; Table 1:** Sample size estimations for Rossmere Golf Course October Cotton trial using a 95% confidence interval.

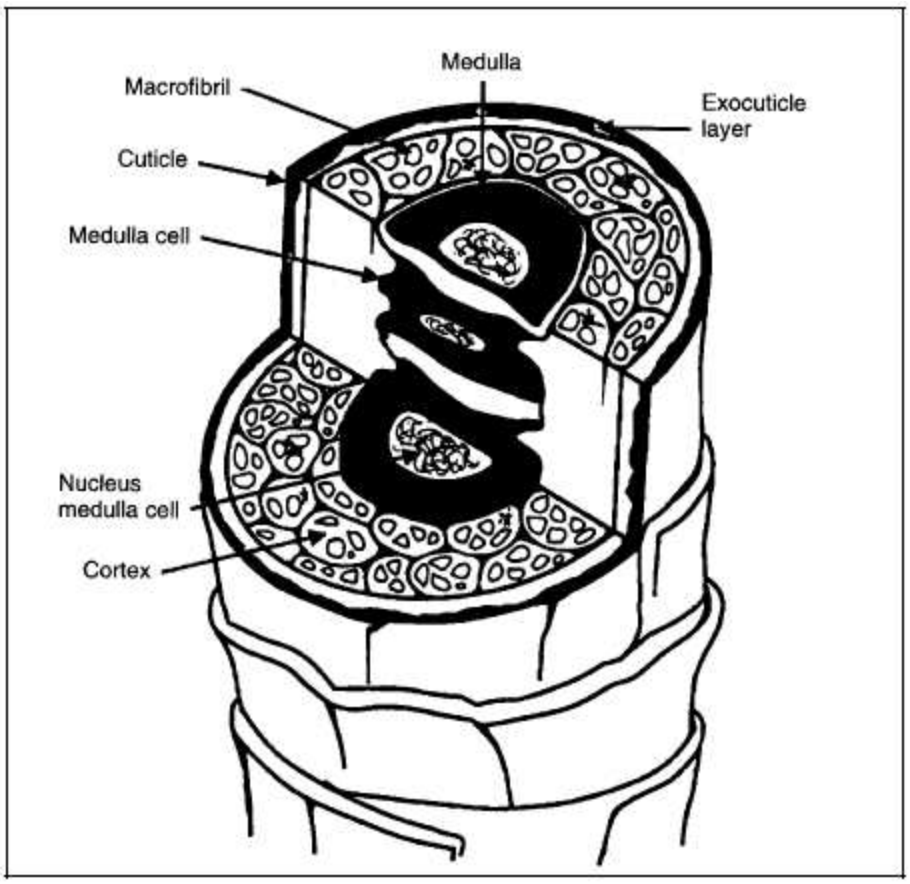
| Day | Average cells/cm$^2$ | Standard deviation | Sample estimate for 5% of the mean | Sample estimate for 10% of the mean | Sample estimate for 20% of the mean |
|---|---|---|---|---|---|
| 1 | 366 | 618.66 | 45734682 | 11433670 | 2858418 |
| 2 | 1446 | 490.47 | 1841183 | 460296 | 115074 |
| 3 | 3204 | 498.32 | 387024 | 96756 | 24189 |
| 4 | 3454 | 695.35 | 648465 | 162116 | 40529 |
| 5 | 5319 | 1808.11 | 1848678 | 462169 | 115542 |
| 6 | 6935 | 526.69 | 92293 | 23073 | 5768 |
| 7 | 9416 | 1297.58 | 303857 | 75964 | 18991 |
| 8 | 8568 | 1326.42 | 383464 | 95866 | 23966 |
| 9 | 11540 | 1842.26 | 407764 | 101941 | 25485 |
| 10 | 14592 | 3142.02 | 741801 | 185450 | 46363 |
| 11 | 15824 | 2464.62 | 388137 | 97034 | 24259 |
| 12 | 15021 | 2306.21 | 377167 | 94292 | 23573 |

**Appendix IV; Table 2:** Sample size estimations for Rossmere Golf Course October Hair trial using a 95% confidence interval.

