THE EFFECTS OF NATURAL AND ANTHROPOGENIC FACTORS ON MICROBES DECOMPOSING THE EMERGING MACROPHYTE *SCIRPUS LACUSTRIS* IN PRAIRIE AQUATIC SYSTEMS

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ABSTRACT

Emergent macrophytes, like *Scirpus lacustris,* are the foundation of the very high biological productivities of wetlands in the Northern Prairies of North America. Fungi and bacteria are the primary organisms that sequester carbon and nutrients from these macrophytes. Almost nothing is known about the process of microbial decomposition and how natural environmental factors and anthropogenic pollutants may impact the microbes associated with *Scirpus* as it decays while standing and after it falls into the water.

Pond 50, located at the St. Denis National Wildlife area, Saskatchewan, Canada, was chosen as the study site because it is a wetland that typifies the prairie ecozone. First, a procedure to extract ergosterol, a molecule used to estimate living fungal biomass, from various environmental matrices was developed. Ergosterol was detected by high-pressure liquid chromatography with a UV detector (HPLC-UV) and corroborated by mass spectrometry analysis. Seasonal variations in fungal biomass and fungal production, the latter measured by the incorporation of [1-^{14}C] acetate into ergosterol, associated with *Scirpus* stems, both above and below water, were determined. Changes in fungal biomass and productivities on freshly cut green *Scirpus* stems decaying in the water under either natural solar radiation (UV+) or protected from ultraviolet (UV-) radiation, were followed over the summer. Several experiments measuring the impact of temperature on fungal biomass and production on *Scirpus* decaying in pond water were conducted in the laboratory. In view of antibiotics being detected in surface waters, tetracycline was chosen to determine its effects on the microbes that decompose *Scirpus*. Prior to this experiment, tetracycline's adsorptive characteristics in distilled, river and pond water were measured. Tetracycline's photolytic and microbial rates of degradation were determined in the three mentioned

waters in the laboratory in the light and dark and also in natural sunlight (UV+) or protected (UV-) from UV radiation in non-sterile waters. The effects of tetracycline on protein production in planktonic bacteria in river and pond water were experimentally measured. Lastly, confocal laser microscopy (CLSM) was used to study the microbial colonization on *Scirpus* stems immediately after submersion in river and pond water, in the absence or presence of 500 $\mu g\ L^{-1}$ and 4000 $\mu g\ L^{-1}$ tetracycline.

Saponification in a hot water bath for 30 mins followed by toluene extraction was the most ideal method to extract ergosterol from environmental samples. HPLC-UV detections of ergosterol, compared to detections by mass spectrometry, were found to be reliable with all matrices except water.

Fungal decomposition began and was the greatest in the spring despite low water temperatures. There was no significant difference in biomass or production on aerial versus submerged portions of *Scirpus*. Water temperature was correlated to fungal production ($r = 0.7$, $P < 0.005$) for aerial stem pieces but not for submerged pieces. However, in laboratory experiments water temperature had a measurable effect on both biomass and production in submerged stem pieces. With respect to *Scirpus* decaying under UV+ or UV- in pond water, there was no significant difference in either fungal biomass ($P = 0.76$) or production ($P = 0.96$) between the two treatments.

There were significant differences ($P<0.05$) in the adsorption of tetracycline between distilled, river and pond waters. Half-lives of tetracycline were significantly shorter ($P<0.05$) in the light than in the dark and in sterile waters compared to non-sterile waters ($P<0.05$) and also were significantly different ($P<0.05$) between the three waters in all experiments. Tetracycline photolysis experiment conducted in natural sunlight showed there were significant differences ($P<0.05$) between the UV+ and UV- light treatments as well as between the three different waters ($P<0.05$) in both light

treatments. Tetracycline bound to the matrix did not undergo photolysis suggesting tetracycline will probably persist in aquatic environments.

Tetracycline significantly ($P<0.05$) inhibited protein production in planktonic bacteria at concentrations ≥ 10 $\mu g\ L^{-1}$ in river water but the inhibition was considerable ($P<0.05$) only at 4000 $\mu g\ L^{-1}$ in pond water. Submersion of dry *Scirpus* stems in river and pond water resulted in dramatic increases in microbial biomass on the stems that peaked about 10 days after immersion. The increase in biomass coincided with the colonization of the external surface of the stem and the decrease with the exhaustion of this substrate. In treatments where tetracycline was added there was a significant decreases ($P<0.05$) in microbial biomass between the control and other treatments in the river experiment but not in pond water due to large variances in data ($P>0.05$). Biofilms tended to be thicker in the 4000 $\mu g\ L^{-1}$ tetracycline treatments in both water experiments suggesting the high concentration of tetracycline may have induced polymer formation so as to limit the toxicity of tetracycline. Fungal biomass tended to be higher at 4000 $\mu g\ L^{-1}$ compared to the control treatments in both water experiments suggesting that in normal conditions bacteria exerted an inhibitory influence on fungi. Total microbial biomass on the stems was greater in the pond water experiment compared to the river water experiment, possibly because of the higher DOC and POM in the pond water, but fungal biomass was greater in the river system compared to the wetland system indicating that fungal dominance may vary in different water ecosystems.

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PUBLICATIONS

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- Headley, J. V., K. M. Peru, B. Verma and R. D. Robarts. 2002. Mass Spectrometric Determination of Ergosterol in a Prairie Natural Wetland. Journal of Chromatography A (958): 149-156.
- Verma, B., R. D. Robarts, J. V. Headley, K. M. Peru and N. Christofi. 2002. Extraction Efficiencies and Determination of Ergosterol in a Variety of Environmental Matrices. Communications in Soil Science and Plant Analysis (33): 3261-3275.

CONFERENCE PRESENTATIONS

- "Ergosterol extraction and detection from a variety of environmental matrices." B. Verma, J. V. Headley, R. D. Robarts, K. M. Peru and N. Christofi at The 38th Annual Western Canada Trace Organic Workshop, Edmonton, Alberta, April 27 - 30, 2003.
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- Invited lecture: Department of Toxicology, University of Saskatchewan "Pharmaceuticals in the Environment" October 2002. B. Verma
- "LC/APCI/MS/MS Determination of Ergosterol in a Prairie Natural Wetland". 50th American Society for Mass Spectrometry Conference 2002 Orlando, FL. J.V. Headley, K.M. Peru, B. Verma and R. D. Robarts.
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- "Ergosterol Detection in a Variety of Environmental Matrices Using LC/UV and Off-Line LC/MS/MS". 7th International conference on Soil and Plant Analysis Edmonton, Alberta 2001: B. Verma, R. D. Robarts, J.V. Headley, K.M. Peru and N. Christofi.

Glossary of Terms

μCi	Micro-Curie
AFDM	Ash Free Dry Mass
ANOVA	Analysis of Variance
APCI	Atmospheric Pressure Chemical Ionisation
CID	Collisional Induced Dissociation
CLSM	Confocal Laser Scanning Microscopy
DAPI	4',6'- diamidino-2-phenylindole
DOC	Dissolved Organic Carbon
DPM	Disintegrations Per Minute
EDTA	Ethylenediaminetetraacetic Acid
EPS	Extracellular Polymeric Substance
ES^+ or ES^-	Electrospray in Positive or Negative Ion Mode
HPLC	High Pressure Liquid Chromatography
HPLC-UV	High Pressure Liquid Chromatography with Ultraviolet Detection
LC-APCI-MS-MS	Liquid Chromatography Atmospheric Pressure Chemical Ionisation with Tandem Mass Spectrometry
MIC	Minimum Inhibitory Concentration
MRM	Multiple Reaction Monitoring
MS-MS	Tandem Mass Spectrometry
ND	Non Detectable
nM	Nanometer
PAR	Photosynthetically Active Radiation
POC	Particulate Organic Carbon
POM	Particulate Organic Matter
PON	Particulate Organic Nitrogen
S.D.	Standard Deviation
S.E.	Standard Error
SIM	Single Ion Monitoring
SPME	Solid Phase Microextraction
SRM	Selected Reaction Monitoring
TCA	Trichloroacetic Acid
UV	Ultraviolet Radiation
UV-A	Ultraviolet Radiation from 320 to 400 nM Wavelegths
UV-B	Ultraviolet Radiation from 280 to 320 nM Wavelengths
Z-Series	A Series or Stack of Sequentially Scanned Optical Sections Along the Z axis

TABLE OF CONTENTS

Table of Figures

LIST OF TABLES

CHAPTER 1

INTRODUCTION

The universe is filled with billions of spheres out of which only the Earth is known to have life. On the Earth there is a great variety of plant and animal life that is readily visible. But if we were to pick up a handful of dirt from a forest floor, a palm full of water from a lake or marsh, there exists in that handful a universe made up of many billions of microbes. Approximately half of the gross global primary productivity is derived from microorganisms (Brock 1966). Microorganisms such as bacteria and fungi are ubiquitous over the whole of the Earth and also play a significant but invisible role in removing carbon and other nutrients from decaying animal and plant litter. Once an organism dies it is a mass of potential energy and nutrition for organisms that have the capacity to assimilate or ingest it. Without this process of decomposition nearly all life on Earth would cease because carbon and nutrients would remain in the dead organisms, unable to be used by any other living things. Bacteria and fungi, therefore, are an essential component of the carbon and nutrient cycle and it can be assumed that anthropogenic compounds, such as pesticides and antibiotics, which may be harmful to these microorganisms, may disrupt the process of decomposition.

Near land water transition zones, such as marshes, streams, lakes and wetlands, there is often a significant amount of plant litter received either allochthonously from forests or from grasses and autochthonously from macrophytes that grow in or near aquatic systems. The Northern Prairies of North America is a region marked by numerous wetlands or ponds that are some of the most productive aquatic systems in the world (Murkin 1989). The productivities of these prairie wetlands, which generate anywhere from 50-80% of North America's duck population (Batt *et al.* 1989) are based and dependent on emergent macrophytes, such as *Scirpus lacustris*, for sources

of carbon and nutrients (van der Valk & Davis 1978). Dead plant litter in these aquatic environments is predominantly stripped of its carbon and nutrients by organisms such as filamentous fungi and bacteria (Gessner & Newell 2002). Researchers have studied macrophyte decomposition in Prairie wetlands but they predominantly measured nutrient and litter mass and did not differentiate between the fungal and bacterial components that are present in decaying litter (Wrubleski *et al.* 1997, Walse *et al.* 1998, Thormann *et al.* 2001).

In other aquatic systems researchers have previously measured bacterial biomass in decaying litter by 4',6-diamidino-2-phenylindole (DAPI), a staining dye which fluoresces when it binds to nuclear DNA of bacteria and other organisms (Baldy *et al.* 2002, Hieber & Gessner 2002) and fungal biomass and production by counting conidia, measuring fungal hyphae or by isolation of fungi on nutrient media (Gessner & Newell 2002). But these methods were always tentative because much of the mycelial network resides within the substrate and is difficult to account for (Gessner & Newell 2002). It was fortunate, therefore, that ergosterol, a cell membrane component largely restricted to fungi (Nielsen & Madsen 2000, Gessner & Newell 2002), was able to be used as an index molecule to estimate eumycotic fungal biomass (Lee *et al.* 1980). Additionally, a method was developed whereby the rate of [1-^{14}C] acetate incorporation into ergosterol could be used to estimate instantaneous rates of fungal growth or production (Newell & Fallon 1991). The use of ergosterol and the [1-^{14}C] acetate into ergosterol incorporation technique to measure fungal production has considerably advanced our understanding of litter decomposition and microbial interactions in a variety of aquatic environments but there have been no such studies conducted in Canada.

Once a macrophyte dies it does not immediately fall into the water and it may remain standing for a considerable time before abscission occurs and the plant enters the water. Scientists have measured fungal biomass and production on dead, standing plant matter, using ergosterol and the acetate incorporation method, (Newell *et al.* 1995, Newell & Porter 2000, Gessner 2001, Newell 2001, Verma *et al.* 2003) and have found that considerable amounts of carbon and nutrients are sequestered while the plant is standing. There have been no such studies conducted on macrophytes or any other litter type in the northern prairies of North America and thus the fungal biomass and productivities associated with standing litter in these regions is unknown. Macrophytes in wetlands are partially under water and partially in the air and it is likely that decomposition is likely to occur simultaneously in plant portions that are submerged as well as above the water. There has thus far been no research conducted that has simultaneously measured fungal biomass and production on dead, naturally standing macrophytes in both the aerial and submerged litter parts. Surprisingly, there have also been no studies published that have regularly measured variations in fungal biomass and production on decaying litter over a season, from spring to fall, so our knowledge of the variability, if any, in fungal biomass and productivities over a season are unknown.

Researchers have measured, over an extended period, microbial biomass and production associated with litter decaying in water (Kominkova *et al.* 2000, Kuehn *et al.* 2000, Verma *et al.* 2003). These studies have shown that in aquatic systems fungi are the dominant microbial decomposers accounting from between 63% to 99% of the total microbial biomass (Baldy *et al.* 1995, Suberkropp & Weyers 1996, Kuehn *et al.* 2000, Baldy *et al.* 2002, Hieber & Gessner 2002). However, there has not been a study thus far that has looked in detail at the patterns of microbial colonization that occurs

immediately upon and following litter submergence. Once litter does fall into the water and becomes colonized, the microbes decomposing the litter are subject, especially in prairies wetlands because these are often shallow, to extremes of temperature and ultraviolet (UV) radiation. Two studies thus far have researched the effects of UV radiation on fungal biomass in decaying litter with one study showing a negative effect (Denward *et al.* 2001) on fungal biomass and the other showing no effects (Denward *et al.* 1999). However, fungal productivities were not measured in either if these studies. Temperature is a very important regulating factor in many microbial processes and it is surprising that there has thus far been no study that has measured the impacts of abrupt temperature changes on fungal biomass and production in decaying litter. As well, there have been no measurements of the long term effects of temperature on fungal biomass and productivities in decaying litter in any system in the world. Our knowledge of the microbial colonization process that occurs immediately after submergence as well as the impacts of both temperature and UV radiation on fungal production and biomass in litter decaying in water, therefore, is limited.

Confocal laser scanning microscopy (CLSM), in conjunction with fluorescent probes, has been utilised to study biofilms on a variety of surfaces such as plastics, metals and soils (Caldwell *et al.* 1992, Wolfaardt *et al.* 1995, Lawrence *et al.* 1997, Lawrence *et al.* 1998). CLSM offers a number of advantages over other methods in detailing the colonization patterns of bacteria and fungi on the surface of decaying plant material in the presence or absence of anthropogenic pollutants. The most important advantage is that the biofilm can be repeatedly scanned, over time, non-destructively and also that optical sections, along the z-axis, of the interior of a biofilm are also able to be visualized. Laser microscopy, in conjunction with molecular probes, therefore, is an attractive and heretofore untried method that can help to visually follow the pattern

of colonization once a plant enters the aquatic environment, in the presence or absence of any anthropogenic pollutants.

In some wetlands of the northern prairies *Scirpus lacustris* forms the base of the food web and is thus the most important source of carbon and nutrients to microbes that inhabit these wetlands (van der Valk & Davis 1978, Murkin 1989). The present thesis was undertaken to answer questions about the microbial process of decomposition of this important macrophyte while it is standing and when it falls and submerges into the water. The effects of temperature and UV radiation in *Scirpus* decaying in pond water were researched. The microbial, photolytic and adsorption behaviour of tetracycline in distilled, river and pond waters was studied. Lastly, the colonizing behaviour of microbes on the surface of *Scirpus* stems, immediately after submersion into both river and pond waters amended or not amended with tetracycline, was studied by CLSM.

In Chapter 2, because ergosterol has not previously been used to measure fungal biomass in Canadian prairie systems, described is a suitable technique to extract ergosterol from plant matrices. This method had to be developed prior to doing any further work. Additionally, a survey was conducted to measure levels of ergosterol, and therefore living fungi, in different environmental matrices such as pond water, pond sediment, decaying poplar leaves, the roots and stems of dead, standing *Scirpus* as well as the stem of living *Scirpus*. Ergosterol was quantified by a previously established high performance liquid chromatography with ultraviolet detection (HPLC-UV) method but the reliability of HPLC-UV analysis was corroborated with more selective analytical instruments such as mass spectrometry.

Once a protocol for the extraction and quantitation of ergosterol was developed, further work was undertaken, in Chapter 3, to measure the role played by fungi in the decomposition of *Scirpus* stems in the field. Seasonal variations in fungal biomass and

production on standing dead *Scirpus lacustris* stems, both below and above water were assessed to determine which environmental factors were dominant in affecting these variations in a typical northern prairie wetland. Additionally, an experiment was conducted where changes in fungal biomass and productivity on freshly cut green *Scirpus* stems decaying in pond water, either exposed to natural solar radiation or protected from UV radiation, were monitored over a summer. To assess the effects of temperature on fungal biomass and productivities, two experiments were conducted in the laboratory. The [1-^{14}C] acetate incorporation into ergosterol method was used to estimate fungal productivities in decaying *Scirpus* while ergosterol was used to quantify fungal biomass.

There has been no reported work on the fate of tetracycline, in terms of adsorption and photolytic or microbial degradation, in freshwater aquatic environments therefore, in Chapter 4, a series of experiments were conducted. First, the absorptive behaviour of tetracycline in river and pond waters were measured because tetracycline that is free in solution is thought to be active against bacteria while the portion that becomes bound to metals or a matrix is thought to be inactive. Since experiments were to be later performed measuring the effects of tetracycline on decomposition of *Scirpus* stems, it was important to know the microbial and photolytic degradation rates or half-lives, of tetracycline inside of the laboratory. Knowledge of the half lives of tetracycline would ensure that the tetracycline amended waters in the effects experiments were replenished in a timely manner so that a small range of concentration of tetracycline could be maintained throughout the course of the experiment. The photolysis experiments in the laboratory were conducted in both sterile and non-sterile distilled, river and pond water. Additionally, the photolytic rates of tetracycline

degradation in natural sunlight conditions, with or without the influence of UV radiation, in non-sterile distilled, river and pond waters were studied.

Lastly, in Chapter 5, experiments were conducted so that the colonizing pattern of microbes on the surface of *Scirpus* stems could be measured immediately after the stem was submerged in river or pond water in the presence or absence of tetracycline. The concentrations used were 500 and 4000 ug L^{-1} tetracycline in both river and pond water. Additionally, because there have not been any previous studies that have precisely followed the exact microbial process that occurs once dry plant litter falls into water, the microbial dynamics of colonization on dry *Scirpus* stems immediately after submersion were followed over 33 days. Confocal laser microscopy, in conjunction with a fluorescent probe, was utilized to measure microbial colonization. The relative incorporation of L-[4,5-^{3}H]leucine, in the presence or absence of tetracycline, into the protein of planktonic bacteria in river and pond water was evaluated.

CHAPTER 2

METHODOLOGY- FUNGAL BIOMASS DETERMINATION

2.1 INTRODUCTION

Fungi are thought to be the predominant decomposers in aquatic environments accounting from 63% to 99% of the total microbial biomass and production (Newell *et al.* 1995, Suberkropp & Weyers 1996, Chauvet & Suberkropp 1998, Kuehn *et al.* 2000). Ergosterol (chemical structure of which is given in Figure 1) is a cell membrane component largely restricted to fungi (Nielsen & Madsen 2000, Gessner & Newell 2002) thus making it an ideal index molecule for this class of organism. Ergosterol is believed to undergo rapid degradation after cell death. Thus levels of ergosterol present is generally considered to be directly correlated to living fungal biomass (Grant & West 1986, Stahl & Parkin 1996). Most research to date has focused on fungal biomass in decaying plant leaves or grasses in salt-marshes and streams (Newell *et al.* 1995, Suberkropp & Weyers 1996, Suberkropp 1997). Little work, however, has been reported for the detection of ergosterol in environmental matrices other than those noted and there have been no previous studies conducted in northern prairie wetlands.

Saponification followed by toluene extraction is the generally accepted extraction procedure for ergosterol though solid phase extraction has also been successfully employed (Gessner & Schmitt 1996). Here a number of techniques are evaluated in order to develop a method most suitable to extracting ergosterol from *Scirpus* litter parts, poplar leaves, pond water and pond sediment: extraction with methanol only, cold and hot saponification with or without a further solvent extraction, and variations of the length of time solvents are in contact with various matrices to potentially increase the extractable ergosterol into a form suitable for detection.