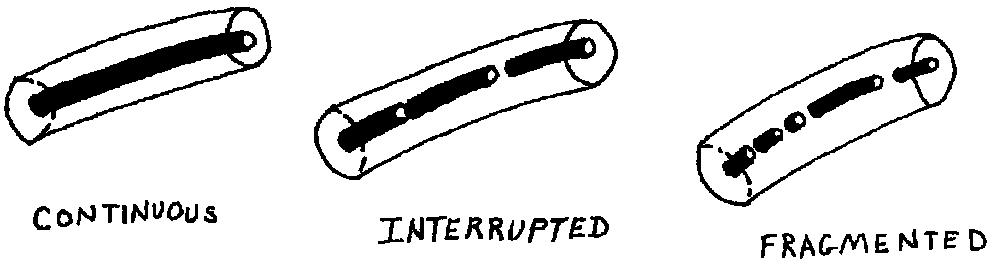
| Day | Average cells/mm | Standard deviation | Sample estimate for 5% of the mean | Sample estimate for 10% of the mean | Sample estimate for 20% of the mean |
|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 |
| 3 | 1 | 1 | 42666667 | 0.001 | 0.002 |
| 4 | 6 | 10 | 40989036 | 0.006 | 0.012 |
| 5 | 11 | 9 | 10746872 | 0.011 | 0.022 |
| 6 | 4 | 3 | 9671111 | 0.004 | 0.008 |
| 7 | 21 | 5 | 931133 | 0.021 | 0.043 |
| 8 | 26 | 9 | 1733149 | 0.026 | 0.052 |
| 9 | 11 | 11 | 17884313 | 0.011 | 0.021 |
| 10 | 23 | 10 | 2837188 | 0.023 | 0.045 |
| 11 | 26 | 5 | 546672 | 0.026 | 0.053 |
| 12 | 19 | 7 | 2082874 | 0.019 | 0.038 |

# Appendix V

Hair strand Anatomy:



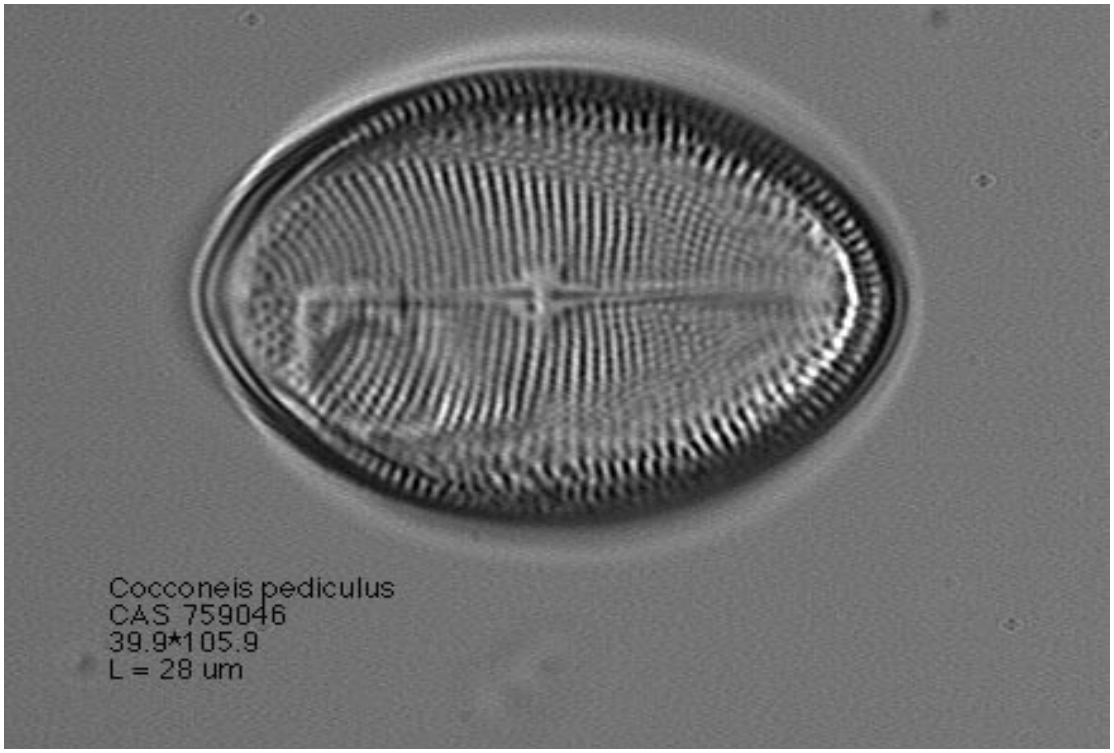

Schematic diagram of the hair shaft, note the outer cuticle, the cortex and inner medulla (Powell and Rogers 1997).



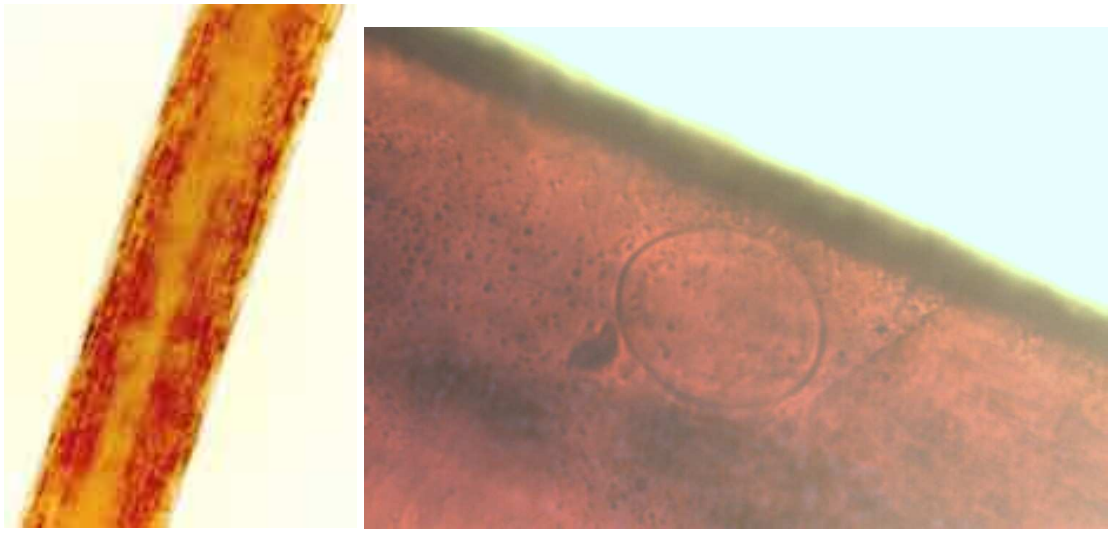

Medulla patterns exhibited in a mature hair shaft

# Appendix VI

Indicator species: *Cocconeis pediculus* Ehr.



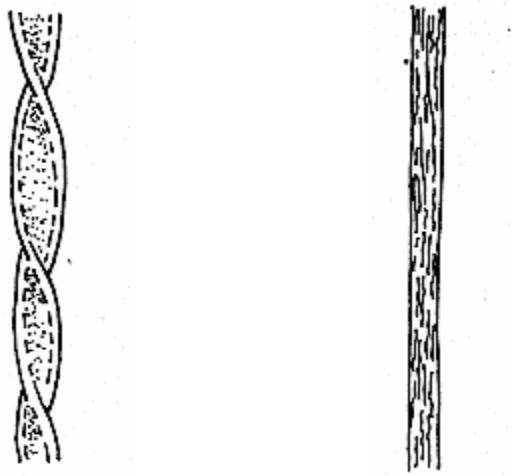

Enlarged photograph of *Cocconeis pediculus sp.* taken with a light microscope (California Academy of Sciences 2001).