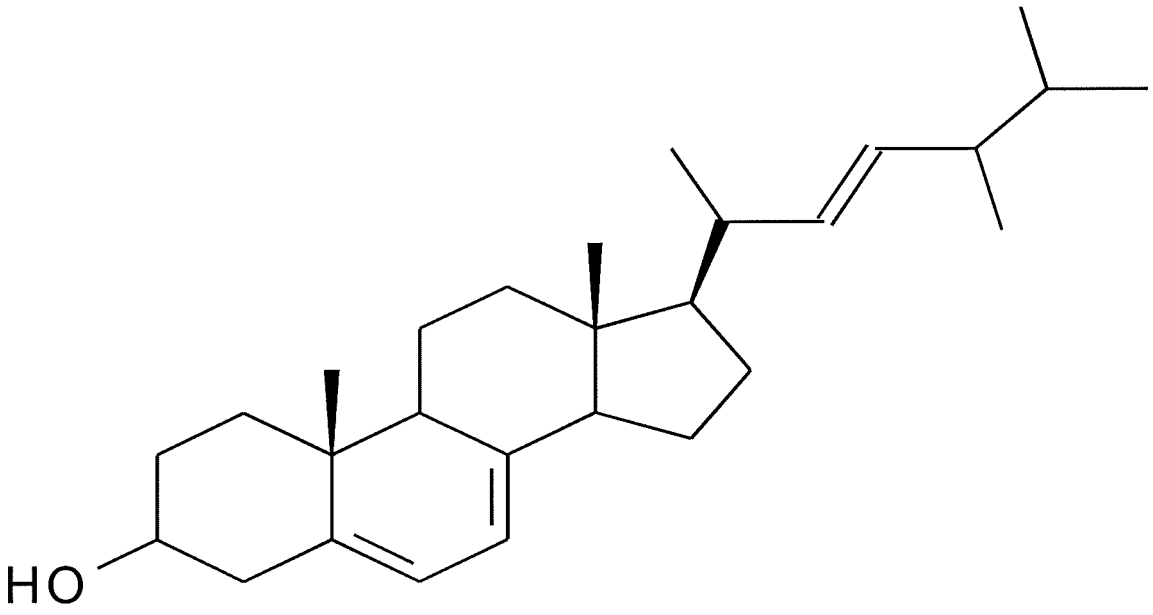


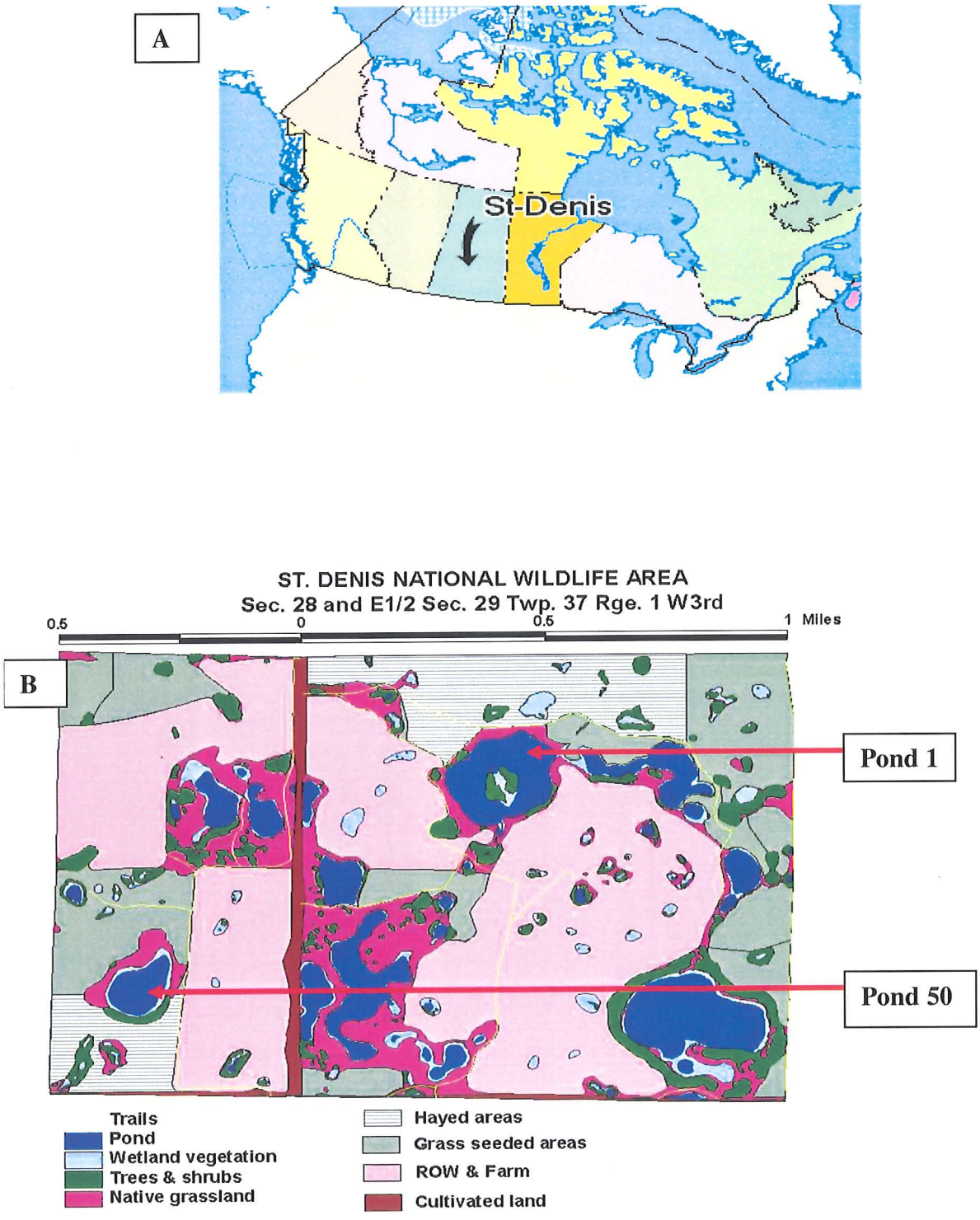
Figure 1. Chemical Structure of Ergosterol

Various methods have been reported for the determination of ergosterol (Arnezeder *et al.* 1985, Rodriguez & Parks 1985, Axelsson *et al.* 1995, Nielsen & Madsen 2000, Saraf *et al.* 2003), most of which are based on conventional high performance liquid chromatography with ultraviolet detection (HPLC-UV) (Rodriguez & Parks 1985) and/or gas chromatography with mass spectrometry detection (Axelsson *et al.* 1995, Saraf *et al.* 2003). For the gas chromatographic methods it is common to form a trimethylsilyl derivative or methyl ester to improve the peak shape and detection limits (Nielsen & Madsen 2000). There has been one application using atmospheric pressure chemical ionization (APCI) with tandem mass spectrometry for the determination of plasma membrane ergosterol of the yeast *Saccharomyces cerevisiae* (Toh *et al.* 2001). However, confirmations were based on infused samples with no liquid chromatographic separation of ergosterol. In the present work a conventional HPLC-UV procedure is applied for quantitation while off-line reverse phase liquid chromatography with positive-ion atmospheric pressure chemical ionisation tandem mass spectrometry (LC/APCI/MS/MS) was utilized for confirmation of ergosterol.

2.2 Materials and Methods

2.2.1 Study Site

Field studies were conducted at a wetland that typifies the prairie ecozone at the St. Denis National Wildlife Area 40 km east of Saskatoon, Saskatchewan, Canada (106° 06′W, 52°02′N) (Figure 2A (The Atlas of Canada 2003) and 2B (Hogan & Conly 2002)).



Figures 2. The location of St. Denis in Canada (A) and Ponds 1 and 50 (B) at the St. Denis National Wildlife Refuge (106° 06′W, 52° 02′N).

2.2.2 Sample Collection and Ash Free Dry Mass Determination (AFDM)

A total of six samples were collected: a triplicate for extractions and another triplicate to determine percent moisture and ash free dry mass (AFDM). All samples were transported on ice to the lab. Samples for extractions were placed in scintillation vials (Fisher Scientific, Canada) containing 10 ml of methanol and extracted for ergosterol the next day (Figure 3). Water was collected in Teflon lined bottles and 10 ml aliquots used for extraction. All materials were stored at 4°C overnight. For AFDM determinations the environmental samples, except water, were placed in zip-lock bags and weighed into pre-weighed aluminium cups immediately upon arrival at the lab. The cups containing the sample were placed in drying ovens for 48 hours at 105°C. The dried samples were then combusted for 4 hours at 450°C (Newell 1993, Kuehn *et al.* 2000). Ash free dry mass was calculated as the difference between the weight before and after combustion. Extraction efficiencies on matrix and blank spikes for all treatments were in the range of 93.9% ± 11.6 S.D. In addition to the spikes, 8 blanks (all non-detect) were run for quality control purposes.

2.2.3 HPLC-UV Conditions

The HPLC-UV was comprised of a Bio Rad (Hercules, California) Model #1350 pump with a reverse phase Supelcosil LC-18 5μm 15 cm X 4.6 mm HPLC column (Supelco, Bellefonte, U.S.A). The mobile phase was HPLC grade methanol with a flow rate of 1.8 ml min^{-1}. The ergosterol retention time was approximately 4.5 minutes and detected by a Dionex (Sunnyvale, California) variable wavelength detector set at 282 nm. External standards were used for quantification which gave a linear calibration (r^2=0.99) (Figure 4). Detection limits under these conditions were approximately 0.33 ng on column and the reproducibility was >95% for all matrices based on triplicate analyses.

2.2.4 LC/APCI/MS/MS Conditions

In order to confirm the presence of ergosterol, a more selective method similar to that described by Toh et al (2001) was employed. However, unlike Toh's application that used simple loop infusion, chromatographic separation was also added to provide retention time as an additional parameter for confirmation. All of the samples, as described below and seen in Table 1, were subjected to LC/APCI/MS/MS analysis. Samples were injected in 15 µl aliquots using a Waters 2690 separations module (Milford, U.S.A). Eluent consisted of 100% methanol at a flow rate of 200µl min^{-1}. Separation was achieved by a Waters Xterrra C_{18} analytical column, 3.5 µm, 2.1 x 100 mm. At these conditions ergosterol eluted at 4.29 minutes. The column effluent was delivered to the APCI interface of a Micromass Quattro Ultima (Manchester, U.K.) triple quadrupole mass spectrometer set to positive ion mode. Interface conditions were as follows: Corona voltage of 7.2 kV and a source and desolvation temperature of 130°C and 500°C, respectively. Nebulizer gas of nitrogen was set to maximum flow rate while the flow rates for the cone and desolvation nitrogen were set to 151 and 181 L hr^{-1}, respectively. Sampling cone voltage was maintained at 11 V. Multiple reaction monitoring (MRM) was employed for confirmation of ergosterol. Monitoring the transition of the precursor ion at 379 m/z to the product ion of 69 m/z (Figure 5), at a dwell time of 0.50 second and an inter channel delay of 0.10 second. The collision cell housing pressure was increased to 3.38×10^{-4} mbar using argon as the collision gas with a collision energy of 78 V applied (laboratory frame of reference).

2.2.5 Ergosterol Stability

Ergosterol standards (Sigma, Canada) of 1, 2, 4 and 10 mg L^{-1} were prepared in methanol and left out on a lab bench for 5 days (21°C± 3°C). Aliquots of the standards were taken daily and compared against freshly prepared standards by injection onto an HPLC-UV system.

2.2.6 Extraction of Ergosterol by Methods Other Than Heating

Homogenised pond bed sediment samples containing decaying plant litter was extracted for ergosterol by a variety of techniques. Equal weights of sample were used for all tests and all were completed in triplicate. The varying extraction liquids (described below) were in contact with the sediment for 30 minutes during the extraction process. Syringe filters were tested for absorbency or reactivity to ergosterol by filtering known standards and measuring the filtrate for loss of ergosterol. No measurable losses were observed due to the filters. For all of the treatments below the mixture volume was evaporated to 1 ml using a gentle stream of nitrogen. 100 µl of the extract was injected into an HPLC-UV system to determine ergosterol concentrations.

2.2.6.1 Methanol

The sediment samples were weighed into scintillation vials containing 20 ml of methanol and agitated periodically for half an hour and then filtered into evaporation tubes using 0.2 µm pore size syringe filters (Acrodisc LC13 PVDF, Sigma Canada).

2.2.6.2 Methanol and 40 g L^{-1} KOH in Ethanol

Samples were weighed into scintillation vials containing 15 ml of methanol and 5 ml of KOH solution, agitated periodically for 30 minutes and then filtered into evaporation tubes using 0.2 µm pore size syringe filters.

2.2.6.3 Methanol Followed by Pentane Extraction

Samples were weighed into scintillation vials containing 20 ml of methanol. After 30 min the mixture was filtered using Whatman #41 paper into separatory funnels where the mixture was extracted with 3 X 10 ml pentane. The pentane was evaporated to dryness and the ergosterol redissolved in 1 ml of methanol.

2.2.6.4 Methanol and 40 g L^{-1} KOH in Ethanol Followed by Pentane Extraction

Samples were weighed out into scintillation vials containing 15 ml of methanol and 5 ml of KOH solution. After 30 minutes the mixture was filtered using Whatman #41 paper into separatory funnels where it was extracted with 3 X 10 ml pentane. The pentane was evaporated to dryness and the ergosterol redissolved in 1 ml of methanol.

2.2.7 Extraction of Ergosterol by a Conventional Method but With Variations in Heating Time

A homogenised sample of decaying poplar leaves was extracted for ergosterol as per Eash *et al* (Eash *et al.* 1996) (see below), with variations of time spent in the water bath in order to determine maximum extraction efficiency. The times tested were 30, 60 and 120 minutes in the water bath. In addition to the heating, the leaves were left to stand at room temperature (21°C± 3°C) in pressure tubes for 24 and 48 hours in a solution of methanol and KOH.

Briefly, the Eash procedure is as follows: environmental samples were placed in 35 ml pressure reaction tubes (Figure 3) (Alltech, Deerfield, IL) containing 15 ml of methanol, 5 ml of 40 g L^{-1} KOH in 95% ethanol followed by vortexing and sonicated ((Branson 5210, Danbury, CT. non-adjustable setting) for 1 minute each. The tubes were placed in an 85°C water bath for 30 minutes and hand mixed after 15 minutes.

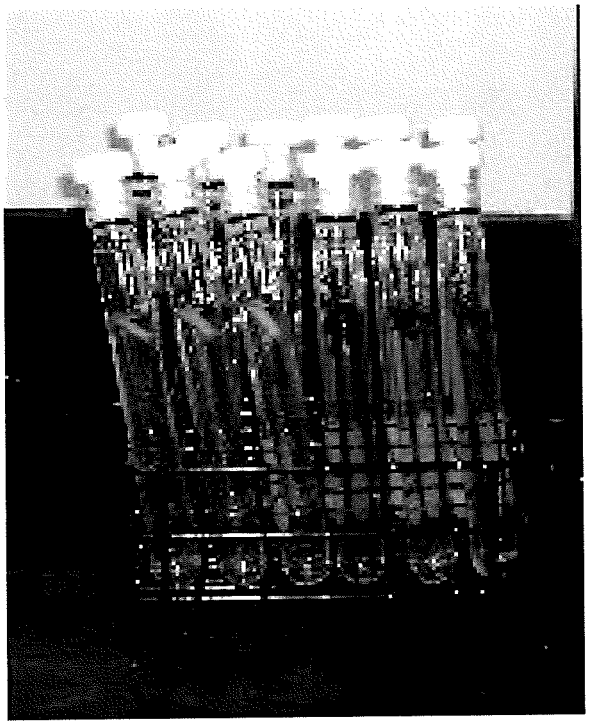


Figure 3. Pressure reaction tubes with *Scirpus* stems inside.

The tubes were allowed to cool and the contents filtered through Whatman # 41 paper into separatory funnels. The filter was rinsed with 5 ml of methanol. The mixture was extracted with pentane three times (3 X 10 ml) and the solvent was evaporated using a gentle stream of dry nitrogen gas. The dried extract was redissolved in 5 ml of methanol, filtered through a 0.2 μm syringe filter and the extract concentrated to 1 ml by evaporation with nitrogen. 100 μl of the extract was injected into an HPLC-UV system to determine ergosterol concentrations.

2.2.8 Extraction of Ergosterol From a Variety of Environmental Matrices with Confirmation Using LC/APCI/MS/MS

A variety of differing matrices (sediment from the sediment/water interface of Pond 50, standing live and dead *Scirpus* stems, *Scirpus* roots, and pond water) were extracted for ergosterol in triplicate as per Eash *et al.* (1996), with the exception samples were concentrated to 2 ml and divided into two 1 ml portions: one for injection into the HPLC-UV and the other for LC/APCI/MS/MS.

2.3 RESULTS AND DISCUSSION

2.3.1 Ergosterol Stability

Ergosterol concentrations of 1, 2, 4 and 10 mg L^{-1} in methanol were found to be stable over the 5 day period (Figure 6). The stability of ergosterol in methanol and in a laboratory setting is not surprising since the methanol solution lacks an oxidising agent, to which ergosterol is susceptible. As well, any photolytic breakdown from a full spectrum of light are minimised in a laboratory. In addition, bacterial degradation of ergosterol would be unlikely to occur in a methanol solution. This data indicated that calibration standards used in quantification of ergosterol are stable for at least 5 days even without refrigeration.

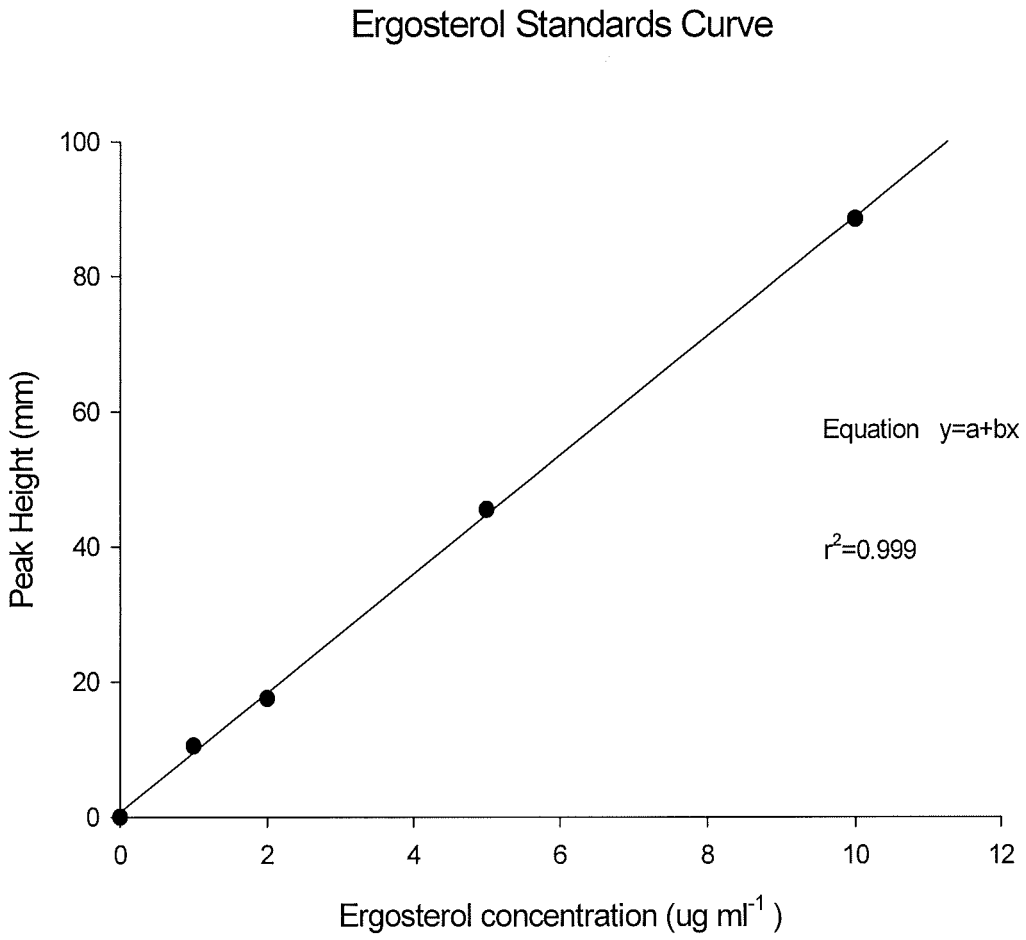


Figure 4. The relationship between peak height and ergosterol concentration. The solid Line represents the linear regression of the data.

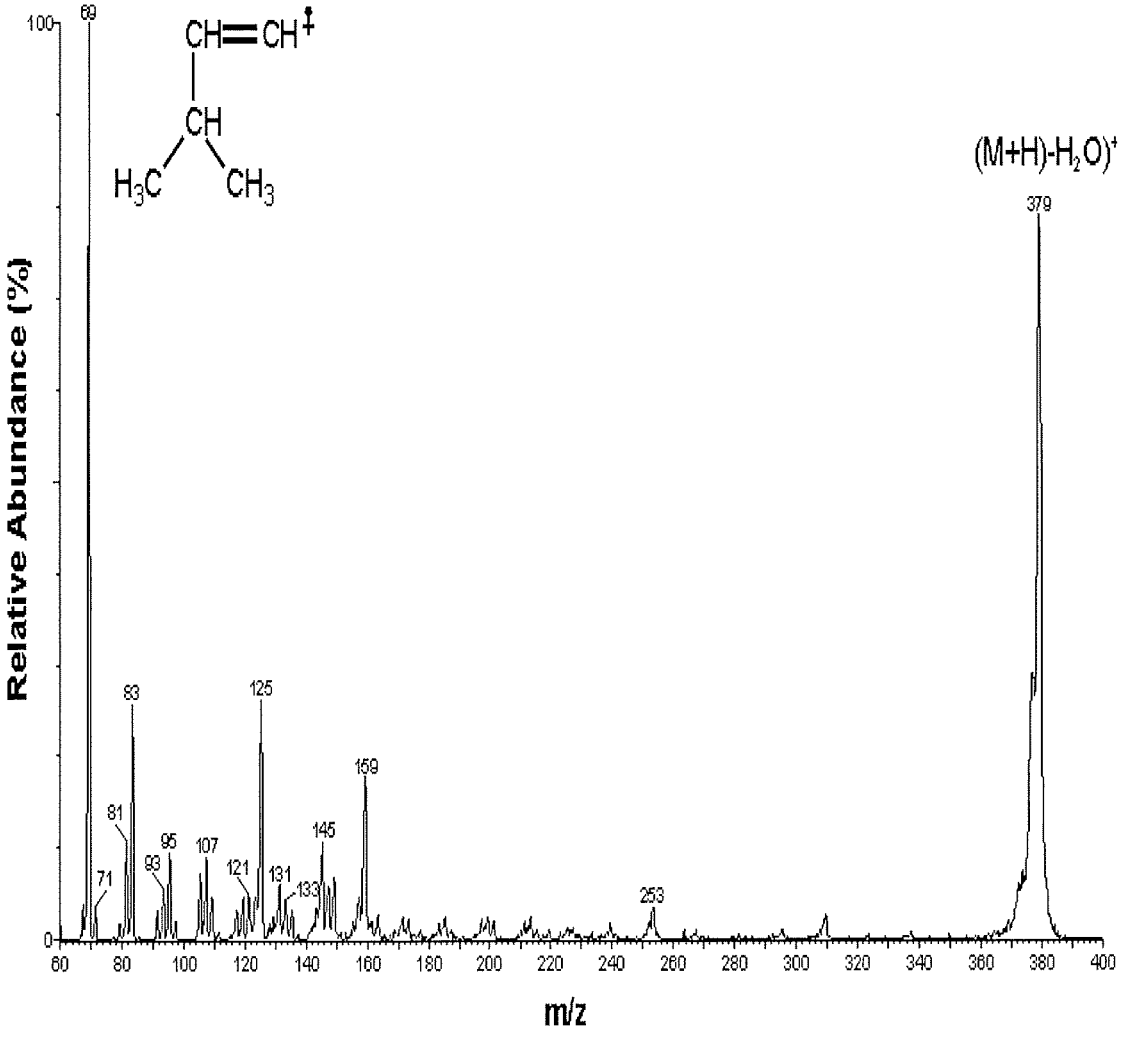


Figure 5. Product ion spectrum of ergosterol m/z 379.