An un-colonized hair strand (left) at 100x magnification and *C.pediculus* colonizing a hair strand (right) at 400x magnification.

# Appendix VII

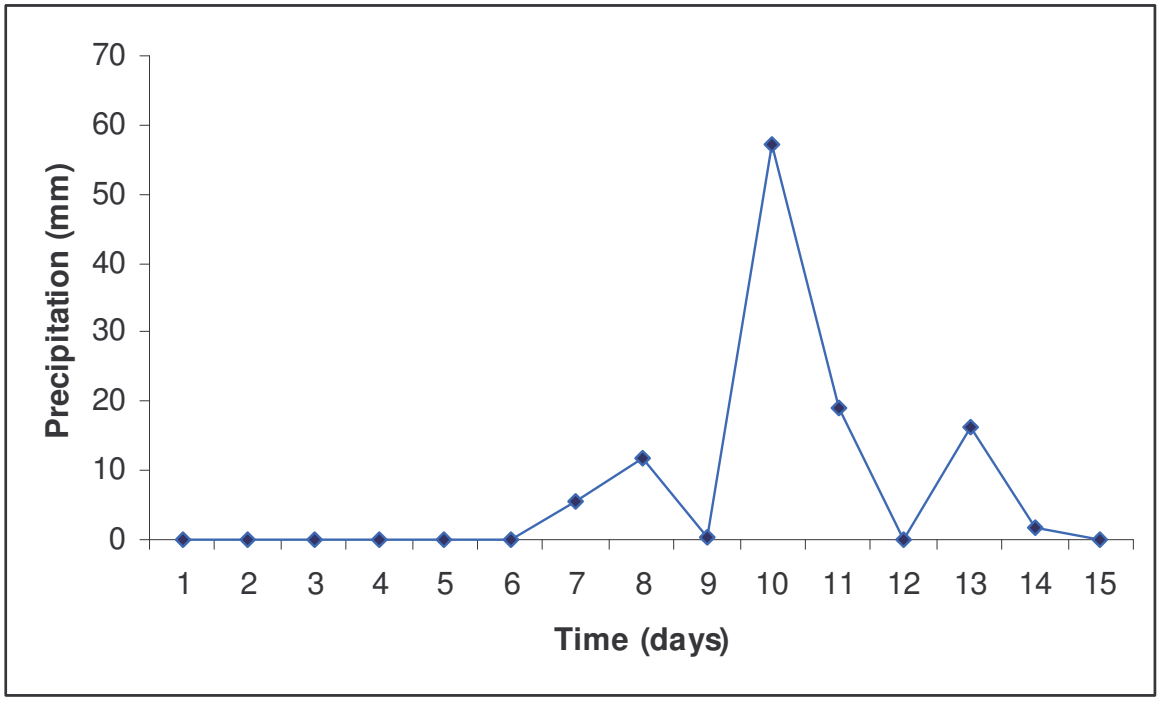
Cloth morphology:



Cotton fiber (left) and nylon fiber (right), modified from the National Institute of Forensic science website (National Institute of Forensic Science 2001). The cotton fiber is twisted, while the nylon fiber is straight and sleek this allows for a tighter weave than cotton.

# Appendix VIII

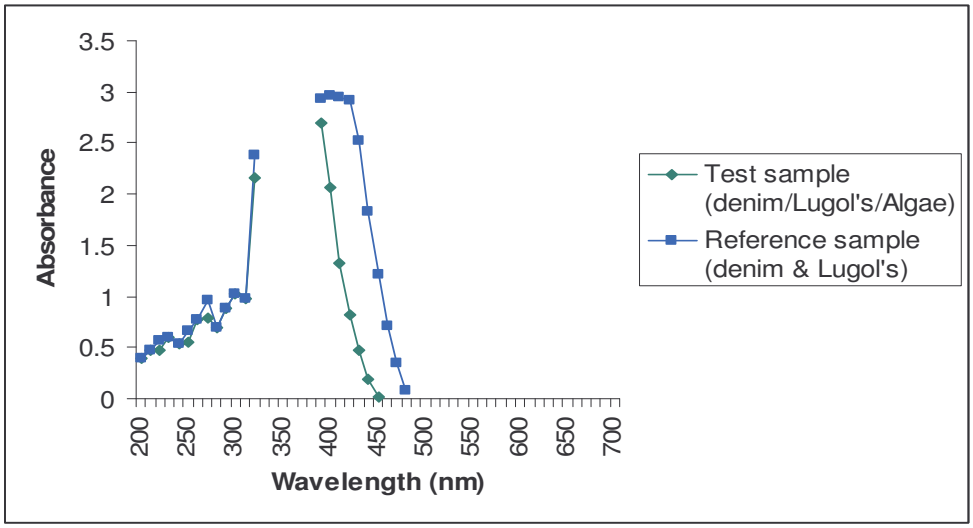
Environmental factors affecting colonization: Precipitation



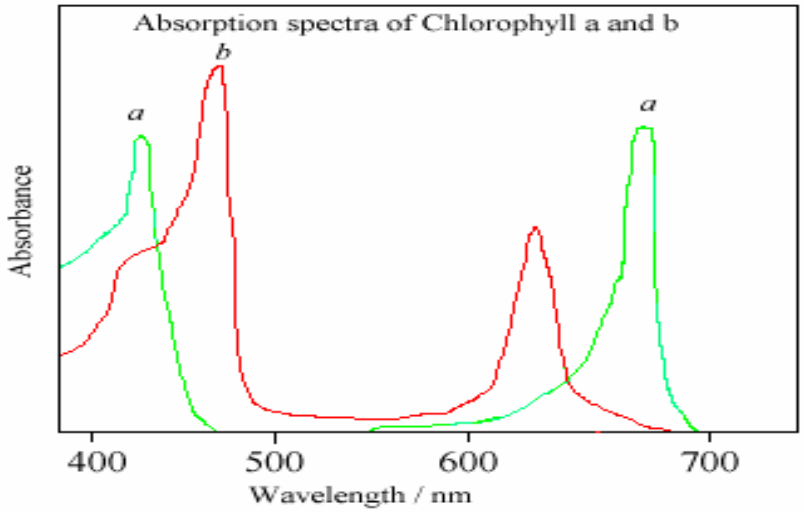

Net rainfall for June 2005. Rainfall experienced on days seven, eight, ten and eleven correspond to days in which algal cell density decreased on the nylon cloth substrate.