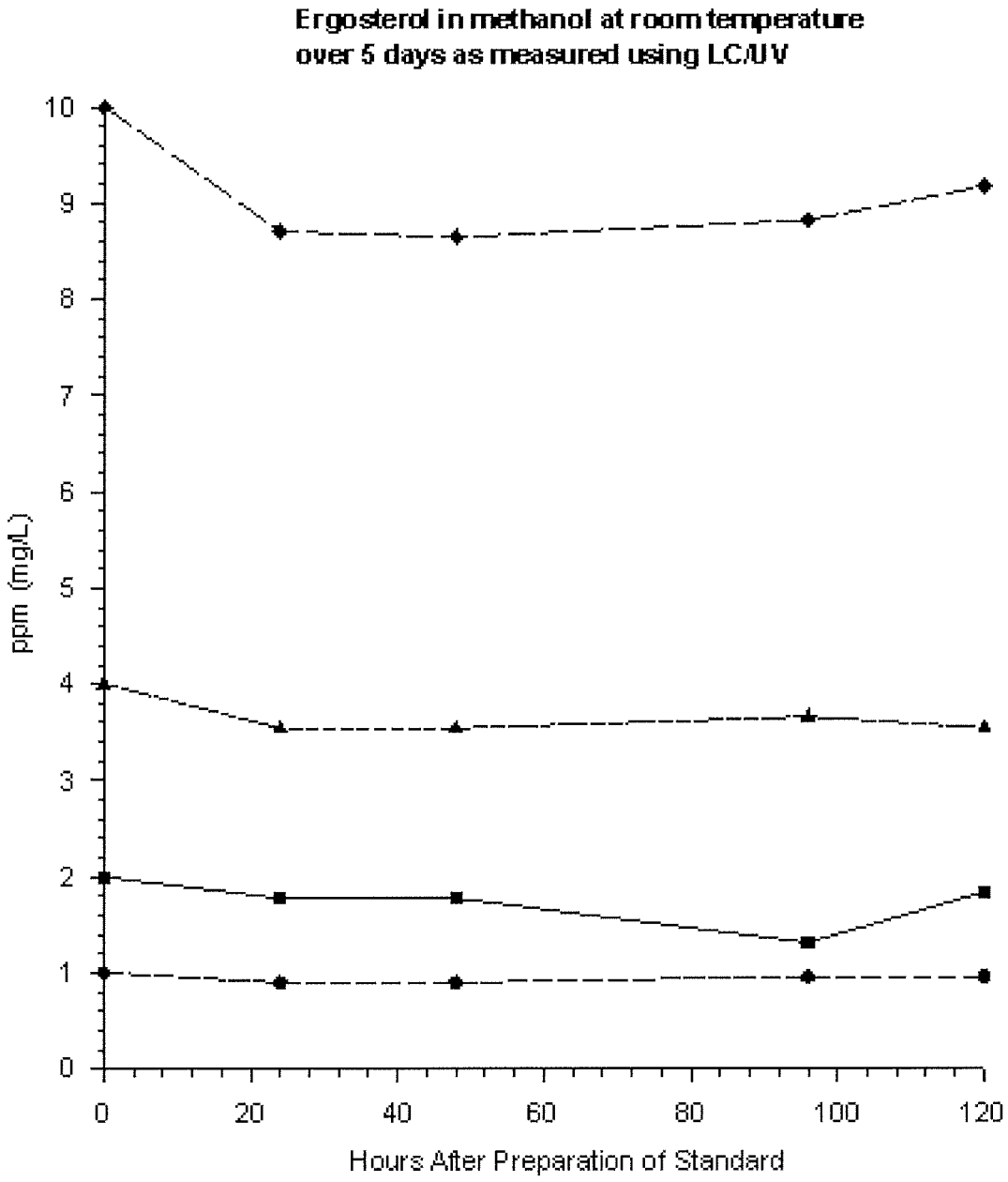


Figure 6. Stability of differing concentrations of ergosterol, in methanol, over 120 hours.

2.3.2 Extraction of Ergosterol from Leaf Litter by Methods Other Than Heating

Four different methods to maximise the extractability of ergosterol and to achieve a relatively clean extract in order to aid HPLC-UV detection were evaluated (Figure 7). The methanol plus KOH mixture followed by filtering resulted in the extraction of 2.43 ± 0.38 S.D μg ergosterol g^{-1} soil wet weight. However, despite the seemingly higher levels of ergosterol when compared to other extractions, the sample loaded the column with large quantities of matrix that resulted in unreliable chromatograms. Overall, in terms of a clean sample extract and good extraction efficiency, the best results were obtained from the methanol plus KOH followed by extraction with 3 X 10 ml of pentane. This latter resulted in 1.45 μg ergosterol g^{-1} soil wet weight. The solvent extraction of the mixture resulted in polar compounds being left behind and thus an overall cleaner sample.

2.3.3 Extraction of Ergosterol by a Conventional Method but With Variations in Heating Time

The extraction amounts for the 24 and 48 h samples (Figure 8) were low and had a large deviation with the latter ranging from 19.2 to 30.6 μg ergosterol g^{-1} leaf wet weight when compared to other treatments. Best extraction efficiencies, 30.1 to 34.3 μg ergosterol g^{-1} leaf wet weight, were obtained from heating the sample in a methanol plus KOH mixture for 30 mins. The results of increased efficiency of ergosterol extraction due to 30 minutes of heating can be explained by the fact that ergosterol may be bound as different esters to solid matrixes (Rezanka 1992). To best liberate bound ergosterol a saponification step followed by extraction in an organic solvent is required. Heating for 30 minutes probably opens the cellular components of plants, and thereby exposing the internally associated fungal hyphae, as well as the fungal plasma membrane thereby freeing the ergosterol. Prolonged heating with one and two hour

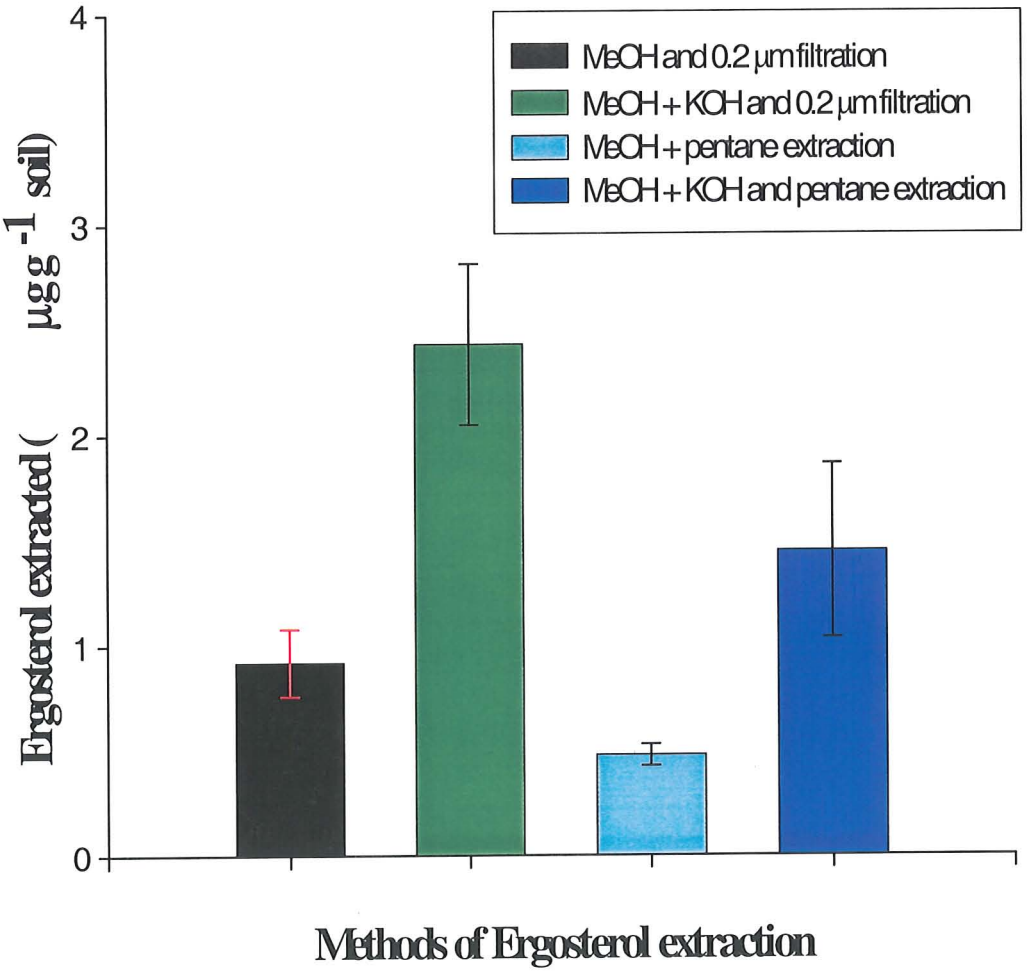


Figure 7. Efficiency in extraction of ergosterol using four cold saponification methods. Error bars are ± S.D.

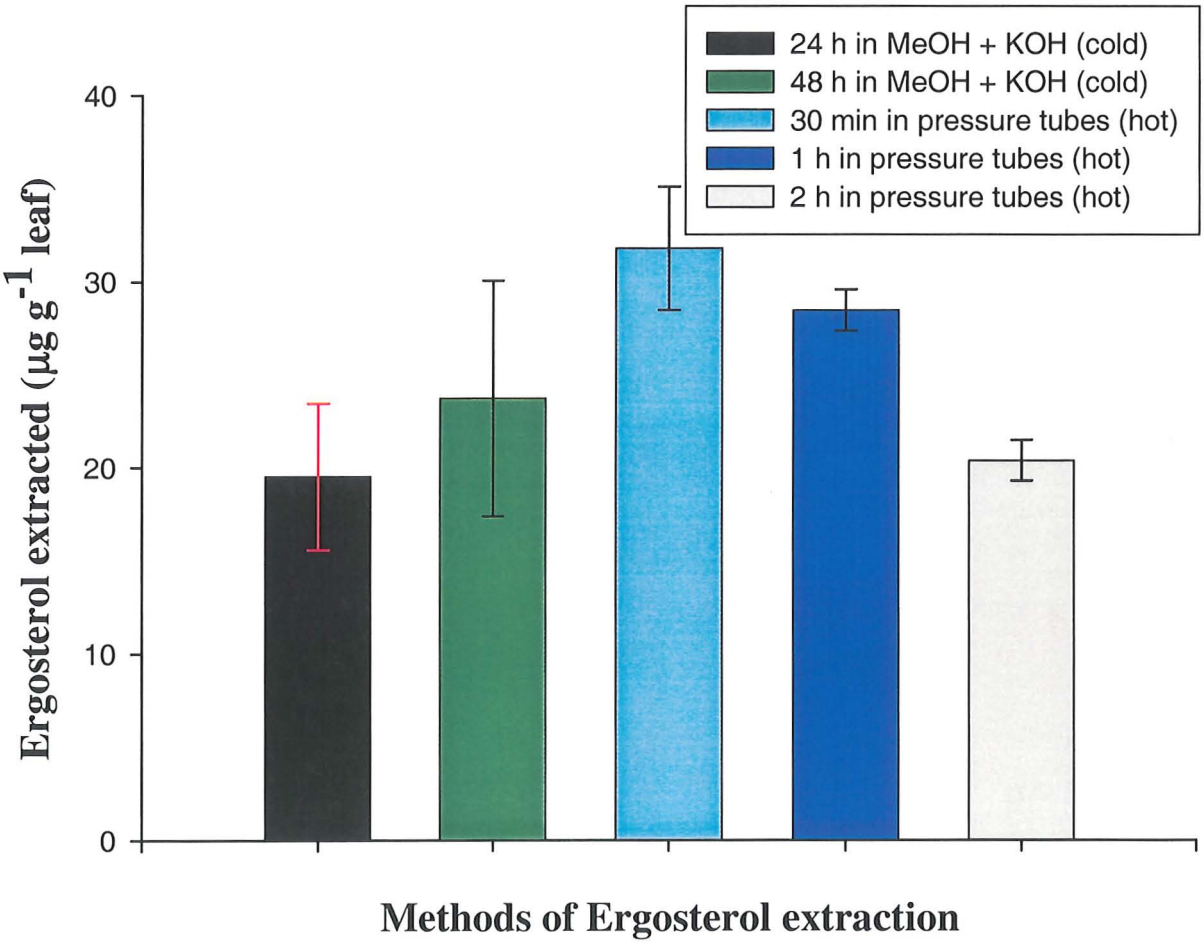


Figure 8. Time dependence in extraction efficiency of ergosterol using cold and hot saponification. Error bars are ± S.D.

treatments seemed to decrease the efficiency of extraction, probably due to a breakdown of the ergosterol molecule.

2.3.4 Extraction of Ergosterol from a Variety of Environmental Matrices with Confirmation Using LC/APCI/MS/MS

Ergosterol was detected using the HPLC-UV in all of the matrices that were sampled (See Table 1). Standing dead *Scirpus* stems had the highest levels of ergosterol with the shoot tips and stems accounting for an average of 302.9 and 90.2 μg g^{-1} AFDM, respectively. These numbers are consistent with what other researchers have found and are indicative of fungi being associated with plants. The presence of fungi also indicated that considerable decomposition and carbon sequestration by the fungi was occurring while the plant was standing (Newell 1993, Verma *et al.* 2002). The sediment from the sediment/water interface also had significant amounts of ergosterol with 38.6 μg g^{-1} AFDM. This may have been associated with the small quantities of plant material which were observed in the sediment and may have been colonized by aquatic fungi. It has been noted that ergosterol levels temporarily decrease once a plant dislodges and falls into the water (Kuehn *et al.* 2000). This is believed to occur because of colonization by aquatic fungi and the dislodging or death of terrestrial species but has not been experimentally confirmed. Persistence of ergosterol in the environment has not clearly been established and it is possible that the ergosterol observed was from dead fungal hyphae. In the laboratory, however, it has been shown that ergosterol, at least in one fungal species, rapidly degrades and becomes photo-oxidised in the presence of light, leading to fungal death (Trigos & Ortega-Regules 2002). In the environment, with complex matrices, the stability of ergosterol requires further study. The occurrence of ergosterol on green stems is

Table 1. Levels of ergosterol in plant tissue, sediment, and water from a prairie wetland at St. Denis, Saskatchewan as detected by HPLC/UV and further confirmed using MS/MS.

Environmental Matrix	**Average Ergosterol (μg g^{-1} wet weight or ml) ± S.D.**	**Average Concentration (μg g^{-1} AFDM or ml) ± S.D.**	**LC/APCI/MS/MS**
Dead *Scirpus* rhizomes	4.26 ± 0.16	27.40 ± 24.20	Confirm
Dead *Scirpus* shoot tips	143.98 ± 76.80	302.86 ± 176.80	Confirm
Dead *Scirpus* stems at sediment/air interface	52.12 ± 24.68	90.20 ± 27.80	Confirm
Green *Scirpus* stem	0.30 ± 0.058	2.42 ± 0.38	Confirm
Ambient pond water	0.014 ± 0.014	0.014 ± 0.014	Non Detect
Sediment at the sediment/water interface	1.80 ± 0.02	38.56 ± 14.62	Confirm

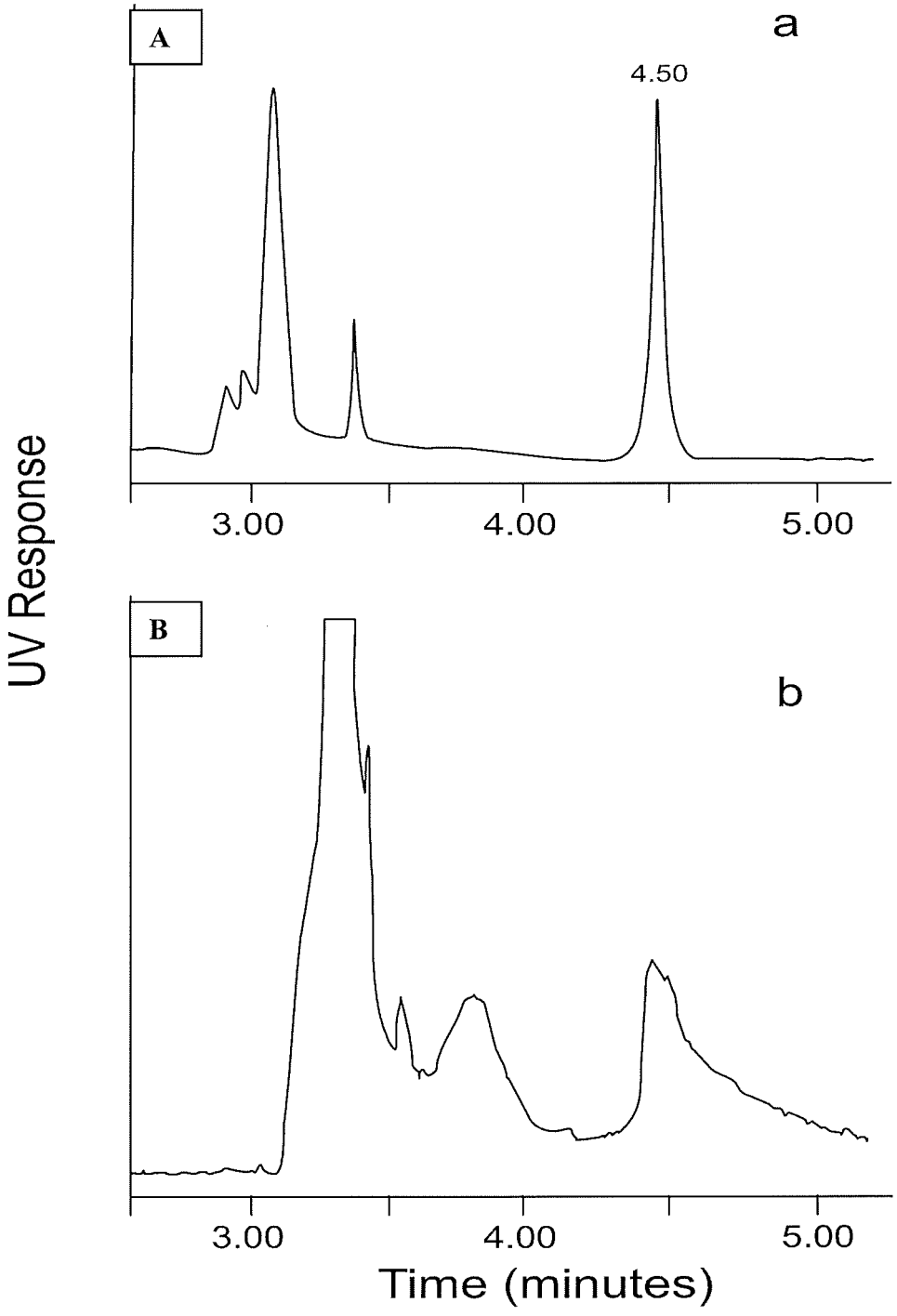
surprising and to the best of the authors knowledge has not been reported elsewhere. This finding may be attributed to remnants of fungal material or yeast on the surface of the plant or of either symbiotic or parasitic fungi (Osono 2002).

Although the detection limits of the conventional LC-UV method were sufficient for the analysis of ergosterol in wetland vegetation, sediments and water, there were cases where the quality of the detections was subject to interferences from co-eluting components. In sediment samples the baseline was elevated due to interference and this in turn could lead to masking of ergosterol. A more critical problem with the HPLC-UV mode of detection can be seen in the case of the ambient water samples where peaks in the range of 0.004 to 0.03 μg ergosterol ml^{-1} were detected (Figure 9B while Figure 9A is of an ergosterol standard). For all three water replicates there was a high peak at a retention time and absorbance of ergosterol standards. The LC/APCI/MS/MS did not detect (Figure 10A is of a ergosterol standard chromatogram using LC/APCI/MS/MS while Figure 10B is of the water extract) what otherwise would have been thought to be ergosterol in the same water samples. This observation illustrates the need for a more selective analysis, such as MS/MS, to confirm ergosterol concentrations in environmental matrices such as water and sediment.

2.4 Conclusions

Ergosterol standards were found to be stable in methanol over five days in a lab setting. Ergosterol was detected in nearly all of the environmental matrices examined. Of the various extraction techniques tested, the one that resulted in a high extraction efficiency of ergosterol, as well as the cleanest sample was that of heating the sample in a water

bath for 30 minutes in a solution of methanol and KOH followed by a solvent extraction. The conventional HPLC-UV method proved amenable to detection of ergosterol in wetland samples. However, as indicated by the artefact created by co-elution in the water samples, the system can be compromised and caution is required. Due to the high degree of complexity of natural matrices, being reliant on an HPLC-UV system for ergosterol detection and quantification may lead to erroneous results. The LC/APCI/MS/MS provided added data quality confirming the prevalence of, or lack of in case of water, ergosterol in a variety of environmental matrices of a prairie wetland. However, because the HPLC mode of detection for ergosterol worked well in plant matrices, as seen in Table 1, it was decided to continue using HPLC for all further work.



Figures 9. (A) Chromatogram of HPLC-UV detection of ergosterol. (B) Matrix interference in water sample-false positive detection of ergosterol by HPLC-UV.