# Appendix IX

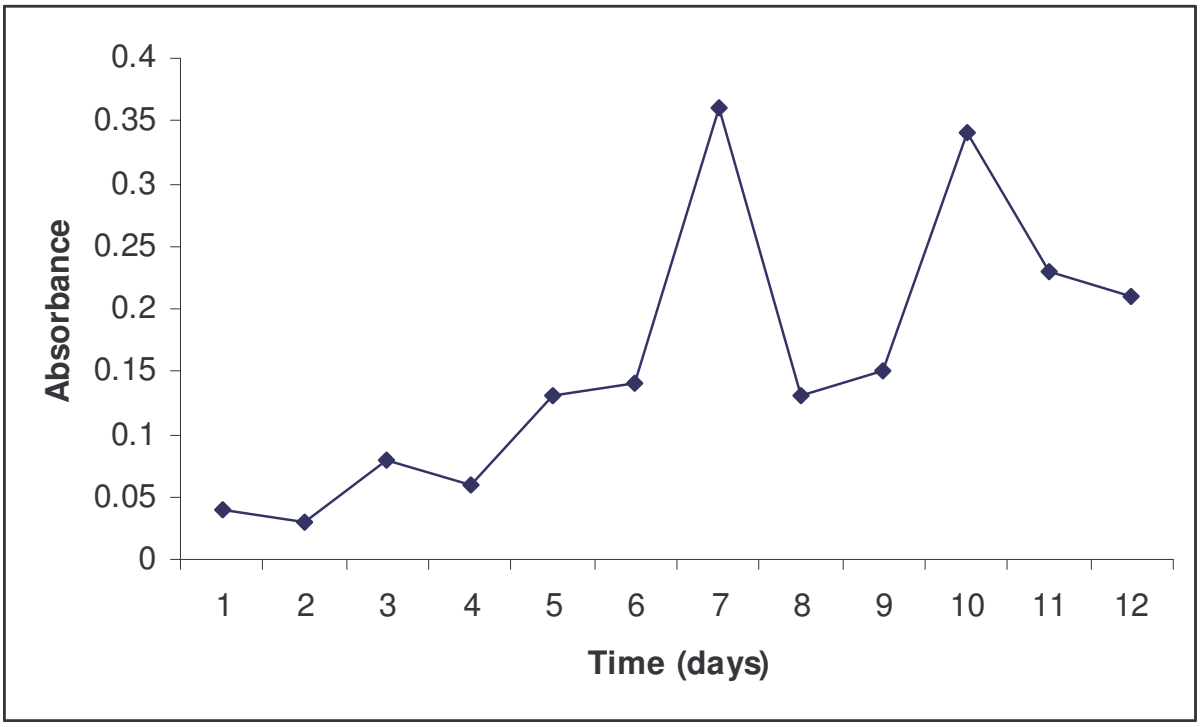
Rejected techniques for algal PMSI estimations:




**Appendix IX; Figure 1:** Absorbance spectrums comparing two samples, the reference sample containing denim and Lugol's Iodine and the test sample containing denim, Lugol's Iodine and algae. There were no peaks experienced in the 600-700nm region which correspond with chlorophyll a and b concentrations (below). Therefore it is assumed that the algal chlorophyll was masked by the Lugol's Iodine and denim dyes.




**Appendix IX; Figure 2:** Typical Absorbance spectrum of chlorophyll a and b (May 2006)




**Appendix IX; Figure 3:** Optical density test for the Fort Whyte Center October cotton trial. The curve experiences large peaks at day seven and ten, followed by troughs on days 8 and 11 respectively. These peaks and troughs do not correspond to visual diatom enumerations and therefore the optical density method was rejected.

# Appendix X

Simulation tests:

**Hair and cloth Data Analysis Sheet: Case 1**

Label: CASE 1 .
Processor: KELLY A. HILLE .
Processing date: MARCH 1ST 2006 .

Collection date: 8:OO AM, JUNE 24TH 2005 .
Collector: DR. KENT SIMMONS .
Location: ROSSMERE GOLF & COUNTRY CLUB .
Victim: MALE 27, 6'1", 185LBS .

**Environmental Conditions:**

| Condition | Description |
|---|---|
| Type of water body (Lake, pond, river,etc.) | POND |
| Water temperature | 22 °C |
| Nutrient status of the water body | EUTROPHIC |
| Current conditions (upon collection) | SLIGHT RAIN SHOWERS; 5-8mm |
| Antecedent conditions (ie: precipitation) | DRY, SUNNY, NO PRECIPITATION |
| Other pertinent information: | NONE |

**Hair Analysis:**

| Characteristic | Description |
|---|---|
| Microscopic field diameter at 400x | 0.037mm |
| Conversion factor | 5.4 |
| Tip or cut end of hair | TIP END |
| Placement in bundle | OUTER EDGE OF THE BUNDLE |
| Color | DARK BROWN/BLACK |

Sample analysis:

| Count 1 Cells/mm | Count 2 Cells/mm | Count 3 Cells/mm | Count 4 Cells/mm | Count 5 Cells/mm | Count 6 Cells/mm | Count 7 Cells/mm | Count 8 Cells/mm |
|---|---|---|---|---|---|---|---|
| 3.24 | 8.64 | 14.04 | 21.6 | 12.96 | 24.84 | 23.76 | 30.24 |

| Average Cells/mm | Standard deviation |
|---|---|
| 17 | 9.2 |

**Cloth Analysis:**

| **Characteristic** | **Description** |
|---|---|
| Microscopic field diameter at 100x | 0.84mm |
| Conversion factor | 446.25 |
| Fabric type | COTTON |
| Type of garment | WHITE COTTON T-SHIRT |
| Placement on garment (where was it cut from) | CHEAST |

Sample analysis:

| Count 1 Cells/$cm^2$ | Count 2 Cells/ $cm^2$ | Count 3 Cells/ $cm^2$ | Count 4 Cells/ $cm^2$ | Count 5 Cells/ $cm^2$ |
|---|---|---|---|---|
| 7898.625 | 7987.875 | 10174.500 | 9058.875 | 11156.250 |

| Average Cells/mm | Standard deviation |
|---|---|
| 9255 | 1409.3 |

(Please use a new sheet for each fabric type)

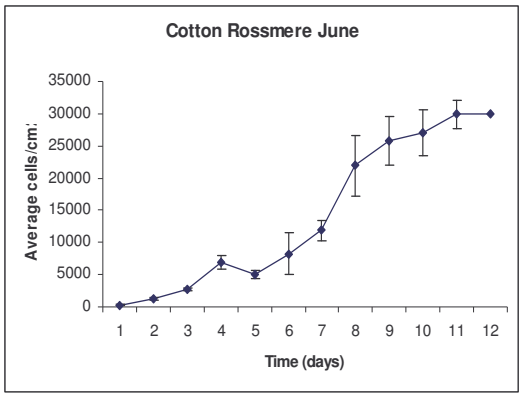
Postmortem Submersion Interval Estimate:

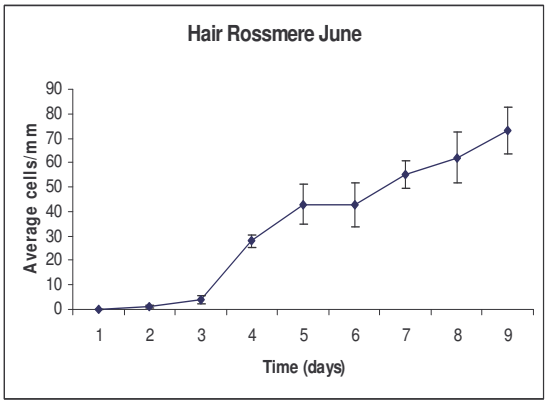
PMSI estimate: FOUR DAYS OF SUBMERSION .

Logic/reasoning (be as thorough as possible):

A PMSI ESTIMATE OF FOUR DAYS WAS DETERMINED BY OVERLAPPING THE HAIR AND COTTON CALIBRATION CURVES. THE COTTON CALIBRATION CURVE PRODUCED AN ESTIMATE OF FOUR OR SIX DAYS POSTSUBMERSION. THE HAIR CALIBRATION CURVE PRODUCED AN ESTIMATE OF THREE TO FOUR DAYS OF SUBMERSION. THE PMSI WAS THEREFORE ESTIMATED AS BEING FOUR DAYS OF SUBMERSION.

Calibration curves:



**Hair and cloth Data Analysis Sheet: Case 2**

Label: CASE 2 .
Processor: KELLY A. HILLE .
Processing date: MARCH 2ND 2006 .

Collection date: 4:00 PM, OCTOBER 17TH 2005 .
Collector: KELLY A. HILLE .
Location: FORT WHYTE CENTER .
Victim: MALE 40, 5'10", 210 LBS .