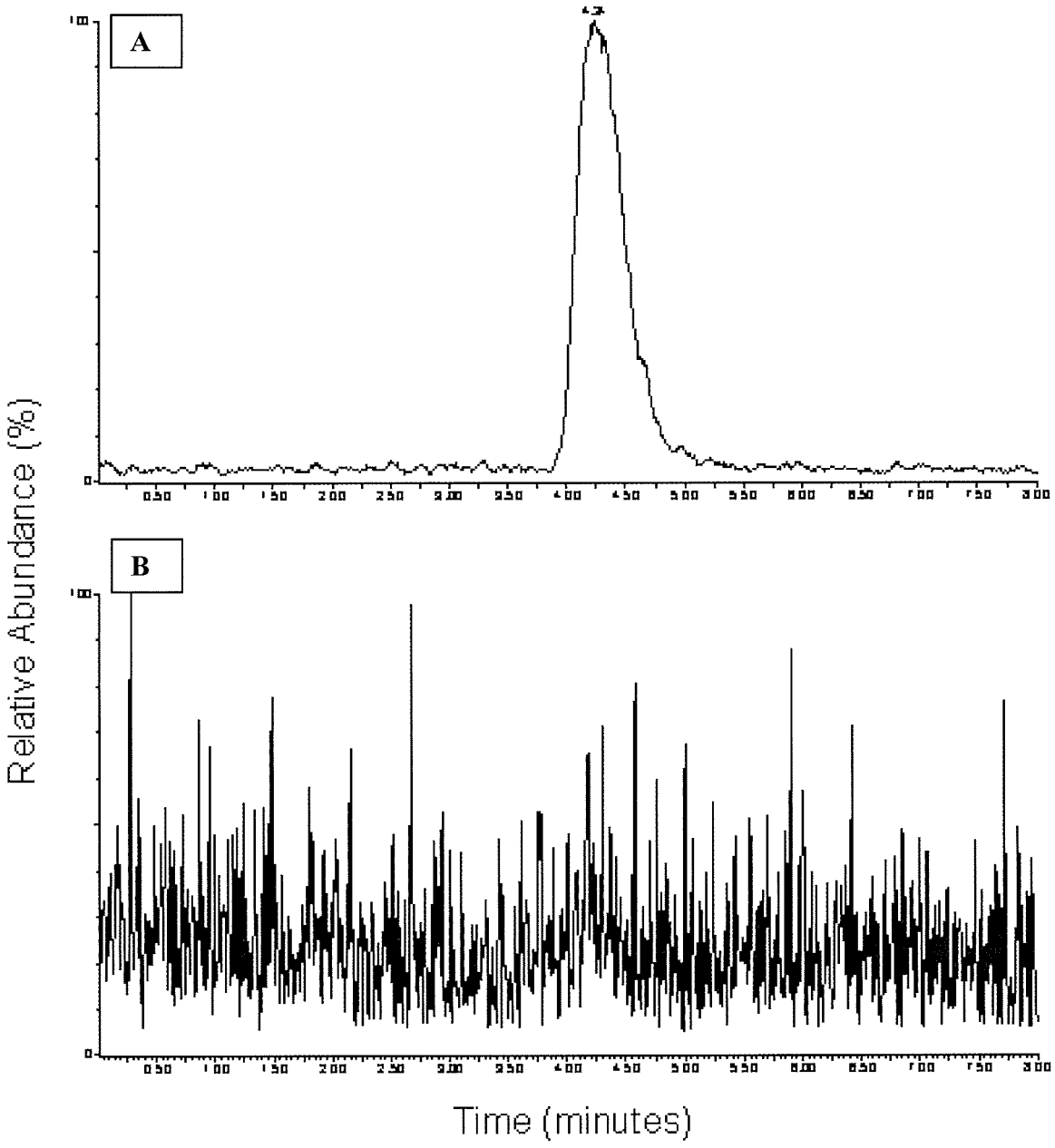


Figure 10. (A) Detection of ergosterol using MS/MS. (B) MS/MS chromatogram of same water sample as 9B indicating no detection of ergosterol.

CHAPTER 3

FUNGAL BIOMASS AND PRODUCTION ON *SCIRPUS* STEMS DECAYING IN THE STANDING POSITION OR IN POND WATER

3.1 INTRODUCTION

Emergent macrophytes are an important source of carbon in wetlands of the northern prairies (van der Valk & Davis 1978). Previous measurements of macrophyte decomposition in these wetlands have had to rely on nutrient and litter mass loss with little differentiation between the fungal and bacterial components responsible for decay (Wrubleski *et al.* 1997, Walse *et al.* 1998, Thormann *et al.* 2001). The use of ergosterol as an index molecule to estimate eumycotic fungal biomass (Lee *et al.* 1980) and of [1-^{14}C] acetate into ergosterol incorporation technique (Newell & Fallon 1991) to measure, in situ, fungal production rates, has resulted in considerable advances in the understanding of litter decomposition and microbial interactions in a variety of environments. Fungi have been found to be the predominant decomposers in some aquatic environments accounting for up to 99% of the total microbial biomass and production (Newell *et al.* 1995, Suberkropp & Weyers 1996, Kominkova *et al.* 2000, Kuehn *et al.* 2000, Hieber & Gessner 2002).

Researchers have measured fungal colonization on aquatic macrophytes in the standing position (Newell *et al.* 1995, Newell & Porter 2000, Gessner 2001, Newell 2001) and in plants submerged in water (Kominkova *et al.* 2000, Kuehn *et al.* 2000). Using the ergosterol labelling technique these studies have provided evidence that a significant portion of carbon from decaying plant material is removed while the plants are in the standing position. Work on standing dead macrophytes that have collapsed into the water column has shown an initial lag phase, due perhaps to the dying of terrestrial fungi or to the conditioning of the plant material, before colonization by

aquatic fungi can occur. As they decompose standing, dead macrophytes in wetlands are often partially under water or under sediment and fungal decomposition does not occur entirely in the standing or submerged positions.

Microbial organisms responsible for decomposition in macrophytes are subject to extremes of temperature and ultraviolet (UV) solar radiation, especially in shallow wetlands, during the season. Surprisingly temperature as a regulating factor of fungal production and biomass in decaying organic material has received little attention (Chauvet & Suberkropp 1998).

UV radiation is known to have damaging effects on bacterial DNA (Herndl *et al.* 1993) and UV may also be debilitating to fungi and retard their decomposition activities in aquatic systems. Despite negative influences by UV radiation the overall effect may actually be beneficial to bacteria through the production of more labile forms of dissolved organic carbon (DOC) (Herndl *et al.* 1997). Likewise, UV radiation may speed up the crumbling process in plant litter (Denward & Tranvik 1998, Anesio *et al.* 1999) thereby making it easier for fungi to extract carbon from plant material. Solar radiation effects on fungi as they decompose litter in an aquatic environment have varied with one study showing negative effects (Denward *et al.* 2001) and the other showing no effects (Denward *et al.* 1999).

There have been no published studies that have followed, with regular sampling from spring to fall, seasonal changes in fungal biomass and production on standing dead litter of emerging macrophytes in an inland aquatic system. In the present study, seasonal changes in fungal biomass and production on standing dead litter of the hardstem bulrush *Scirpus lacustris* in a northern prairie wetland were assessed. Also examined were how solar radiation and water temperature effected fungal colonization and decomposition of *Scirpus* using the [1-^{14}C] acetate into ergosterol technique.

3.2 Materials and Methods

3.2.1 Study Site

Field studies were conducted at a wetland that typifies the prairie ecozone and situated at the St. Denis National Wildlife Area 40 km east of Saskatoon, Saskatchewan, Canada (106° 06'W, 52°02'N). Pond 50 is a permanent, slightly saline (Mean Total Dissolved Salts = 11.1 ± 4.66 g L^{-1}) mesotrophic wetland with a normal maximum mean July depth of 1.4 meters. During the present study pond depth declined from a maximum of 58 cm in May to < 20 cm by late October. The major water loss is via evaporation hence the dissolved organic carbon concentration in the pond on May 5 was 40.8 mg L^{-1} and by September 28 it was 120 mg L^{-1} (Mean = 74 ± 24 mg L^{-1}) (Waiser 2001b). The plant flora contributing the largest fraction of organic matter production to Pond 50 is *Scirpus lacustris* L. p.p., a hardstem bulrush (Figures 11A (Lindman 2003) and 11B).

3.2.2 Fungal biomass

Standing dead *Scirpus* was sampled biweekly for fungal biomass and production. Seven stems were chosen at random on each sampling date and the air/water interface was marked. Two cm pieces of stem from below the interface were used for the in-water measurements; aerial measurements were made with 2 cm stem pieces from 5 cm above the interface. As the water receded during the summer to the sediment surface, the "in- water" incubations done on and subsequent to July 7 (Julian day 194) involved stem pieces taken from the upper part of the sediments, which were loosely consolidated.

Figure 11. Illustration of *Scirpus lacustris* L. p.p. (A) while photograph (B) is of Pond 50 at St. Denis with standing live and dead *Scirpus* in the foreground.

Fungal biomass was measured as ergosterol concentration. A conversion factor of 10 μg of ergosterol (Kuehn *et al.* 2000, Gessner & Newell 2002) was used as indicating one milligram of living fungal carbon and is based on the assumption that the carbon content of fungi is 50% (Kuehn *et al.* 2000, Gessner & Newell 2002). Variations in optimum extraction procedure for ergosterol were performed, as noted in Chapter 2, (Verma *et al.* 2002) and the method established by Eash *et al.* (Eash *et al.* 1996) was found to be the most suitable for plant matrices. Contents of the incubation tubes used to measure fungal productivity were extracted and detected by HPLC analysis exactly as indicated above in Chapter 2. However, instead of 100 μl injections as was done previously to measure ergosterol concentration, a 500 μl aliquot of the extract was injected into the HPLC system to determine ergosterol concentrations as well as to separate the [1-^{14}C] labelled ergosterol from other constituents in the extract. Suspect ergosterol samples were confirmed by mass spectrometric analysis according to Headley *et al.* (Headley *et al.* 2002). In addition to the samples, one glassware blank (all non-detectable) and one spiked blank for percent recovery/loss (average recovery of 85%) were run for quality control purposes at every extraction date.

3.2.3 Fungal Production

Fungal production was measured from the rate of Na [1-^{14}C] acetate uptake. Stem pieces were incubated in triplicate in 10 ml polycarbonate tubes (VWR Canada) containing 5 ml of 0.2 μm filtered pond water, 1 mM Na [1-^{14}C] acetate with a specific activity of 58 to 59 mCi mmol^{-1} (Amersham Pharmacia Biotech, Montreal, Canada) for 3 to 4 hours. Incubations for both the submerged and aerial stem pieces were performed under natural light conditions in the standing *Scirpus* canopy. The production values on aerial stem portions are only estimates of potential production since the incubation

method involves wetting the stems. (Kuehn *et al.* (Kuehn *et al.* 1998) have noted significant increases in microbial activity, as measured by CO_2 (fungal + bacterial) evolution, within 5 minutes of wetting dry *Juncus effusus* litter). The submerged-stem tubes were placed in the pond water during incubations while the tubes with aerial stem pieces were placed on the ground. One killed control per sampling date, in the presence of 2% formalin, was done at every incubation date (Newell 1993). All incubations were stopped with the addition of formalin to a final concentration of 2%. Samples were stored at 4°C and extracted the following day. Radiolabelled ergosterol fractions, once run through the HPLC for ergosterol quantification, were collected in scintillation vials, dried under N_2 gas and redissolved with 5 ml of a universal liquid scintillation cocktail (Canberra Packard, Canada). Disintegrations per minute (DPM) were determined on a Packard scintillation counter using an external standard.

In preliminary experiments radioisotope dilution that occurred when incubating *Scirpus* stems in Pond 50 water was measured. The concentration of cold (unlabelled) Na acetate was varied while keeping the labelled acetate constant. The concentrations of combined cold and hot Na acetate used were 1, 2, 4, 6 and 12 mM and were applied to duplicate samples. Isotope dilution was established using a non-linear equation to analyse the rate of uptake versus total added acetate concentration (Robarts & Zohary 1993). Fungal production was calculated (Newell 1993) using a conversion factor of 19.3 μg fungal biomass $nmole^{-1}$ of acetate incorporated (Suberkropp & Weyers 1996, Suberkropp 1997, Kuehn *et al.* 2000) and the mean isotope dilution value.

The remaining three stems were cut exactly as above and used to determine percent moisture, after being dried at 60°C for 48 hours, and ash free dry mass (AFDM) by being combusted for 4 hours at 450°C (Newell 1993, Kuehn *et al.* 2000).

3.2.4 Solar Radiation Experiment

A floating, two compartment chamber constructed of plexiglass with a mesh bottom (Figure 12), to allow water exchange while keeping out macro grazers, had two different plexiglass covers: one allowed the penetration of full sunlight including UV radiation (UV^+), while the other excluded UV radiation (UV^-) but allowed the penetration of all other wavelengths. Scan measurements for the UV^- cover, done with a Optronics scanning spectroradiometer (Model OL-754), showed that the plexiglass allowed no UV-B (280-320nm) through, 0.1% UV-A (320-400nm) and 89% Photosynthetically Active Radiation (PAR) (400-700nm). The full spectrum plexiglass allowed through 76 % UV-B, 85 % UV-A, and 88 % PAR. The chamber was allowed to acclimatise in Pond 50 for 10 days before the introduction of plant material. Living *Scirpus* plants were harvested and selected for similar diameter size and cut into 2 cm pieces in the lab. Twenty-four hours after harvesting equal amounts of cut stems were placed on both sides of the chamber. In addition, stem samples were taken at the start and termination of the experiments to measure particulate organic carbon (POC) and particulate organic nitrogen (PON). POC and PON were analysed using a CHN analyser.

Water level in the chambers was maintained at a depth of 4 cm throughout the length of the experiment. Though the following results will vary over the course of a season, the average values for 1% penetration depths for UV-A and UV-B were 13 and 4 cm, respectively (Waiser 2001b). For the first two weeks the majority of stems floated on the surface of the water and therefore were exposed to the radiation penetrating the plexiglass. Subsequent to this incubation samples were always taken from stems lying on the screen which meant that in the UV^+ treatment the stems were exposed to about 88% PAR, UV-A ranging from 13-27% and < 1% UV-B irradiance

Figure 12. Photograph of the floating two compartment chamber used in the solar radiation experiment to study *Scirpus* decomposition in the water, as affected by solar radiation.

from June to late August. Due to increasing DOC concentrations, which rose sharply in late August (Waiser 2001b), at the end of September UV-A exposure had dropped to about 6%. While no underwater radiation data are available for October because the water levels had dropped so low that the spectrometer could not be used, it can be assumed that the stem pieces were exposed to PAR and UV-A of < 6%.

On sampling days six stem pieces of *Scirpus*, plus one for a killed control, were randomly selected from each chamber. Fungal biomass and production measurements, as well as percent moisture and AFDM parameters were determined as described above. The incubation vials were placed in the pond water for the duration of the incubation. A Campbell Scientific 107B thermistor and CR10X data logger were used to measure and record water temperature.

3.2.5 Temperature Experiments

3.2.5.1 Short Term Temperature Experiment

Two temperature experiments were conducted in the laboratory. For the first experiment, Pond 50 water and stems from the UV^- portion of the incubation chamber were brought to the laboratory. Five ml of 0.2 μm filtered pond water, along with the stems, were placed in polycarbonate incubation vials to equilibrate for 24 hours in Perceival incubators set at 4, 10, 15, 21 and 30°C. After acclimatization the stems were incubated with 1 mM Na [1-^{14}C] acetate to measure fungal production. All incubations were done in triplicate and included one killed control.

3.2.5.1 Long Term Temperature Experiment

For a longer-term temperature experiment two 20.8 L (A-3002 Hagen, Canada) glass aquaria were used. Standing, dead *Scirpus* stems were harvested in November,

cut into 2 cm pieces and placed in the aquaria along with 10 L of a fresh supply of 20 μm filtered Pond 50 water. The filter pore size was used to remove large grazers but allow the majority of the microbial population to pass through. One aquarium was maintained at 4°C and another at room temperature (21°C ± 3°C). Air was constantly supplied to the aquaria through an air pump (A-799 Elite Hagen, Canada). Both aquaria were agitated periodically to mix the contents and additional 20 μm filtered pond water was added as required to replenish the system through the course of the experiment. Both aquaria were kept in the dark to prevent algal growth. Fungal production and biomass for both treatments were measured as indicated above except estimates of production were made in quadruplicate along with one killed control. The same quality control standards, except a glassware blank (as all previous blanks had been non-detectable), of one ergosterol spike (average recovery of 98.3% ± 7.7%) at every extraction date were included.

SigmaStat 2.03 (SPSS Inc., U.S.A.) was used to perform all statistical analysis including analysis of variance (ANOVA). Parameter means given in the text are ± standard error.

3.3 Results

3.3.1 Isotope Dilution

In both of the Na [1-^{14}C] acetate isotope dilution experiments conducted, there was a decrease in DPM counts with increasing concentration of total sodium acetate. The amount of labelled isotope was held constant at 15 μCi while that of cold acetate was increased. As the ratio of labelled to cold carbon from added acetate decreased, this resulted in a decrease in labelled ergosterol, therefore a decrease in DPM's, indicating

that there was a dilution of ^{14}C occurring. To quantify the amount of dilution occurring, DPM's were plotted against the total sodium acetate concentrations (Figure 13) and fit the non-linear equation y =a + b/x to the data (Robarts & Zohary 1993) where a is the dilution pool size in dilution units and b is the slope. Solving the equation for "a" calculates the degree to which the labelled isotope participated in the synthesis of ergosterol. Isotope dilution was 283 in the first experiment and 284 in the second experiment done six months later. A factor of 284 was used in the calculation of fungal production.

3.3.2 *Scirpus* stem percent moisture

Aerial stem percent moisture, based on a wet weight basis, ranged from a high of 51.8% in July to a low of 9.8% in November. Percent moisture for submerged stems ranged from a low of 41.4% in May to a high of 78.1% in August. Surprisingly, stems that were submerged were not saturated with water even though the plants had died during the fall of the previous year. There was a statistically significant difference between the moisture content of in-water versus the aerial stem portions ($P < 0.001$). Ash free dry mass for 2 cm stem pieces were similar ($P > 0.06$) over the course of the sampling season with mean values of 0.11 ± 0.02 g for the submerged stem pieces and 0.098 ± 0.03 g for the aerial stem pieces.

3.3.3 Fungal Biomass On Standing Dead *Scirpus* litter

On May 10th (Julian Day 192) an initial survey of Pond 50 was conducted to assess the variations in fungal biomass on standing dead *Scirpus* litter. An average fungal biomass of 4.9 ± 0.8 mg C g^{-1} AFDM was measured at the tips of standing dead *Scirpus* with a percent moisture of $13.7\% \pm 4.4\%$; a biomass of 3.79 ± 0.60 mg C g^{-1}

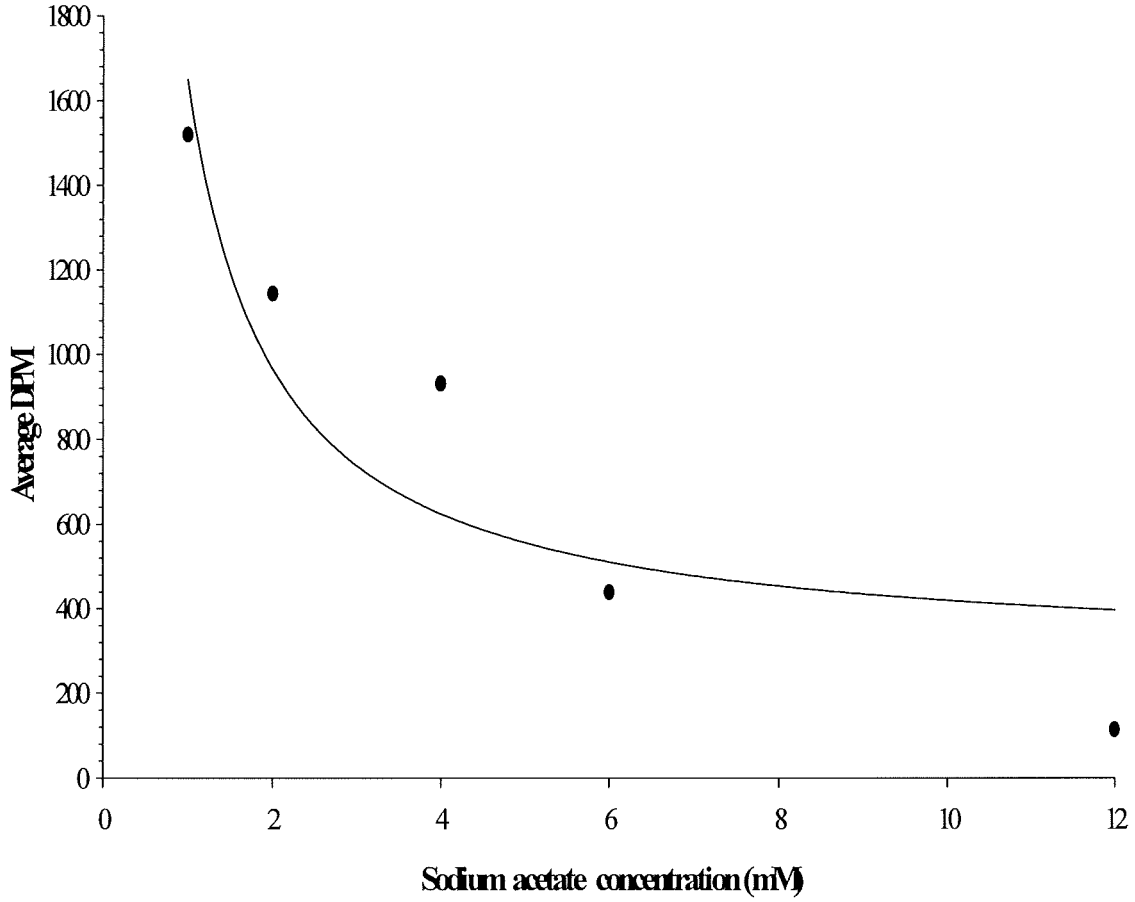


Figure 13. Relationship between sodium acetate concentration and DPM counts to measure [1-^{14}C] acetate isotope dilution. Line represents the non-linear regression of the data.

AFDM at above the water line with a moisture content of 28% ± 5%. Biomass on *Scirpus* that had dislodged and fallen into the water was 2.41 ± 0.15 mg C g^{-1} AFDM. No acetate uptake experiments were performed at this time. During the ice-free season biomass on aerial sections of dead litter showed two peaks; the first of 1.5 mg C g^{-1} AFDM in July (Julian Day 194) and the second peak in late September of 1.3 mg C g^{-1} AFDM (Figure 14A and Table 2). Fungal biomass for submerged stem portions had much higher and more sustained biomass values (Figure 14A and Table 3). For submerged litter a biomass of 2.2 mg C g^{-1} AFDM was measured at the end of March (Julian Day 144) followed by a general decrease to 0.11 mg C g^{-1} AFDM by July 12 Julian Day 194). It then increased to a peak in August to 5.8 mg C g^{-1} AFDM before waning to non-detectable in October. Surprisingly, biomass increased again to 4.91 mg C g^{-1} AFDM in November (Julian Day 306). The differences in fungal biomass in submerged and aerial *Scirpus* stems on the different sampling dates, however, were not significantly different ($P > 0.05$). The mean fungal biomass over the sampling time for the aerial portions was 0.69 ± 0.2 mg C g^{-1} AFDM while for submerged portions it was about three times higher with a mean of 1.95 ± 0.56 mg C g^{-1} AFDM. Mean biomass values between the aerial and submerged stem portions were statistically different ($P < 0.04$).

3.3.4 Fungal Production

Fungal production also varied over the season. The highest production values for aerial stem portions were measured in July (Julian day 194) at 376 μg C g^{-1} AFDM day^{-1}, 253 μg C AFDM day^{-1} in late August (Julian day 236) and 159 μg C AFDM day^{-1} in October (Figure 14 and Table 2). The lowest production rate in aerial stems was measured at 12.7 μg C AFDM day^{-1} in early October (Julian day 278). There was no

significant difference between the peaks. The second and third peaks were significantly higher ($P < 0.01$) than the surrounding values whereas the first peak was not ($P > 0.05$) due to high replicate variation. The highest production values for submerged *Scirpus* stem portions were on 23 May (Julian day 144) with 372 μg C g^{-1} AFDM day^{-1} and 275 μg C g^{-1} AFDM day^{-1} on 5 September (Julian day 249) (Figure 14 and Table 3) while the lowest was 1.8 μg C AFDM day^{-1} in July (Julian Day 194). These variations in fungal production in submerged *Scirpus* stems were not significantly different among the sampling dates ($P > 0.05$). The potential mean production rates were 121.4 ± 41.1 μg C g^{-1} AFDM day^{-1} for the aerial stem portions and 91.8 ± 32.7 μg C AFDM day^{-1} for submerged portions, and these values were not statistically different ($P > 0.35$). On an annual basis, from ice melt in spring to freeze-up in the fall, potential production on aerial stem portions was 36.2 mg C g^{-1} AFDM y^{-1} while on submerged litter it was 27.7 mg C g^{-1} AFDM y^{-1}. This difference may not be real because fungal production in aerial stem parts was not measured until six weeks after commencing fungal production measurements for submerged *Scirpus*.

3.3.5 Statistical Correlation's and Relationships on Standing *Scirpus* Stems

For aerial stem parts there was a positive correlation between the production to biomass ratio and temperature ($r^2 = 0.7$, $P < 0.05$) (Figure 15) and production to increasing solar radiation ($r = 0.66$, $P = 0.05$). The optimum percent moisture for fungal production on aerial portions of *Scirpus* stems appeared to be in the 15-22% range (Figure 16). For submerged stem portions a stem moisture content of 45-50% (Figure 17) was more favourable for fungal biomass while there was a negative correlation ($r = -0.6$ $P = 0.05$) between biomass and increasing solar radiation.

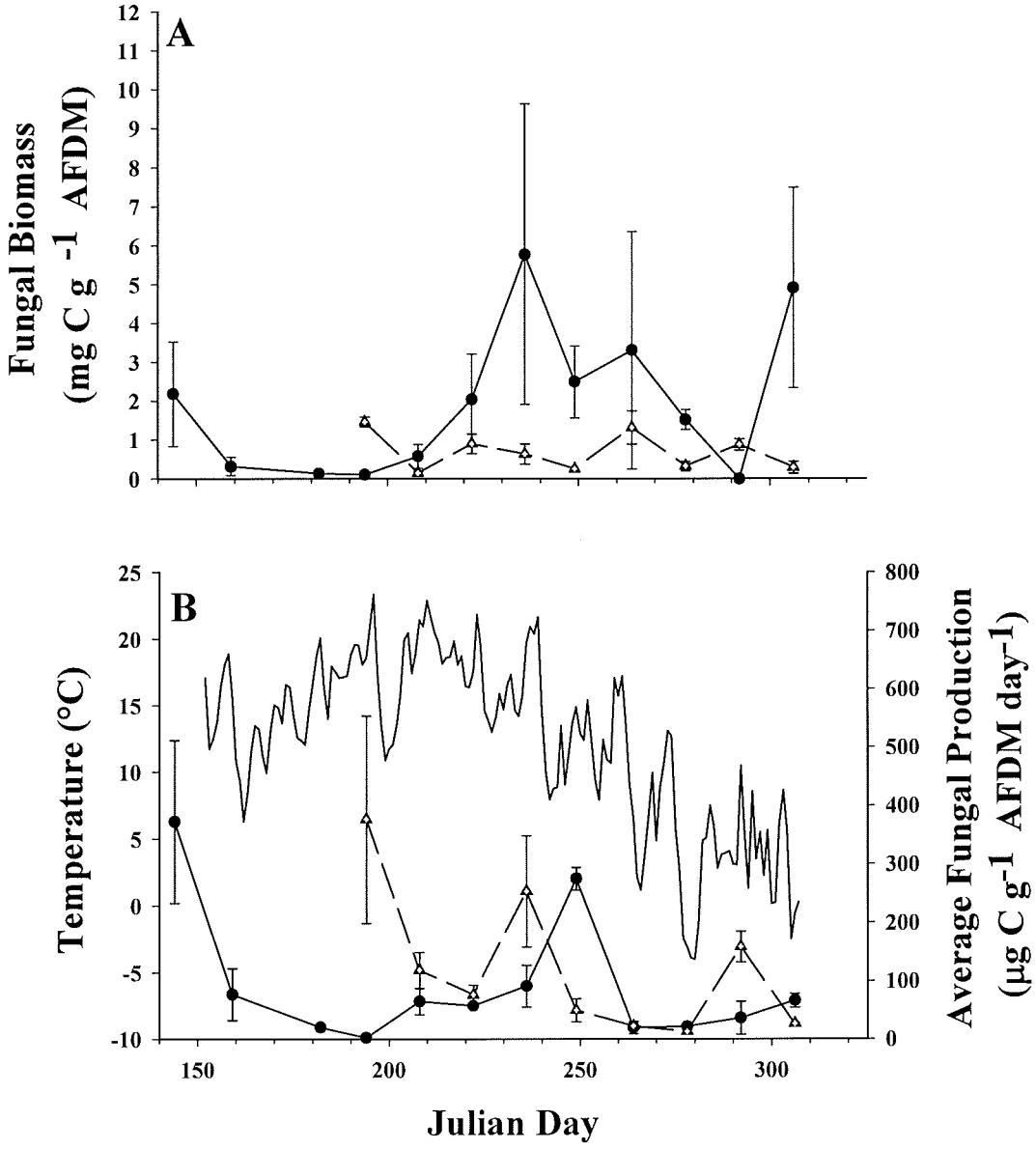


Figure 14. Seasonal changes in fungal biomass (A) and production (B) on *Scirpus* in aerial (Δ) and submerged (•) parts of stems. Solid line is average air temperature in °C. Error bars are standard error.

Table 2. Average fungal production and biomass in aerial portions of *Scirpus* stems.

Julian Day	Average Fungal Biomass (μg C g AFDM^{-1}) ± S. E.	Average Daily fungal Production (μg C g^{-1} AFDM D^{-1}) ± S. E.
194	1460± 130	376 ± 177
208	148± 36	118± 31
222	899± 250	76± 16
236	634± 260	253± 95
249	257± 0	49± 20
264	1315± 429	22± 6
278	321± 122	13± 0.0
292	878± 150	159± 26
306	290± 150	26± 2.0

Table 3. Average fungal production and biomass in submerged portions of *Scirpus* stems.

Julian Day	Average Fungal Biomass (μg C g $AFDM^{-1}$) ± S. E.	Average Daily fungal Production (μg C g^{-1} AFDM D^{-1}) ± S. E.
144	2180± 1350	372± 139
159	320± 230	77± 45
182	134± 0	20± 6
194	108± 54	1.80 ± 0.0
208	578± 302	65± 22
222	2039± 1170	57± 0.00
236	5770± 3860	91± 36
249	2490± 930	275± 19
264	3303± 3052	19± 11
278	1515± 257	21± 6
292	Non Detect	36± 28
306	4910± 2580	66± 12

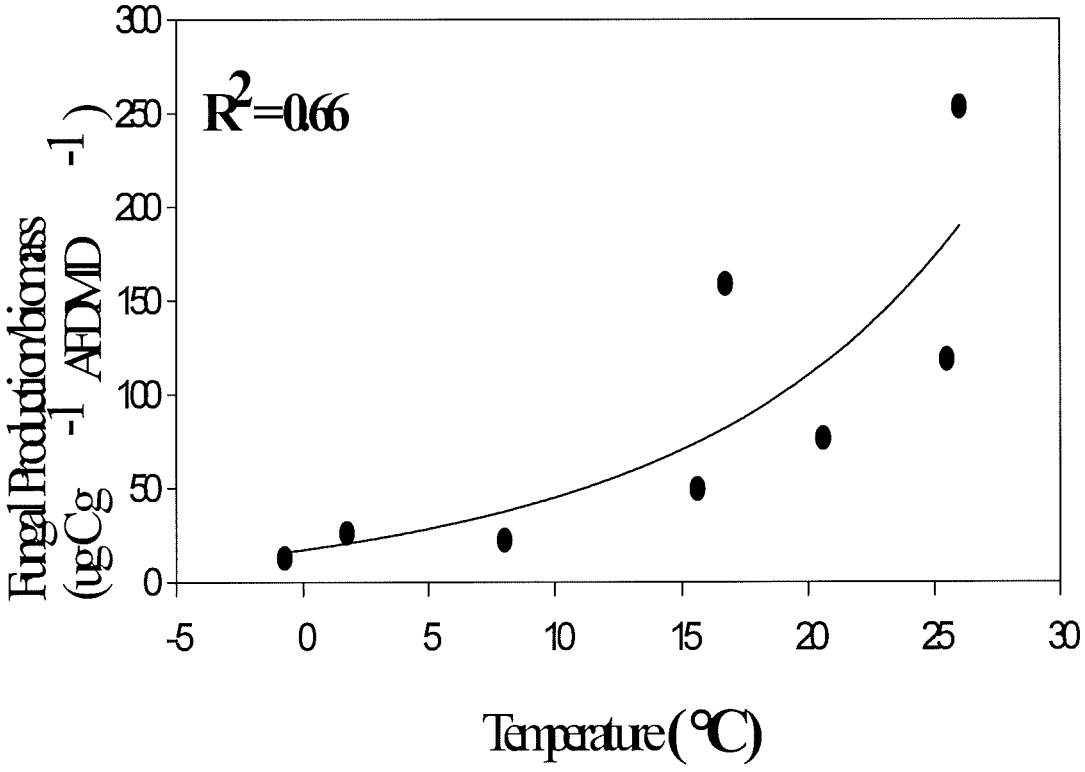


Figure 15. Relationship between aerial fungal production/biomass and air temperature.

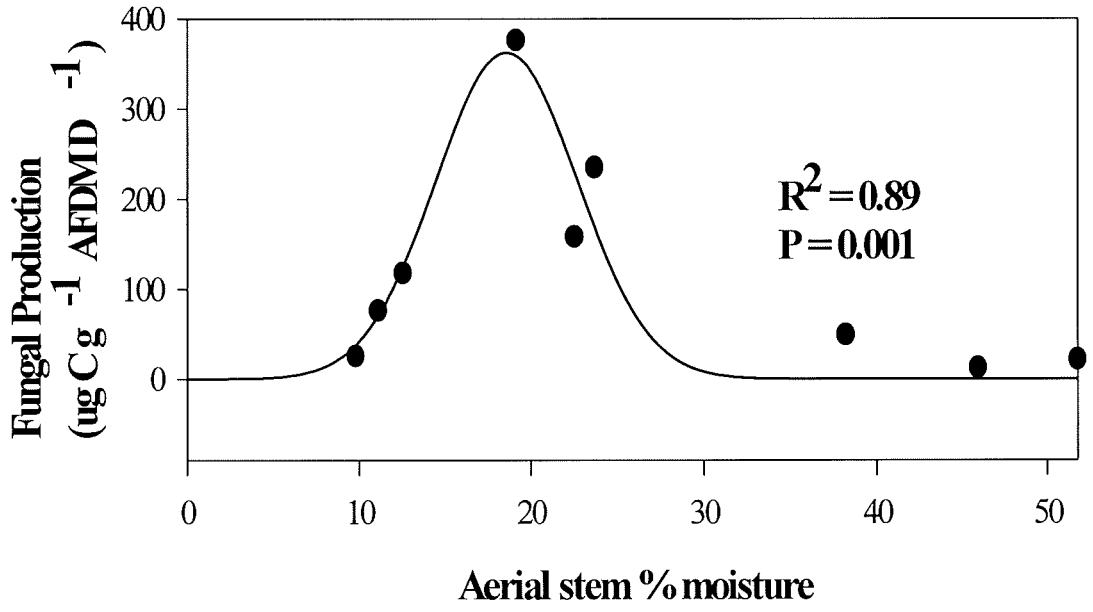


Figure 16. Relationship between fungal production and percent moisture in aerial portions of *Scirpus* stems.

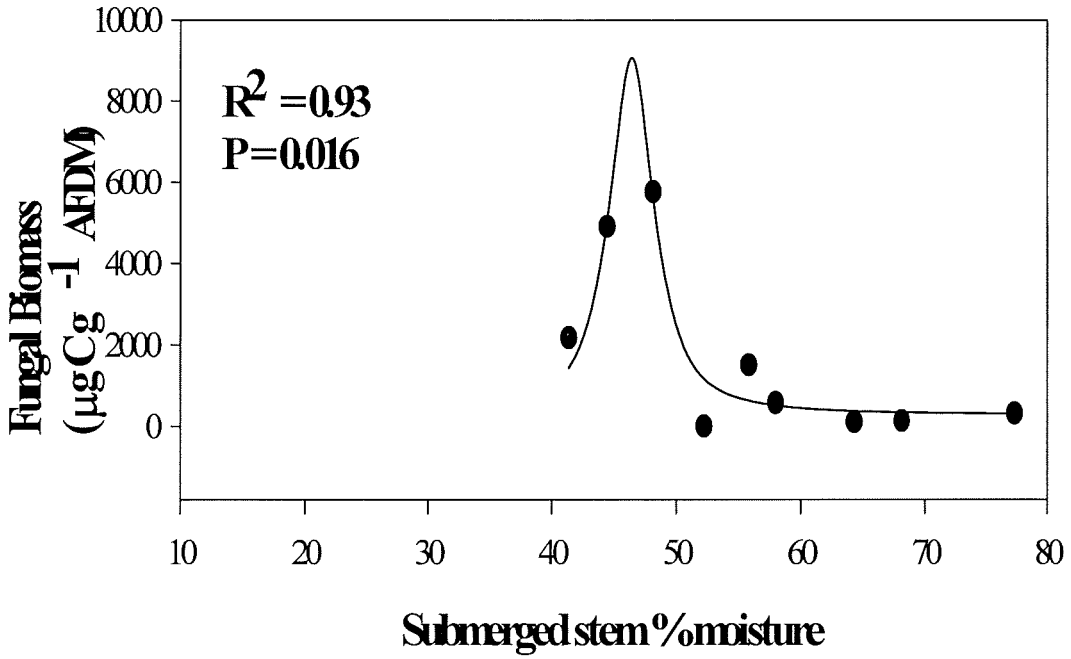


Figure 17. Relationship between biomass and stem percent moisture for *Scirpus* stem portions below the water.

3.3.6 Solar Radiation Experiment

3.3.6.1 Percent Moisture

On the day they were placed in the incubation chambers the moisture content of *Scirpus* stems was 77%. From July to November the percent moisture in the stems, for both the UV^- and UV^+ treatments, was 93.6 and 93.8 %, respectively. The average AFDM in both treatments was 0.079 ± 0.02 g C. There were no differences in percent moisture ($P > 0.64$) or in AFDM ($P > 0.99$) between the two treatments.

3.3.6.2 Solar Radiation Experiment Fungal Biomass

Fungal biomass and production rates were measured 24 h after harvesting the live *Scirpus*. Surprisingly I measured a fungal biomass of 0.16 mg C g^{-1} AFDM and a production rate of 384 ± 202 μg C g^{-1} AFDM day^{-1}. Twelve days later the biomass in the UV^+ stems (Figure 18A and Table 4) had increased to a seasonal high of 4.2 mg C g^{-1} AFDM and this was followed by a slow decline to 0.36 mg C g^{-1} AFDM by November 1st (Julian Day 306). Stems in the UV^- treatment showed a similar trend but reached a biomass peak of 3.7 mg C g^{-1} AFDM (Figure 18A and Table 5) 26 days after the start of the experiment (Julian Day 208). Thereafter, there was a considerable drop in biomass to 0.55 in August (Julian Day 222 or 40 days after the start of the experiment) a slow rise to 2.7 mg in September before the final decline to 0.46 mg C g^{-1} AFDM by November 1. The mean fungal biomass in the UV^+ treatment was 1.5 ± 0.4 mg C g^{-1} AFDM while in the UV^- treatment it was 1.6 ± 0.4 mg C g^{-1} AFDM. There were no significant differences in biomass ($P > 0.76$) between the two treatments or within treatments ($P > 0.05$) over the course of the experiment.

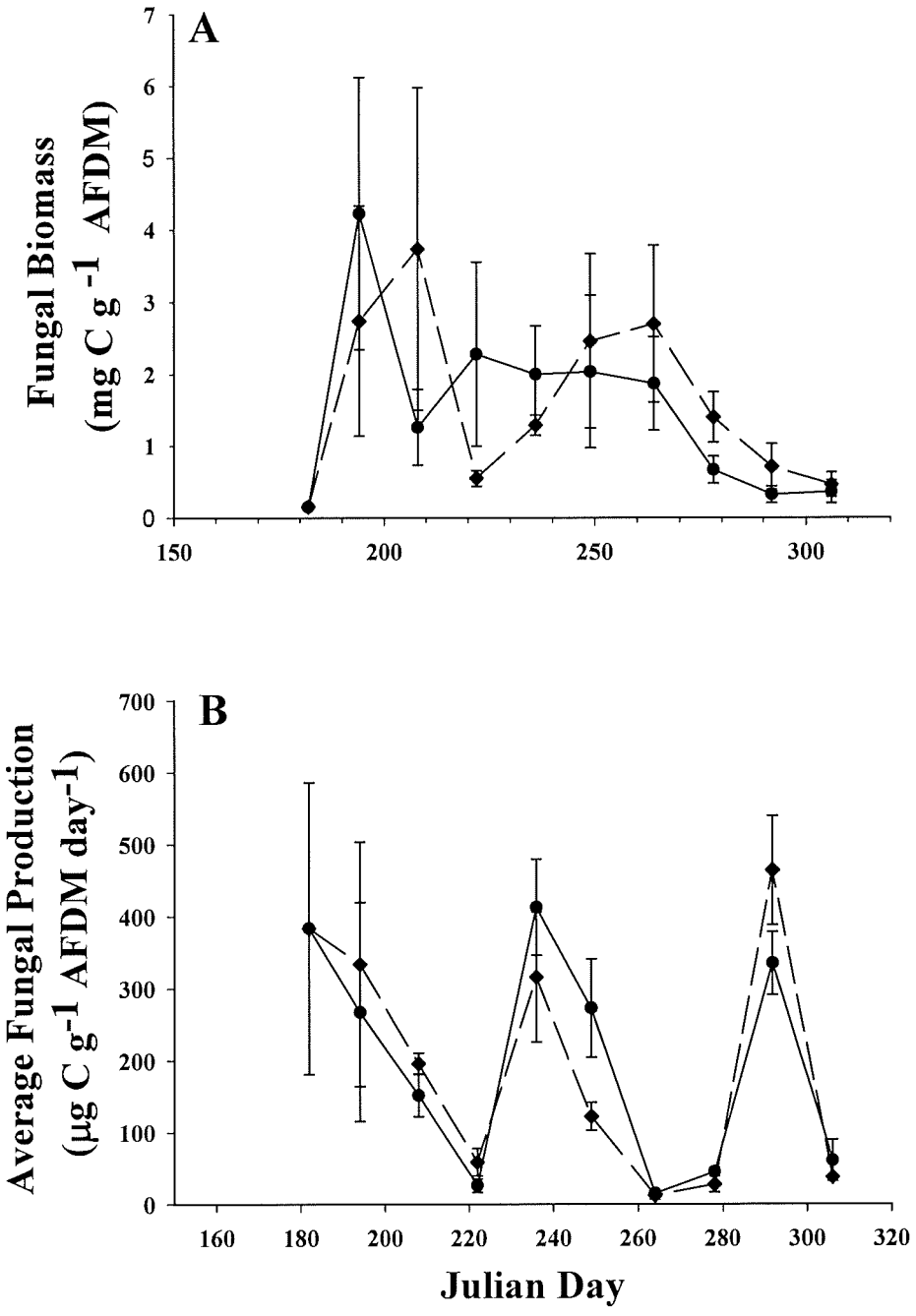


Figure 18. Seasonal changes in fungal biomass (A) and production (B) on *Scirpus* stems exposed (•) or protected (♦) from UV radiation. Error bars are standard error.

Table 4. Average fungal production and biomass in U. V. exposed (UV+) *Scirpus* stems decaying in Pond 50.

Julian Day (days from start of experiment in brackets)	Average Fungal Biomass (μg C g $AFDM^{-1}$) ± S. E.	Average Daily fungal Production (μg C g^{-1} AFDM D^{-1}) ± S. E.
182 (0)	155± 0	384± 202
194 (12)	4233± 1890	268 ± 152
208 (26)	1260± 530	152± 30
222 (40)	2280± 1280	26± 9
236 (54)	1998± 670	413± 67
249 (67)	2035± 1060	273± 68
264 (82)	1870± 650	15± 1.4
278 (96)	667± 190	45± 6.4
292 (110)	323± 117	335± 44
306 (124)	360± 160	61± 29

Table 5. Average fungal production and biomass in U. V. protected (UV-) *Scirpus* stems decaying in Pond 50.

Julian Day (days from start of experiment in brackets)	Average Fungal Biomass (μg C g $AFDM^{-1}$) ± S. E.	Average Daily fungal Production (μg C g^{-1} AFDM D^{-1}) ± S. E.
182 (0)	155± 0	384± 202
194 (12)	2740± 1600	334± 169
208 (26)	3740± 2240	196± 14
222 (40)	550± 110	59± 19
236 (54)	1290± 140	316± 90
249 (67)	2460± 1210	122± 19
264 (82)	2700± 1090	12± 6
278 (96)	1400± 350	28± 11
292 (110)	710± 320	464± 76
306 (124)	460± 170	38± 2

3.3.6.3 Solar Radiation Experiment Fungal Production

Production measurements for the UV+ stems (Figure 18B and Table 4) ranged from a high of 413 ± 66.7 μg C g^{-1} AFDM day^{-1} on 23 August (Julian Day 236) to a low of 15.1 ± 1.4 μg C g^{-1} AFDM day^{-1} on 20 September (Julian Day 264 or 82 days after the start of the experiment) (Figure 18B). The production data for the UV- stems (Figure 18B and Table 5) followed the same general rise and fall pattern of the UV+ exposed stems. The highest production value on the UV- stems was 464 ± 75.5 μg C g^{-1} AFDM day^{-1} on 18 October and the lowest on 20 September (Julian Day 264) of 12.4 ± 5.6 μg C g^{-1} AFDM day^{-1}. Over the course of the experiment the average daily fungal production on the UV+ stems was 197 ± 49 μg C g^{-1} AFDM day^{-1} while for the UV- stems it was 195 ± 53 μg C g^{-1} AFDM day^{-1}. There was no statistical difference in production between the UV+ and the UV- treatments ($P > 0.96$) or among sampling dates within the treatments ($P > 0.05$). The total fungal production from beginning to end of the radiation experiment was very similar for both the UV+ and the UV- treatments with 42.6 and 42.5 mg C g^{-1} AFDM, respectively. There was a positive correlation between temperature and production in both the UV+ ($r^2 = 0.65$, $P < 0.05$) and the UV- ($r^2 = 0.67$, $P < 0.004$) treatments.

3.3.6.4 Solar Radiation Experiment *Scirpus* Stem POC and PON levels

At the beginning of the experiment the POC content was 40 mg g^{-1} and PON was 0.78 mg g^{-1}. Results at the end of the experiment for the UV^- stems were 24.3 mg g^{-1} POC and 1.98 mg g^{-1} PON; for the UV^+ stems the POC content was 23.0 mg g^{-1} and the PON content was 0.81 mg g^{-1}. The C:N ratio at the start of the experiment was 51:1. At the end of the experiment the C/N ratio for the UV^- stems was 12:1 and the C/N ratio for the UV^+ stems was 28:1.

3.3.7 Water Temperature Experiments

3.3.7.1 Short Term Temperature Experiment

In the short-term experiment there were no differences ($P > 0.05$) in the production rates at 4 and 10°C (Fig 19) or at 15, 21 and 30°C. However, the production rates at 4 and 10°C were significantly lower ($P > 0.05$) then the production rates at 21 and 30°C, but not significantly lower than the production rates at 15°C ($P > 0.05$).

3.3.7.2 Long Term Temperature Experiment

3.3.7.2.1 Percent Moisture

The percent moisture in the stems the day they were placed in the aquaria for the long-term temperature experiment was 6%. Twelve days later in the 4°C chamber the percent moisture had increased to 69% and by Day 35 had stabilized at 76% where it remained for the duration of the experiment. For the 21°C treatment the moisture content reached 81.9% by Day 12 and stabilized in the 80-85% range for the remainder of the experiment. The stem percent moisture was significantly higher in the 21°C treatment compared to the 4°C treatment ($P < 0.001$).

3.3.7.2.2 AFDM, Biomass and Production

The mean AFDM for stems in the 4 and 21°C treatments was 0.069 ± 0.013 g C and 0.064 ± 0.007 g C, respectively and was not different ($P > 0.08$). The biomass of 0.22 mg C g^{-1} AFDM on Day 1 initially dropped to 0.056 mg C g^{-1} AFDM but then rose tenfold to 0.52 mg C g^{-1} AFDM by Day 35 (Figure 20A and Table 6). After this there was a gradual decline and then a sudden increase to 1.56 mg C g^{-1} AFDM on Day 197. Fungal production rate on the cut stems at the start of the experiment were 92 µg C g^{-1} AFDM day^{-1}. The production rate declined dramatically in the 4°C treatment to 10.0 µg C g^{-1} AFDM day^{-1} by Day 55 and then rose to an experimental high of 21.9 µg C g^{-1}

AFDM day^{-1} on Day 134 (Figure 20B). On day 255, with a biomass of 0.43 mg C g^{-1} AFDM, there was no measurable uptake of radiolabelled Na acetate in the *Scirpus* stems at 4°C. Over the duration of the experiment, excluding Day 1, the mean production was 13.3 ± 2.5 μg C g^{-1} AFDM day^{-1} and the biomass 0.43 ± 0.17 mg C g^{-1} AFDM.

In the 21°C experiment the biomass increased to 1.21 mg C g^{-1} AFDM by Day 35, then decreased to 0.056 mg C g^{-1} AFDM by Day 55 and thereafter was stable at near 2.0 mg C g^{-1} AFDM for the duration of the experiment (Figure 20A and Table 7). The production rates declined gradually from the start of the experiment to 9.92 μg C g^{-1} AFDM day^{-1} by Day 55 (Figure 20B) and then rose sharply, by Day 134, to 269.4 μg C g^{-1} AFDM day^{-1}. The mean production was 76.5 ± 32.6 μg C g^{-1} AFDM day^{-1} and the biomass 1.46 ± 0.34 mg C g^{-1} AFDM. The mean biomass ($P < 0.008$) and the mean production rate ($P < 0.05$) were significantly higher in the 21°C treatment than in the 4°C treatment.

3.3.8 Pooling of Data to Illustrate the Significance of Temperature to Both Fungal Biomass and Production

Out of interest, and to illustrate the significance of temperature on fungal production and biomass, all of the biomass and production data from the seasonal measurements and the UV experiment were pooled with respect to temperature. Because there was a distinct rise in production between 10 and 15°C (Figure 19) in the short-term temperature experiment, 15°C was pegged as a defining temperature and the total seasonal data split into >15°C and < 15°C. Significantly, 83.4% of total production measured occurred at temperatures above 15°C ($P < 0.001$). For biomass, however, 49% of total measured fungal biomass was detected at temperatures > 15°C and 52 % below.

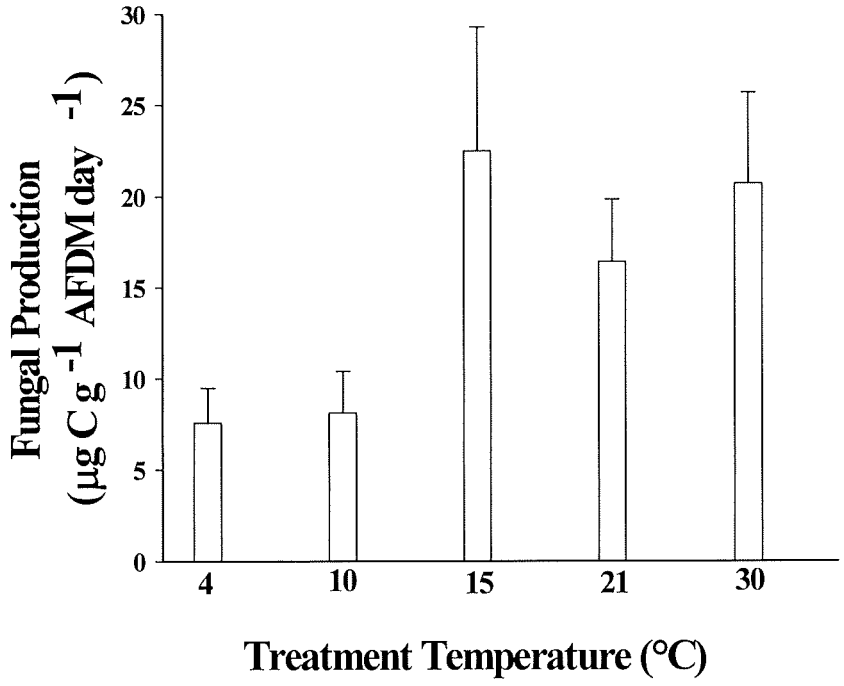


Figure 19. Temperature versus fungal production in *Scirpus* stems in the short-term temperature experiment. Error bars are standard error.

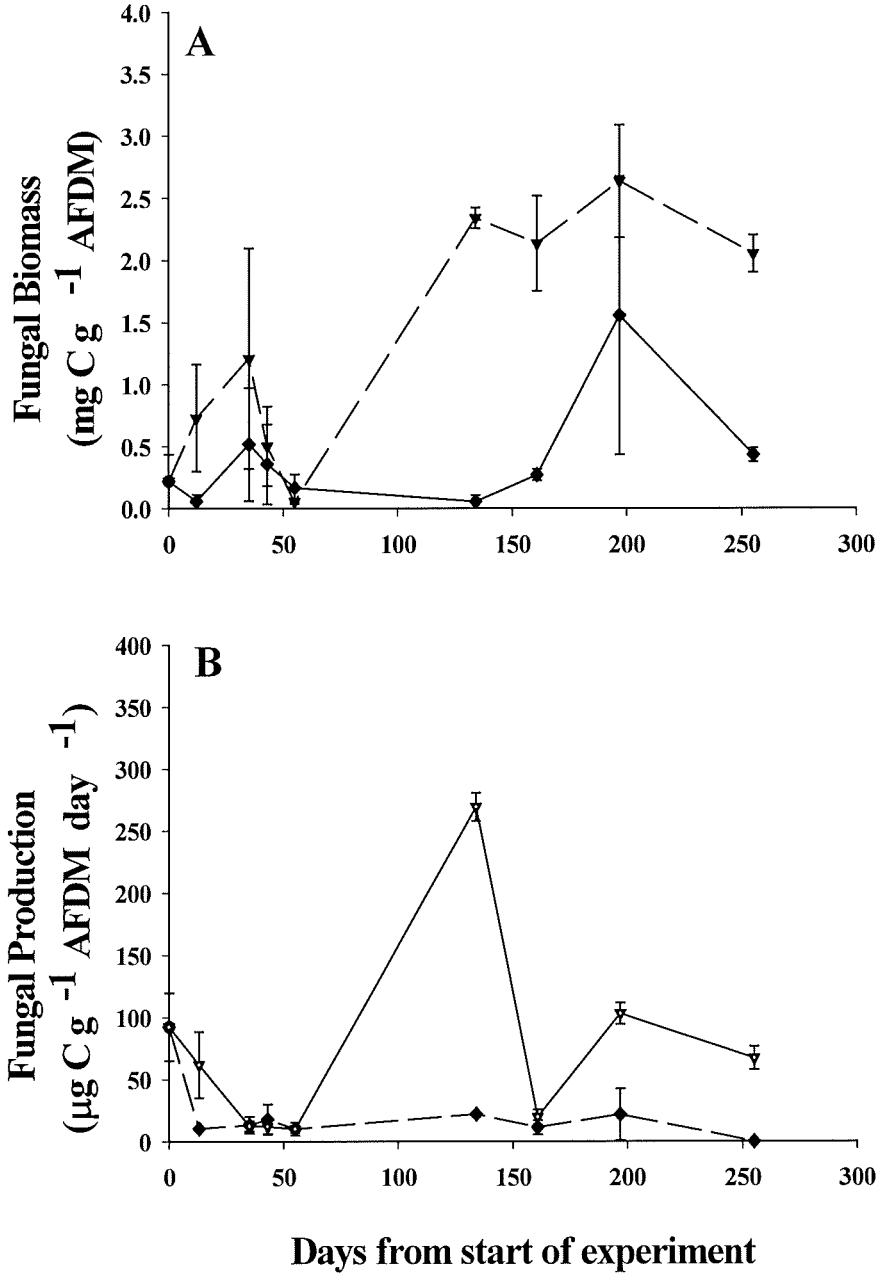


Figure 20. Fungal biomass (A) and production (B) on *Scirpus* stems over time at 4 (♦) and 21°C (▽). Error bars are standard error.

Table 6. Average fungal production and biomass in the long-term 4°C experiment.

Days from start of experiment	Average Fungal Biomass (μg C g $AFDM^{-1}$) ± S. E.	Average Daily fungal Production (μg C g^{-1} AFDM D^{-1}) ± S. E.
1	217± 0	92± 27
13	56± 56	10± 0.69
35	517± 457	13± 6.7
42	358± 323	18± 12
55	165± 110	10± 5.1
134	54± 54	22± 2.0
161	271± 46	22± 2.0
197	1555± 1122	11± 5.8
255	432± 55	0± 0

Table 7. Average fungal production and biomass in 21°C experiment.

Julian Day (days from start of experiment in brackets)	Average Fungal Biomass (μg C g AFDM^{-1}) ± S. E.	Average Daily fungal Production (μg C g^{-1} AFDM D^{-1}) ± S. E.
1	217± 0	92± 27
13	730± 432	62± 27
35	1209± 890	12± 4.2
42	503± 322	12± 5.7
55	56± 19	10± 3
134	2341± 83	269± 11
161	2136± 382	20± 6
197	2637± 452	103± 9
255	2054± 150	67± 9

3.4 Discussion

3.4.1 Fungal Biomass and Production on Standing Dead Litter of *Scirpus*

On the first sampling date of May 10 with an average daily temperature of 8°C, a high ergosterol content was detected in dead stems at water level and at the tips of the shoots where, in the latter, there was only 13% moisture. This observation suggests the process of plant breakdown begins to occur, and production in fact peaks, very early in the spring in prairie wetlands despite the relatively cold temperatures. Though the moisture levels in the stem tips were low at the time of sampling, snow, rain, fog and dew would have provided sufficient moisture to sustain fungi (Newell 2001). As well, the relationship between aerial fungal production and stem % moisture (Figure 16) suggests that 13% moisture may not be low in terms of fungal sustainability and activity. The highest fungal production values of the growing season for the submerged portions of *Scirpus* stems were observed in May and in June in the aerial stem portions. Unfortunately, no data for earlier dates closer to ice-out is available for both aerial and submerged stems and it is possible that production would have been higher. While the high spring production rates were not approached later in the season, there were smaller peaks in August and later in the fall.

In the present study it was found that water temperatures and stem percent moisture are strong variables affecting fungal biomass and production (Figures 15, 16 and 17). This suggests that fungal response to temperature and moisture fluctuations is relatively sudden under optimum environmental conditions with rapid biomass accumulation and increases in production followed by prolonged declines. Kuehn *et al.* (Kuehn *et al.* 1998) noted significant increases in CO_2 (bacterial and fungal) evolution within five minutes of wetting dry standing litter of the macrophyte *Juncus effusus* and the reverse occurred when wet litter was exposed to drying conditions. Similar rapid

increase in both fungal and bacterial biomass were observed in arid soils after rainfall events (Vishnevetsky & Steinberger 1997). Suberkropp (Suberkropp 1991) has noted that biomass accumulation and sporulation occurs rapidly during the initial colonization of leaves in streams and that this phase is followed by a decrease in sporulation as well as respiration rates similar to what occurred in Pond 50 in the spring. Temperature fluctuations and moisture availability based on rainfall events would partly explain the rise and fall in fungal production and biomass on decaying *Scirpus* at Pond 50.

3.4.1.1 Role of Fungal Succession

Details of fungal species succession in the course of litter breakdown, especially in lentic systems, remain sketchy due to the difficulty in identifying fungi (Newell & Porter 2000, Sridhar & Bärlocher 2000). Gessner *et al.* (Gessner *et al.* 1993) followed the colonization patterns of aquatic hyphomycetes during leaf litter breakdown in a stream in the French Pyrenees. They reported the occurrence of five fungal species 2 weeks after litter placement in a stream, 13 species at 28 days and by 8 weeks fungi dominant earlier had declined in abundance. As well, sampling in streams for fungal conidia has also shown seasonal fluctuations indicating the possibility of fungal succession (Fabre 1998, Barlocher 2000). Osono demonstrated successional patterns of colonization by following changes in fungal species composition on beech leaves while they were attached to the tree, as soon as they had fallen to the ground and as they decomposed (Osono 2002). It may be that fungi that decompose plant material in the prairie spring are more tolerant of cooler weather conditions and less of higher temperatures and may, as the season advances, be displaced by fungi more tolerant of higher temperatures and desiccating conditions of summer. These in turn are succeeded by species better able to function in colder and wetter conditions of fall.

Previous studies have shown how seasonal temperatures can affect the presence or absence of particular species of fungi with species dominant in summer disappearing in the fall (Gessner *et al.* 1993). Additionally, it may be possible that fungi in the spring may degrade the more labile portions of bound plant carbon in *Scirpus* and are later followed by fungi with specialized enzymes for digesting the more refractive stem constituents. In leaf litter decay in woodlands, it has been proposed that initially the leaf is colonized by sugar fungi that are able to utilize sugars and these fungi then sporulate and become inactive (Garrett 1951). Next to develop on the leaves are fungi that are able to utilize hemicellulose, and are followed by cellulose degraders and finally by lignin degraders. In decomposing terrestrial beech leaf litter it has been reported that different fungal species selectively utilized either the lignocellulose or the holocellulose portions of leaf carbon (Osono & Takeda 1999). In lotic aquatic systems, there is evidence that different types of plant litter (leaves, stems, roots) support relatively specific aquatic fungal species thus resulting in selective fungal colonization of plant parts (Gulis 2001). This suggests that fungi are not all equipped with a broad array of enzymes able to degrade all portions of plant material and there may be limitations on fungi imposed by the carbon profile of the plants. Such scenarios of fungal succession, based on temperature and carbon utilization limitations, would account for the fungal production and biomass peaks observed in summer and fall in decaying *Scirpus* in Pond 50. Laboratory studies, in conjunction with species specific DNA or RNA fluorescent probes, examining the process of *Scirpus* decomposition over time may further elucidate the rise and fall in fungal production and biomass on decaying *Scirpus*.

3.4.1.2 Role of Invertebrate Grazing

Invertebrate grazing of fungi may also lead to the loss of fungal biomass and productivity and partially explain the variability of the results in Pond 50. Previous researchers have shown the importance of pre-conditioned plant material as a nutrient source for organisms at higher trophic levels (Barlocher 1985, Barlocher *et al.* 1989). Not only would fungi provide a more enriched source of nutrition than the plant material itself (Newell 1996), but the fungi would also have partially pre-digested plant components which organisms at higher trophic levels would have been unable to do on their own. Newell and Porter (Newell & Porter 2000) have shown significant decreases of fungal biomass due to mycophagy by periwinkle and molluscs. Though no organisms that might have been fungivores were observed on any *Scirpus* stems incubated, microscopic mycovores such as amoebae and mites (Newell 1996, Newell & Porter 2000) and nematodes (Santos *et al.* 1981) may have caused a reduction in fungal biomass during the sampling season. Fungal mycelial loss could have also occurred by fragmentation and weathering thus resulting in decreased fungal biomass.

3.4.1.3 Role of Inorganic Nutrient Availability

Inorganic nutrient availability may also have played a role in influencing fungal productivity and biomass in both the aerial and submerged *Scirpus* stem portions. Nitrogen is possibly an important nutrient in controlling fungal production associated with decaying smooth cordgrass in salt marshes (Newell 2001) and the same may be the case in *Scirpus* stems. Unfortunately, concentrations of nitrogen, phosphorus and other nutrients in the stems were not measured and therefore one cannot surmise what role they may have played in the measured fungal biomass and production. However, Waiser (Waiser 2001a) assessed possible nutrient limitations of bacterial and algal

populations in the water and growing on *Scirpus* in Pond 50. She found no evidence of either P or N limitation. The PON results from the UV+ treatment where the C: N ratio was 28:1 at the end of the study may be indicative of nitrogen being slightly limiting, at least in the submerged portions of standing *Scirpus*.

3.4.1.4 My Study Results Compared to Other Similar Studies

The data for fungal production in decomposing *Scirpus* in Pond 50 ranged from 1.8 to 376 μg C g^{-1} AFDM day^{-1} and biomass from non-detectable to 5.8 mg C g^{-1} AFDM. These numbers are well within the range of what other researchers have reported, though comparisons may be not be entirely equivalent due to large differences in the nature of the decomposing litter. Newell *et al.* (Newell *et al.* 1995) reported fungal biomass of 0.15 mg C g^{-1} AFDM with production values ranging from 116 to 665 μg C g^{-1} AFDM on standing dead leaf blades of *Carex walteriana* in freshwater. These estimates for fungal production and biomass by Newell *et al.* were later revised upwards by one and half times due to a fault found in an HPLC (Newell & Porter 2000, Newell 2001) but are still close to the ranges measured in the present *Scirpus* study. Kominkova *et al.* (Kominkova *et al.* 2000) measured fungal biomass of approximately 10 to 80 mg C g^{-1} AFDM and fungal production ranging from 72 to 1224 μg C g^{-1} AFDM day^{-1} on submerged leaves of an emergent macrophyte in a hardwater Swiss lake. Kuehn *et al.* (Kuehn *et al.* 2000) measured fungal biomass of 7 to 42 mg C g^{-1} AFDM and production ranging from 73 to 2,836 μg C g^{-1} AFDM d^{-1} on decaying leaves of an emergent macrophyte in a freshwater wetland in Alabama. The highest biomass and production values in both the Kominkova et al's and Kuehn et al's studies are considerably greater than mine but may be partially due to the *Scirpus* stem being a less labile source of carbon (Walse *et al.* 1998, Gessner 2001) than leaves. In addition,

the lower temperatures associated with northern prairie conditions probably also account for the lower production and biomass values relative to other systems.

The conversion rates for fungal biomass and production used in the present *Scirpus* study were obtained from Kuehn *et al.* 2000, Suberkropp and Weyers 1996 and Kominkova *et al.* 2000 (but see Gessner and Newell 2002 for both fungal biomass and production estimates used in other studies) and not specifically determined for the primary fungal species responsible for decomposition in the aerial and submerged stems of *Scirpus* at Pond 50. The use of non-specific conversion values could greatly alter the estimates of both fungal production and biomass in *Scirpus* at Pond 50. However, observances made in the present study also corroborate other researchers (Kuehn *et al.* 2000, Newell & Porter 2000) in showing that a significant amount of carbon is sequestered, as indicated by fungal colonization and production on dead, standing *Scirpus*, while in the standing position and before abscission and submergence into water.

3.4.1.5 Hypothesis of a Fungal Niche Intermediate Between Terrestrial and Aquatic

Though fungi are well adapted to fluctuations in temperature and stem moisture (Kuehn *et al.* 1998), these parameters seem to have a profound effect on fungal biomass and production in dead *Scirpus* stems regardless of whether they are above or beneath the water. For aerial *Scirpus* stem pieces the optimum temperature for fungal production seemed to be in the 20-25°C range (Figure 15) with an ideal moisture content of between 15 to 22% (Figure 16). Increasing solar radiation was also beneficial to production rates in aerial fungi. For submerged parts of *Scirpus* optimum biomass occurred at the 45-50% moisture levels (Figure 17) and biomass was hindered

by increasing solar radiation. This variation in optimum moisture content and solar radiation effects between the aerial and submerged fungal constituents hints at the possibility of a niche intermediate between the aquatic and terrestrial. *Scirpus* stem moisture levels in the aquatic solar radiation study were > 90%, therefore, aquatic fungi which colonized the stems would be unaffected by a high moisture content while a stem moisture content >30% inhibited terrestrial fungal colonization. For fungi inhabiting the submerged zone optimum conditions are in the mid-range between that of the terrestrial and the aquatic in terms of moisture content. As well, the slight negative effect of solar radiation intensity on submerged fungal biomass also is suggestive of a fungal species distinct from the terrestrial. Further research may elucidate the validity of this hypothesis.

Additionally, in both the aerial and submerged *Scirpus* stems a moisture content higher or lower than the optimum range seemed to be detrimental to fungal production and biomass. This is in agreement with Newell (Newell 2001) where he observed decreased fungal productivity and biomass in standing smooth cordgrass both when rainfall was high and when he artificially increased water saturation by misting. Newell attributed this to increases in leaching of fungal digestate as well as greater number of bacterial competitors under higher moisture levels relative to drier conditions. This trait of higher moisture content negatively affecting productivity may be peculiar to terrestrial fungi, less so to the fungi living on submerged standing stems and, as already discussed, should have little to no effect on aquatic fungi. The lag phase seen in fungal colonization when plant detritus is introduced to a submerged environment, noted by others (Newell *et al.* 1989, Kuehn *et al.* 2000), may in part be due to the low moisture content of the plants when they initially submerge. This may be because the litter degrading enzymes discharged by the aquatic fungi may require a high degree of water

content in the substrate in order to be effective. In terrestrial fungi a much lower range of moisture levels, as seen in Figures 16 and 17, may be required in order for ideal enzymatic activity for plant breakdown to occur. Though terrestrial fungi are known to be highly adapted to varying moisture conditions by regulating their internal concentrations of osmolytes (Kuehn *et al.* 1998), at extremes of water availability, extreme desiccating conditions or water saturation, physiological survival tactics other than osmolyte concentration may become more critical thus resulting in decreased fungal biomass and production. As an example, as the moisture content in the environment decreases, the fungi is able to vary its osmolyte concentration in response; but if the environment becomes extremely dry, beyond what can be buffered by osmolyte concentration, the fungi may be forced to produce resting spores in order to survive the very low moisture conditions (Professor. Brian Flannigan, University of Napier, Scotland, personal communication).

3.4.2 Solar Radiation

The solar radiation experimental conditions were an attempt to mirror conditions for *Scirpus* litter in a typical prairie wetland and to measure the effects of solar radiation on aquatic fungal colonization. Dead litter from a previous season stands in a new season after ice out before falling into the water. Dead litter sinks to the bottom of a wetland after becoming water logged. Besides being shallow, the waters in prairie wetlands have very high concentrations of DOC that rapidly attenuate UV-radiation (Arts *et al.* 2000). Therefore, fungal exposure to solar radiation on standing dead litter, and to UV-radiation in particular, is limited to a very narrow band beneath the water surface. Once the litter falls into the water and sinks to the sediments, much of it will only be exposed to PAR because of the rapid attenuation of UV. To mimic

these events in my experiment there were three distinct types of exposure. First, for about 2 weeks the *Scirpus* pieces were exposed to PAR (88%), UV-A (85%) and UV-B (76%) in the UV^+ treatment. Second, after the stems sank to the screens at 4 cm (by day 194), the average depth of 1% penetration of UV-B (46), they were primarily exposed to UV-A ($\bar{x}$ = 23%) + PAR from mid-July to mid-September (days 195 to 270). Third, the UV^- and UV^+ treatments were essentially the same with only PAR exposure, after day 270 to the end of the experiment. Unlike in other studies, where fungi were exposed to the UV flux of almost full sunlight (Denward & Tranvik 1998, Anesio *et al.* 1999), exposures in the present study were closer to the levels of exposure experienced by most of the underwater fungal population except in the upper few centimetres of the water column.

Over the incubation period there was no significant difference in fungal production or biomass between the UV^+ and UV^- treatments. In the two-week exposure to UV-B at the beginning of the experiment there was no impact on fungi. During the next 75 days when the stem pieces were only exposed to PAR + UV-A there was still no significant difference in biomass and production between the two treatments, even though there seemed to be a possible stimulation of fungal production in the UV^+ treatment from days 236 to 249. Though much work has focused on UV-B, as it is the more energetic form of UV radiation, UV-A has been found to be as or more important than UV-B on microbial processes. Aas *et al.* (Aas *et al.* 1996) observed equal inhibition in the incorporation of labelled thymidine by bacteria under UV-A and UV-B conditions. Sommaruga *et al.* (Sommaruga *et al.* 1997) recorded a 70% decrease in bacterial thymidine and leucine uptake in both freshwater and marine systems and cautiously attributed it entirely to UV-A radiation. The influence of UV-A radiation on fungi has been less studied than for bacteria but there are some indications that fungi,

for the most part, are inured to this form of radiation. Denward *et al.* (Denward *et al.* 2001) saw no decrease in aquatic fungal biomass on aquatic macrophytes exposed to PAR + UV-A levels similar to those in the present study. Moody *et al.* (Moody *et al.* 1999) observed that UV-A effects, as measured by mycelial extensions in fungi grown on agar, were largely beneficial to terrestrial fungi. Newsham *et al.* (Newsham *et al.* 1997) however, reported the disappearance of a single fungal species under UV-A treatment and also that UV-A accelerated the decomposition rate of leaves in terrestrial litter. These types of UV-A effects may have occurred during the experiments with *Scirpus* and helped mask any differences between the two treatments and is an area for future research.

The lack of differences between the treatments may also have been due to several other factors. It is possible that aquatic fungi may differentially colonize plant material with some inhabiting the surface and others preferring to tunnel into decomposing material as occurs in terrestrial litter with some inhabiting the phylloplane of detritus and others burrowing more deeply into plant litter (Newsham *et al.* 1997, Moody *et al.* 1999, Osono 2002). If this occurs with aquatic fungi there may have been little or no affect by the UV radiation on fungi penetrating the stems because they would have, once inside, been shaded while fungi on the surface would have been affected. Additionally, it is possible that if there was an increased breakdown of the *Scirpus* stems in the UV^+ treatment (Denward & Tranvik 1998) and this may have offset any inhibitory effects on the fungi thereby accounting for the similar POC levels at the end of the experiment in both treatments.

3.4.2.1 Role of Fungal Species Composition Change

Another possibility is a change in species composition in the UV^+ treatment. In work done by Moody *et al.* (Moody *et al.* 1999) it was shown that phylloplane species were largely unaffected by UV-B radiation whereas other species were usually negatively affected. Similarly, Gehrke *et al.* (Gehrke *et al.* 1995) and Newsham *et al.* (Newsham *et al.* 1997) observed differences in terrestrial fungal species composition between those subjected to an artificial 30% increase above ambient levels of UV radiation and those under ambient solar radiation in decomposing terrestrial litter. Unfortunately, the biomass and production measurements in Pond 50 would not detect a change in species composition between the two treatments. Ultraviolet radiation in the UV^+ treatment may have led to death or debilitation of some fungi while favouring others more tolerant of the exposure conditions. Though the work by Newsham *et al.* and Gehrke *et al.* was done on terrestrial fungi the same may apply to fungi in an aquatic setting albeit less strongly since organic compounds in water rapidly attenuate UV radiation, especially in prairie wetlands.

3.4.2.2 Roles of POC and PON

The decrease in POC levels in both treatments during the experiment in the present study indicated that organic carbon from the decomposing *Scirpus* either leached into the water or was utilized by fungi and other microorganisms. The increase in PON in the UV- treatment was probably associated with an increase in microorganisms, possibly bacteria and algae, since fungal biomass declined. Conversely, in the UV+ treatment PON decreased indicating that bacteria and algae may have been inhibited since fungal biomass did not change. Therefore, the high C:N

ratio measured in the UV+ treatment at the end of the experiment was possibly due more to the harmful effects on microorganisms other than fungi. Newell and Porter (Newell & Porter 2000) and Battle and Golladay (Battle & Golladay 2003) have found similar results in decomposing cordgrass and leaf litter, respectively, and attributed the lower C:N ratios to higher levels of available nitrogen and robust microbial activity. Denward *et al.* (Denward & Tranvik 1998) found no change in the C:N ratio of decomposing aquatic macrophyte litter, placed in buckets of tap water under treatments blocking UV-B or both UV-A and UV-B, possibly because their experiment only ran for 60 days. Additionally, the increased leaching of organic material into water would increase the DOC concentrations, leading to greater UV attenuation and decreasing the amount of radiation impacting the microorganisms over time.

The initial measurement of fungal biomass and production on aquatic plant material within 24 hours of harvesting living *Scirpus* is surprising and to the best of the author's knowledge has not been reported before. The simplest explanation may be that the ergosterol and fungal production can be attributed to yeast and/or symbiotic or parasitic fungi that are part of the natural microbial flora of living plants (Osono 2002). However, there is also the possibility that decomposition and colonization by fungi begins to occur very quickly, within hours, after plant death. Both of these ideas would explain the little amount of biomass observed and the high uptake of Na [1-^{14}C] acetate into ergosterol so quickly after harvesting of living plant material.

3.4.3 Temperature Experiments

It is surprising that temperature as a regulating factor in aquatic fungal dynamics has gone largely unstudied. Suberkropp and Weyers (Suberkropp & Weyers 1996) observed an exponential increase in fungal carbon production with increasing

temperature from 10 to 25°C on yellow poplar leaves. Chauvet and Suberkropp (Chauvet & Suberkropp 1998) assessed the influence of temperature (15, 20 and 25°C) on sporulation of eight aquatic hyphomycetes. Optimal temperature for sporulation varied for five species, two produced similar amounts of conidia at 20 and 25°C and one sporulated equally at all three temperatures.

There was a positive correlation between temperature and fungal production in the solar radiation experiments as well as in the aerial stem portions in the seasonal study. In both the ideal temperatures for fungal production were in the 20-25°C range. The results of the short term experiment only indicated that fungal production was lower at ≤10°C than it was ≥15°C. However, in the long term study there was a significant difference in both the biomass and production which were both higher at 21°C compared to 4°C. From these experiments it is apparent that fungi are sensitive to temperature fluctuations and there are repercussions on the rate of dead macrophyte decomposition in northern prairie wetlands.

3.5 Conclusions

Fungal decomposition of standing, dead *Scirpus* in a northern prairie wetland began and was greatest in the spring despite relatively low water temperatures and percent moisture in the aerial portions of stems. Fungal biomass and production on both aerial and submerged parts of *Scirpus* stems occurred at similar levels. This research corroborates other studies in showing considerable sequestration of carbon from macrophytes by fungi occurs while the plants are standing and before collapsing into the water. In Pond 50, stem percent moisture along with water temperature, seemed to play key roles in accounting for seasonal changes in fungal biomass and production. It

is possible that a different species of fungi or fungus, as indicated by different optimum moisture levels, were responsible for decay in the submerged portions of *Scirpus*. Exposing dead *Scirpus* to realistic levels of PAR + UV radiation in Pond 50 did not affect fungal biomass or production when compared to non-exposed stems. However, large differences in PON between treatments suggested other microorganisms colonizing dead *Scirpus* litter may have been impacted, but this preliminary observation needs confirmation. Therefore, while exposure to solar radiation may have induced changes at the organism (algae and bacteria) or species level (fungal), which were not measured, based on the results it is likely that PAR + UV radiation does not have a significant impact on fungal breakdown of dead *Scirpus* litter in Pond 50. Annual fungal production on *Scirpus* in this northern prairie wetland was at the lower end of rates reported elsewhere as a result of a colder climate and the possibly lower lability of *Scirpus* compared to the leaves and softer macrophytes examined in other studies at lower latitudes. However, it may also be that unlike other systems where fungi seem to dominate litter breakdown, bacteria may also play a significant role in *Scirpus* decomposition in the northern prairies thereby accounting for the lower fungal production and biomass measured. Macrophyte plant decomposition begins, under favourable conditions, while plants are in the standing position and continues once abscission occurs and plants collapse into the water. The initial process of fungal and bacterial colonization after a plant enters the aquatic environment has not been studied in detail thus far. Additionally, the process of fungal and bacterial litter decomposition in aquatic systems can be affected by a variety of organic contaminants such as fungicides and antibiotics that may be present in aquatic systems.

CHAPTER 4

TETRACYCLINE BEHAVIOUR IN DISTILLED, RIVER AND POND WATERS

4.1 INTRODUCTION

Alexander Fleming discovered penicillin, an antibiotic produced by the fungus *Penicillium notatum*, in 1929. However, it was not until the early 1940's that penicillin began to be used to treat bacterial infections in humans (Nester *et al.* 1998). Since the 1940's a few new antimicrobials such as flouroquinolones and sulfonamides have been synthesized in the laboratory while hundreds of naturally occurring antibiotics have been discovered and commercialized for use not only in the treatment of bacterial diseases in people, plants and animals, but also as additives in feed as a prophylactic and to promote growth in animals (U.S.D.A 1999). In the 40 year period between the commencement of antibiotic use and 1984, it has been estimated that over one million tonnes of antibiotics have been released into the biosphere (Mazel & Davis 2003) by sewage effluents, manure runoff from agricultural fields and by disposal. This represents an average of 25 million kilograms of antibiotics released into the environment per year. However, the combined human and agricultural use of antibiotics in 1999 in the United States alone, which is said to represent approximately 40 to 60 % of total world-wide antibiotic use (European Union 1999, Mellon *et al.* 2001), was estimated to be 25 million kg (Meyer *et al.* 2000).

In the early 1980's some antibiotics belonging to the tetracyclines family along with erythromycin and sulphamethoxazole were found in the River Lee, United Kingdom (Halling-Sorensen *et al.* 1998). Subsequently antibiotics have been detected in surface and ground waters in many countries around the world (Halling-Sorensen *et al.* 1998, Hartmann *et al.* 1998, Daughton & Ternes 1999, Hartig *et al.* 1999, Heberer

2002, Kolpin *et al.* 2002). It has been generally assumed the antibiotics being detected are anthropogenic in source.

The study of the fate and chemical behaviour of antibiotics in aquatic environments are rare (Halling-Sorensen *et al.* 1998, Daughton & Ternes 1999, Ingerslev *et al.* 2001). Some possible fates of antibiotics in aquatic ecosystems may be similar to that of other organic compounds: persistence (Winckler *et al.* 2001), adsorption and re-mobilisation from water matrices, sediment and living biota (Wolfaardt *et al.* 1995, Headley *et al.* 1998, Wolfaardt *et al.* 1999), breakdown into smaller metabolites, and hydrolysis or mineralization to CO_2 and H_2O by photolytic or bacterial degradation (Headley *et al.* 1995, Wolfaardt *et al.* 1995).

Tetracyclines are a group of naturally occurring and semi-synthetic antibiotics that inhibit protein synthesis in a wide range of Gram-positive and Gram-negative bacteria. Tetracyclines as a whole are one of the more commonly utilised antibiotics in the world and have been used for both human and animal therapy as well as a prophylactic and for growth promotion (U.S.D.A 1999, Oka *et al.* 2000, Halling-Sorensen *et al.* 2002). Tetracycline (Figure 21A), as may be inferred by its name, belongs to the tetracyclines family and is an antibiotic produced by a variety of soil bacteria in the *Streptomyces* family (Figure 21B and 21C) (The Merck Index 1983, Chopra & Roberts 2001). Tetracycline is known to chelate to metals (Halling-Sorensen *et al.* 2002) and to bind to humic and bentonite clay particles under certain conditions (Sithole & Guy 1987a, Sithole & Guy 1987b). Oka *et al.* (Oka *et al.* 1989), identified photodecomposition products generated from tetracycline when it was exposed to UV radiation in aqueous solution but provided no details as to the nature of water used. Peterson *et al.* (Peterson *et al.* 1993) studied paints containing tetracycline which were applied to boat hulls to inhibit fouling, and the rates at which tetracycline leached

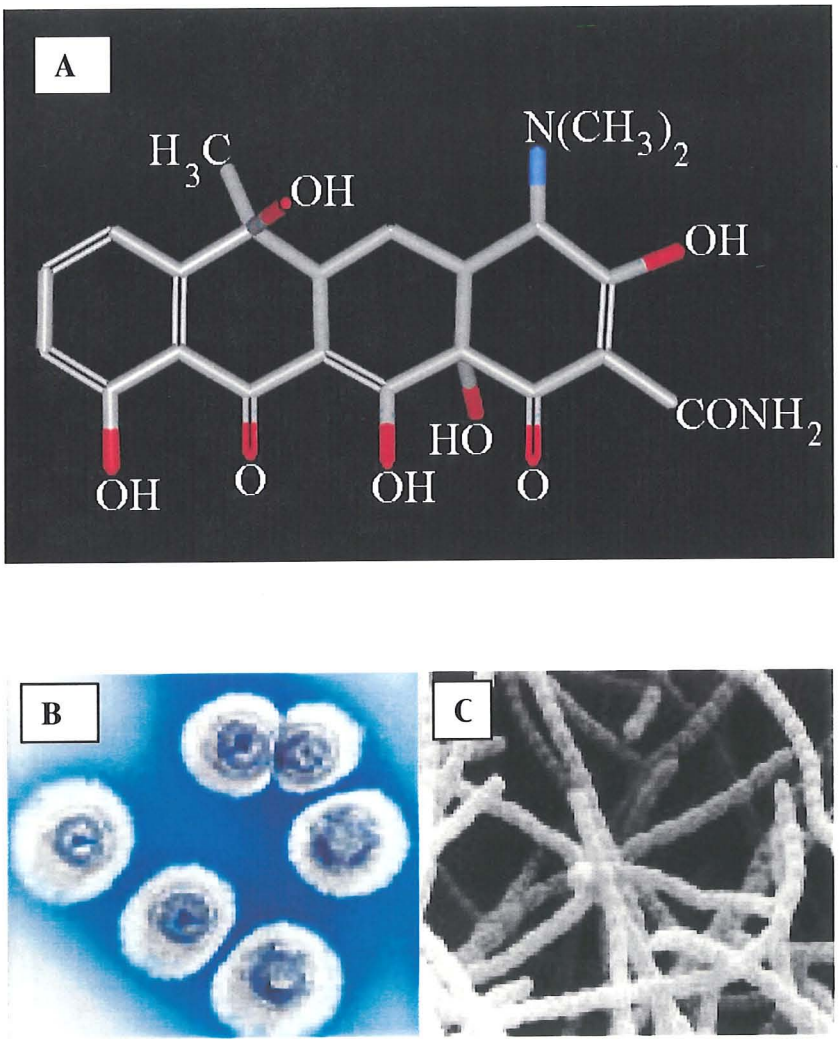


Figure 21. Schematic diagrams of the molecular structure of tetracycline (A), *Streptomyces* colonies (B) and a microscopic image showing the filamentous nature of *Streptomyces* bacteria. (C).

from the painted surface into seawater under laboratory conditions was measured. There has been one laboratory study that has researched the removal of the parent tetracycline from hog manure and Ringers solution (Kuhne *et al.* 2000). A recent study examined the persistence of tetracycline in a field sprayed with liquid manure (Hamscher *et al.* 2002).There has been no reported work on the fate of tetracycline, in terms of adsorption and photolytic or microbial degradation, in freshwater aquatic environments.

Part of the reason for the lack of study has been the unavailability of suitable extraction procedures as well as instrumentation that can precisely, and at trace levels, identify tetracycline from the many compounds that are present in complex environmental matrices. Because experiments to measure the effects of tetracycline on microbial populations involved in *Scirpus* decomposition were to be performed, the objective of the present work was to characterize the behaviour of tetracycline in natural waters with respect to how that behaviour might directly influence microorganisms. Tetracycline that becomes bound to metals or a matrix is thought to be inactive against bacteria (Halling-Sorensen *et al.* 2002). Therefore, the portion of spiked tetracycline that is free and the portion that becomes bound to the matrix in distilled, river and pond waters were studied. Additionally, the disappearance rates of tetracycline in both laboratory and natural sunlight conditions, with or without the influence of UV radiation, in sterile (photolytic degradation) and non-sterile (microbial plus photolytic degradation) distilled, river and pond waters were studied. Owing to the low concentrations of tetracycline being studied, it was felt necessary to extract tetracycline from the water, prior to detection, thus a recently developed procedure that utilizes solid phase microextraction (SPME) columns (Lindsey *et al.* 1998) for the extraction of tetracycline from distilled, river and pond water was evaluated. High-

performance liquid chromatography combined with electrospray ionization tandem mass spectrometry (HPLC-ESI-MS-MS) was used for tetracycline confirmation and quantitation (Kamel *et al.* 1999).

4.2 Materials and Methods

4.2.1 Water Collection, Filtration and Bacterial Enumeration

River water was obtained from the South Saskatchewan (SSK) River, Saskatoon, Saskatchewan, Canada (106° 38'W, 52° 07'N) whereas pond water was obtained from Pond 1 situated at the St. Denis National Wildlife Area (See Figure 2b, Chapter 3). Water from these sites was collected in 10 and 20 L plastic containers and, once at the laboratory, filtered through Whatman glass fibre (GF/C-1.2 μm pore size) filters. The three waters, including reverse osmosis distilled water, were stored at room temperature and allowed to acclimate to laboratory conditions before being used in experiments. When required, the waters were filter sterilized by, first, using a tangential filtering Pall Filtron Omega (Sin-Can, Calgary, Canada) open channel system equipped with a 0.16 μm pore-size polyethersulfone filter pack (Waiser 2001b) and, second, by being filtered through sterile 47 mm 0.1 μm pore-size Sartorius cellulose nitrate filters. All water samples were sub-sampled before and after filtration in order to determine bacterial numbers (Table 8) by using 4',6'- diamidino-2-phenylindole (DAPI) and epifluorescent microscopy (Tumber *et al.* 1993) . Water chemistry (Table 8) was performed by suppressed conductivity ion-exchange chromatography on a Dionex 400 Ion Exchange chromatograph (Sunnyvale, California). All laboratory experiments were conducted at room temperature (21 ± 3°C) and under laboratory light conditions.

4.2.2 Glassware Cleanup

In view of previously published reports of tetracycline chelating with metals as well as binding to glass and plastic surfaces (Peterson *et al.* 1993, Halling-Sorensen *et al.* 2002), all glassware was detergent washed and soaked in 20% nitric acid overnight to remove organic impurities and metals (Talbot & Weiss 1997). The glassware was rinsed with distilled water (3X) and Milli Q water (3X) before being oven dried. Glassware sterilization, if required, was done by autoclaving.

4.2.3 Tetracycline Extraction

Initially, thinking the tetracycline spiked into distilled, river and pond waters in experiments would have to be extracted prior to injection and detection by HPLC-ESI-MS-MS, the Lindsey (Lindsey *et al.* 1998) procedure was utilized to extract for tetracycline. In this procedure 100 ml of water samples are prepared for extraction by adding 75 μL of 40% H_2SO_4 and 700 mg of EDTA. Waters Oasis solid-phase hydrophilic-lipophilic balance (HLB) 60 mg columns (Milford, U.S.A.) were used to extract tetracycline from water. The HLB column was pre-conditioned by washing with 3 ml of MeOH followed by 3 ml of 0.5 N HCl and then rinsed with 3 ml of Milli Q water. The ambient water sample containing tetracycline was passed through the columns at a rate of $\cong$ 10 ml min^{-1}. The column was rinsed with 1 ml of Milli Q water, to remove excess EDTA, and the tetracycline eluted with 5 ml of MeOH. A gentle stream of N_2 gas was used to evaporate the methanol and concentrate tetracycline. Concentrated samples were placed in Waters 12 X 3 mm amber vials (Milford, U.S.A.) prior to mass spectrometric analysis for detection and quantitation. These amber vials were used for storage of water samples in all experiments.

4.2.4 Instrumentation Parameters

Samples containing tetracycline were injected in 20 µl aliquots using a Waters 2690 (Milford, U.S.A.) separations module consisting of a Waters X Terra C_{18} (Milford, U.S.A.) analytical column, 2.1 X 100 mm with a 3.5 µm particle size. The column temperature was 25°C. Eluent A consisted of 90:10 water: methanol and eluent B of 100% methanol. Both eluents contained 10 mM ammonium formate and 4 ml L^{-1} formic acid. Under isocratic conditions, 50% A and 50% B were used for chromatographic separations at a flow rate of 0.20 ml min^{-1}. The column effluent was delivered to the atmospheric ionisation (API) interface of a Micromass Quattro Ultima (Manchester, U.K.) triple quadrupole mass spectrometer utilising electrospray ionisation in positive ion mode (ES^+). Interface conditions were as follows: the capillary was set at 2.99 kV with source and desolvation temperatures of 90°C and 220°C, respectively. Nebulizer gas of nitrogen was set to maximum flow rate while the flow rates for the cone and desolvation nitrogen were set to 145 L hr^{-1} and 488 L hr^{-1}, respectively. Argon was used as the collision gas with a collision energy of 35 V (lab frame of reference) and the detector multiplier set at 650 V. Selective reaction monitoring (SRM) was utilised for the determination of tetracycline by monitoring the transition of the protonated molecule (m/z 445) to m/z 410 $[M+H-H_20-NH_3]^+$ (Figure 22) at a dwell time of 1.0s and an inter-channel delay of 0.10s. At these settings the limit of quantitation was 10 ng ml^{-1} (10 ppb) while the detection limit, signal to noise ratio of 2, was ≅ 5 ng ml^{-1} (5 ppb).

The calibration curve (Figure 23) for tetracycline standards (Sigma Chemical Company, Toronto, Canada) ranging from 10 ng ml^{-1} to 4 mg L^{-1} was linear, with r^2 = 0.99. Quantification was obtained by comparing the peak areas of the samples with the peak areas of the external calibration curves.

4.2.5 Tetracycline Extraction and Direct-Injection Experiments

Owing to poor tetracycline extraction efficiencies when using the Lindsey *et al.* (1998) procedure (see section 5.2.3), variations in EDTA and pH were tried to enhance tetracycline recoveries. Variations of EDTA and pH were conducted with pond water because pond water was considered to have the most complex matrix of the three types of water investigated. Duplicate 20 ml pond water samples were spiked with tetracycline to a concentration of 100 $\mu g\ L^{-1}$. The tetracycline spikes were added before EDTA or pH adjustments were made to ensure interaction and binding between the matrix in the water and tetracycline. The Erlenmeyer flasks containing the water were swirled and allowed to stand for 10 min before extraction of tetracycline was begun. A sub-sample of 10 ml was HLB extracted and was also varied as follows: first EDTA was added and then the pH adjusted and second, pH was adjusted and then EDTA was added. There was no difference between these two procedures in terms of tetracycline recovery ($P > 0.05$). The remaining 10 ml of spiked pond water sample was used for direct injection into the Quattro Ultima to test if HLB extraction was even necessary to detect and quantify tetracycline. (It was found there was no difference in tetracycline detected between the HLB extraction and direct-injection experiment and therefore all subsequent samples were direct injected for detection.)

4.2.6 Tetracycline Adsorption Experiment

Duplicate samples (100 ml) of distilled, river and pond water in 125 ml Erlenmeyer flasks were incrementally spiked with tetracycline from 500 $\mu g\ L^{-1}$ to 4000 $\mu g\ L^{-1}$. At each incremental addition (500, 1000, 1500, 2000, 2500, 3000 and 4000 $\mu g\ L^{-1}$), the water was hand mixed and two 1 ml aliquots withdrawn with a Pasteur pipette and placed into amber vials for detection. There were two background control flasks for

each type of water to measure the background levels of tetracycline. If tetracycline was detected in the two background controls, the mean of the amount detected in the controls was subtracted from the amount detected in the treatments. This correction was done in all of the experiments.

4.2.7 Tetracycline Degradation or Photolysis Experiments

4.2.7.1 Photolysis in the Laboratory in Sterile and Non-sterile Waters

Tetracycline photolysis experiments were conducted in the laboratory in both light (L^+) and in darkness (L^-), at 4000 μg L^{-1} concentration in non-sterile distilled, river and pond water. Duplicate 100 ml samples of each type of water were placed into 125 ml Erlenmeyer flasks. For the L^- treatment, tetracycline was spiked in subdued light and the flasks placed in a box with an opaque lid. As a further precaution, the entire box was covered with aluminium foil. Sub-samples from the flasks in the dark treatment were withdrawn in subdued light, placed in amber vials and the vials covered with aluminium foil while being transported to the Quattro Ultima. For the L^+ treatment the water was spiked with tetracycline and the flasks were placed on a cart with the laboratory lights continuously on. The waters from all treatments were sub-sampled and run on the Quattro Ultima for quantitation immediately after spiking. The waters were sub-sampled and mixed by hand every 24 hours during the duration of the experiment, which was 9 days. The radiation flux acting on the flasks in the laboratory was 19.3 μE m^{-2} s^{-1}.

Another experiment, identical to the one above, was conducted except that 0.1 μm filter sterilized distilled, river and pond waters (Table 8) were used and the duration of experiment was 7 days. The mouths of the flasks were covered with aluminium foil to minimize bacterial contamination. All glassware (flasks, pipettes and vials) for this

second experiment was autoclaved prior to use and the waters aseptically sub-sampled in a safety cabinet.

4.2.7.2 Photolysis in Natural Sunlight

Duplicate samples (100 ml) of non-sterile distilled, river and pond water were placed in Erlenmeyer flasks and spiked with tetracycline to a final concentration of 1000 $\mu g\ L^{-1}$. The flasks were placed in a water bath (21± 3°C) under natural light conditions (Figure 24). There were two light treatments that utilised two different plexiglass covers: one allowed the penetration of full sunlight including UV radiation (UV^+), while the other excluded UV radiation (UV^-) but allowed the penetration of all other wavelengths. The nature and light transmission properties of the plexiglass have been described in Chapter 3 and in Verma *et al.* (Verma *et al.* 2003). The waters were sub-sampled every 15 min, placed in amber vials, and tetracycline concentration determined with the Quattro Ultima. The experiment was conducted on July 12, 2002. At the time of the experiment the radiation flux was ≅ 417 $\mu E\ m^{-2}\ s^{-1}$, the ambient temperature was 33°C and humidity was 37% (Data Courtesy of Patrick Kyle, Environment Canada and Saskatchewan Research Council, Saskatoon). Additionally, absorbance measurements of river and pond water, with and without tetracycline amendments, were performed at 2 nm intervals from 1100 to 190 nm using a Shimadzu UV-1601PC UV-Visible spectrophotometer (Shimadzu Corporation, Japan) in order to determine the portion of the light spectrum in which light radiation was most absorbed.

4.2.8 Statistical Analysis and Calculation of Tetracycline Decay Rates and Half-Lives

SigmaStat 2.03 (SPSS Inc.) was used to perform all statistical analyses. Parameter means ± standard deviations are given.

For all photolysis experiments first order kinetics was not assumed and therefore the rate constants were calculated (Atkins & Jones 1997). The integrated rate law for a first-order or pseudo first-order reaction is:

$$[A]_t = [A]_0 \text{ X } e^{-kt}$$

where $[A]_t$ is the concentration of reactant at time *t*, the initial concentration of the reactant is $[A]_0$, *k* is the rate constant and t is the elapsed time. This equation can be simplified to the linear equation:

$$y = \text{intercept} + \text{slope X } x$$

Plotting Natural log $[A]_t$ as a function of time will give a straight line with the slope – *k* if the decay rate follows first or pseudo first order degradation kinetics. To determine rate order the natural log of the concentration of tetracycline [concentration at *t* /concentration initial] versus time was plotted. All of the graphs were straight lines thus indicating that the decay rate, or reaction rate, was first order or pseudo-first order for tetracycline. The rate constant, *–k,* is the slope of the straight line on the linearized graphs. The half-life of a first-order reaction is related to the rate constant because the higher the *k* (steeper the slope) the faster it will disappear from a system and will have a shorter half-life. The half-life ($T_{1/2}$) was:

$$t_{1/2} = 0.693/k$$

In first order or pseudo first order reactions, the slope, *k*, will stay constant regardless of the initial concentration of chemical being studied.

4.3 Results and Discussion

4.3.1 Tetracycline Extraction

4.3.1.1 Extraction According to Lindsey *et al.*

Using the Lindsey *et al.* (1998) procedure, and the Quattro in ES^+ SRM mode, tetracycline extraction efficiencies for distilled, river and pond water were (units of measurements can be either $\mu g\ L^{-1}$ or percent because the waters were fortified to 100 $\mu g\ L^{-1}$) 33 ± 17, 37 ± 16 and 36 ± 5.7, respectively. (The tetracycline standards were also run in ES^+ SRM mode and the addition of tetracycline did not alter the pH of the three waters.) Overall the extraction efficiency was 35% with approximately 65% of the added tetracycline being lost or unrecoverable from the water matrix.

For distilled water, Lindsey *et al.* (1998) reported extraction efficiencies from 80 to 98%. Several reasons can account for the disparity between the present results and that of Lindsey *et al.* First, Lindsey *et al.* used an LC/MS in positive ion mode with cone-induced collision. Under cone-induced collision all of the ions present in the water are induced to undergo fragmentation and ions at m/z ratio 428 and 410 are used for quantitation and confirmation of tetracycline. However, it is possible that some ions may not undergo fragmentation and there may be interferences giving rise to other ions with m/z ratio 428 and 410. With the Quattro in ES^+ SRM mode the 445 molecular ion at m/z is selected and undergoes collision induced dissociation to form product ions 428 and 410, characteristic for tetracycline. The latter method is more selective and less prone to interfering moieties from the matrix when compared to the Lindsey *et al.* LC/MS procedure. For comparison, it was noted that interferences were evident in single ion monitoring (SIM) mode (tetracycline standards also were run in SIM mode).

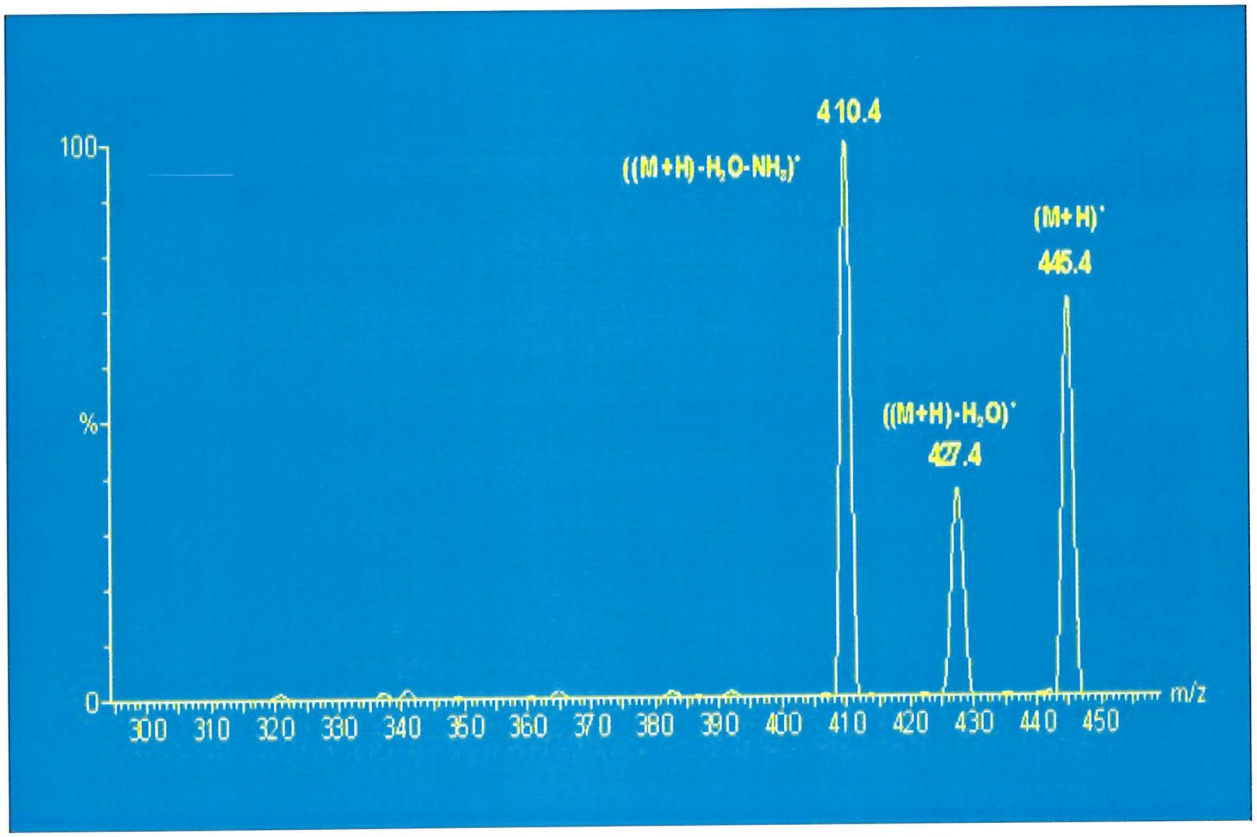


Figure 22. Product ion spectrum of m/z 445.

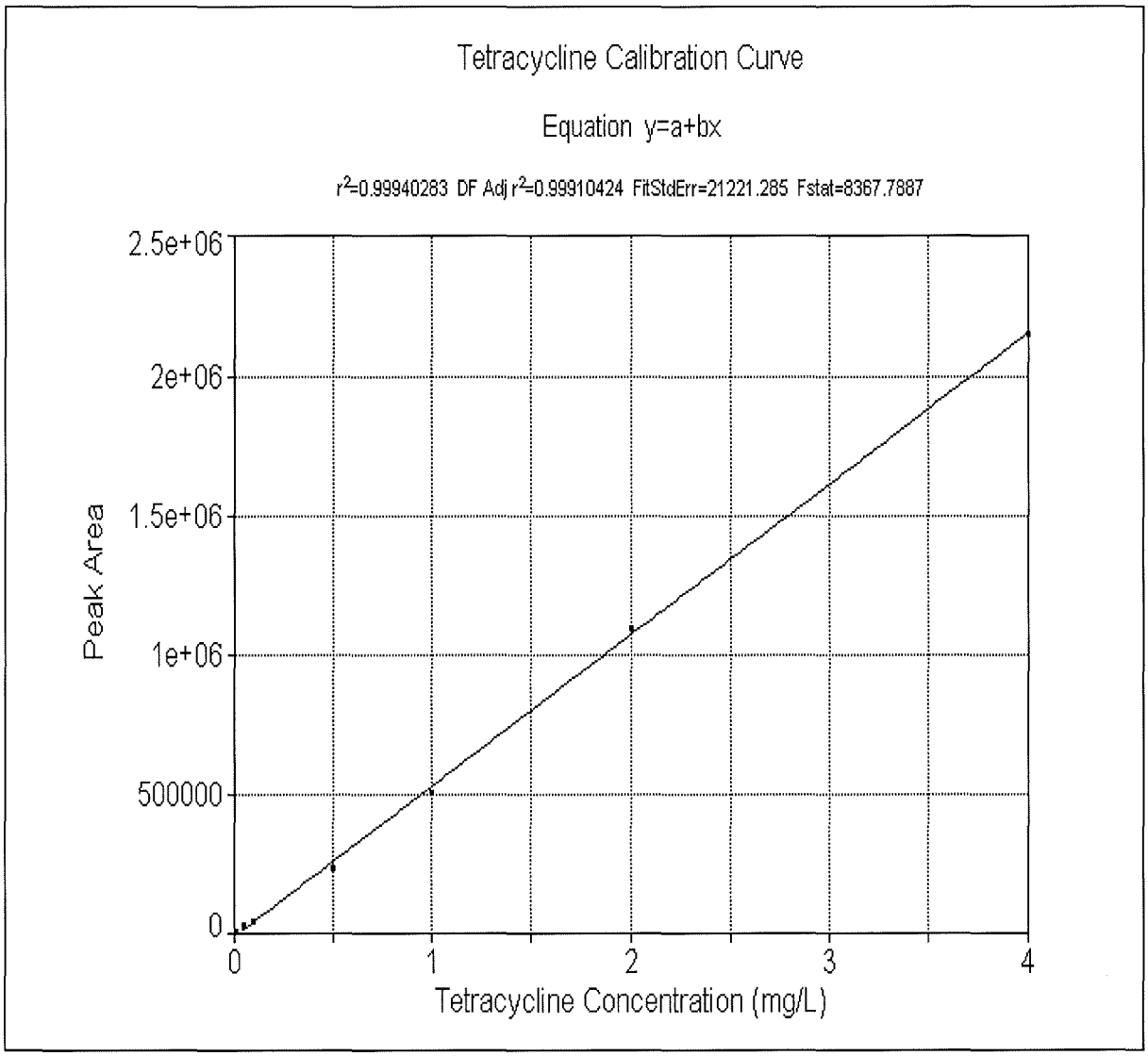


Figure 23. The relationship between peak height and tetracycline concentration. Line represents the linear regression of the data.

Figure 24. Photograph of apparatus used to conduct the tetracycline photolysis in natural sunlight experiment.

Table 8. Water chemistry and bacterial counts (bacteria ml^{-1}) as determined by DAPI and epifluorescent microscopy. N.D. is for non-detectable.

Water	Cl	NO_3	SO_4	pH	Alkalinity	DOC (mg ml^{-1})	DAPI counts before filtration	DAPI counts after filtration
Distilled	N.D.	N.D.	N.D.	7.9	N.D.	0	4.91×10^5	0
SSK	7.50	0.4	71	8.3	96	3.3	5.53×10^6	0
River Pond 1	76.0	1.5	4065	7.5	149	48.8	7.54×10^6	0

In SIM mode, the extraction efficiency in distilled water was approximately 67%, which is closer to that reported by Lindsey *et al.* Indeed, Lindsey *et al.* reported matrix interference for tetracycline spiked ambient water samples. They found that ambient waters spiked at 2 $\mu g\ L^{-1}$ gave, relative to tetracycline standards in distilled water, calculated concentrations of 30 $\mu g\ L^{-1}$. In addition, the nature of the distilled water, whether it was glass distilled or reverse-osmosis generated, can play a significant factor in tetracycline binding in that any impurities in the water can greatly affect the extraction of tetracycline. Lastly, if the extractions were begun immediately after spiking this may result in insufficient time for the tetracycline to interact with the matrix. Lindsey *et al.* did not report the length of time given for tetracycline to interact with distilled water before beginning the extraction procedures.

4.3.1.2 Variations in EDTA and pH to Improve Extraction Efficiencies

4.3.1.2.1 HLB Extractions and Direct-Injections

Because of the very low extraction efficiencies in the initial spiking experiments, the pH and amounts of EDTA added were varied to try to increase tetracycline recoveries from tetracycline amended pond water.

In general, the addition of EDTA (Figures 25 and 26), regardless of the pH of the pond water, nearly always increased tetracycline recoveries compared to when no EDTA was added to the water. There was a statistically significant difference ($P < 0.05$) between no EDTA added versus 200 mg added at pH of both 5 and 8 but not at pH 3 ($P > 0.05$) in the direct injection experiments. In the experiment using HLB extraction the difference between no EDTA and 200 mg EDTA was not significant ($P = 0.8$) due to large data variability, but was significant between 0 and 100 mg ($P < 0.05$) EDTA. This result of EDTA promoting extraction efficiencies of tetracycline is in

agreement with what has been observed by other researchers working with tetracycline in matrices such as water, soil and food (Peterson *et al.* 1993, Lindsey *et al.* 1998, Oka *et al.* 2000, Hamscher *et al.* 2002). The reason for higher extraction efficiencies is because EDTA has a greater tendency to combine with metals than does tetracycline. EDTA out-competes tetracycline by binding to metals, displacing bound tetracycline and therefore increasing the amount of free tetracycline in the water. The results also indicate that a significant portion of tetracycline was bound and not degraded, at least in pH 8 pond water, since the tetracycline recovery doubled, from 25% to 50%, when 200 mg EDTA versus when no EDTA was added in both the HLB and direct-injection studies.

Decreasing the pH of the pond water reduced the amount of tetracycline extracted in both the HLB extraction and in the direct injection experiments (Figure 25 and 26). There was a statistically significant difference ($P = 0.006$) between the extraction efficiencies at pH 3 versus pH 8 when 200 mg EDTA was added in the direct injection experiment but not with HLB extraction ($P > 0.05$) due to large variability of the data in the latter experiment. However, a significant difference in tetracycline extraction ($P = 0.03$) occurred at 40 mg EDTA between pH 3 and pH 8 in the HLB extraction experiment.

There are several possible explanations accounting for the decreasing extractability of tetracycline from acidified pond water. Firstly, tetracycline may be increasingly absorbed to the matrix in the water as the pH decreases. Sithole and Guy (Sithole & Guy 1987a, Sithole & Guy 1987b) have noted that the adsorption of tetracycline to clay and peat increased in acidic conditions but was reduced with increasing pH. This suggests that tetracycline binds to acidic portions of organic material by possibly weak hydrogen bonding since increasing the pH (Figure 27)