**Environmental Conditions:**

| Condition | Description |
|---|---|
| Type of water body (Lake, pond, river,etc.) | POND |
| Water temperature | 10 °C |
| Nutrient status of the water body | MESOTROPHIC |
| Current conditions (upon collection) | CLOUDY, DRY; NO PRECIPITATION |
| Antecedent conditions (ie: precipitation) | DRY; NO PRECIPITATION |
| Other pertinent information: | NONE |

**Hair Analysis:**

| Characteristic | Description |
|---|---|
| Microscopic field diameter at 400x | 0.037mm |
| Conversion factor | 5.4 |
| Tip or cut end of hair | TIP END |
| Placement in bundle | OUTER EDGE OF THE BUNDLE |
| Color | DARK BROWN/BLACK |

Sample analysis:

| Count 1 Cells/mm | Count 2 Cells/mm | Count 3 Cells/mm | Count 4 Cells/mm | Count 5 Cells/mm | Count 6 Cells/mm | Count 7 Cells/mm | Count 8 Cells/mm |
|---|---|---|---|---|---|---|---|
| 15.12 | 5.40 | 4.32 | 3.24 | 15.12 | 4.32 | 11.88 | 6.48 |

| Average Cells/mm | Standard deviation |
|---|---|
| 8 | 5 |

**Cloth Analysis:**

| **Characteristic** | **Description** |
|---|---|
| Microscopic field diameter at 100x | 0.84mm |
| Conversion factor | 446.25 |
| Fabric type | COTTON |
| Type of garment | WHITE COTTON T-SHIRT |
| Placement on garment (where was it cut from) | BACK |

Sample analysis:

| Count 1 Cells/$cm^2$ | Count 2 Cells/ $cm^2$ | Count 3 Cells/ $cm^2$ | Count 4 Cells/ $cm^2$ | Count 5 Cells/ $cm^2$ |
|---|---|---|---|---|
| 981.75 | 803.25 | 1115.62 | 1428.00 | 981.75 |

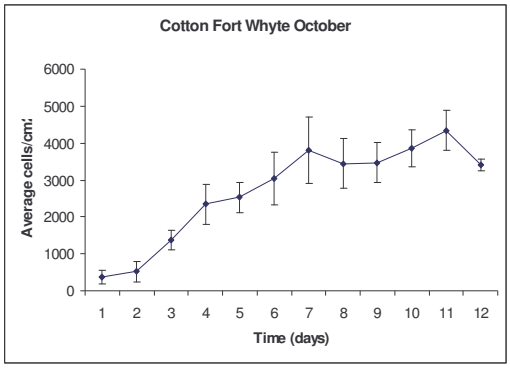
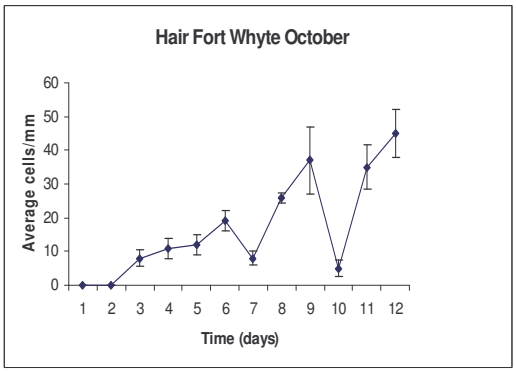
| Average Cells/mm | Standard deviation |
|---|---|
| 1062.00 | 232.70 |

(Please use a new sheet for each fabric type)

Postmortem Submersion Interval Estimate:

PMSI estimate: THREE DAYS OF SUBMERSION .

Logic/reasoning (be as thorough as possible):

A PMSI ESTIMATE OF THREE DAYS WAS DETERMINED BY OVERLAPPING THE HAIR AND COTTON CALIBRATION CURVES. THE HAIR CALIBRATION CURVE PRODUCED AN ESTIMATE OF THREE, FOUR, FIVE OR SEVEN DAYS OF SUBMERSION. THE COTTON CALIBRATION CURVE PRODUCED AN ESTIMATE OF THREE DAYS OF SUBMERSION. THE PMSI WAS THEREFORE ESTIMATED AS BEING THREE DAYS OF SUBMERSION.

Calibration curves: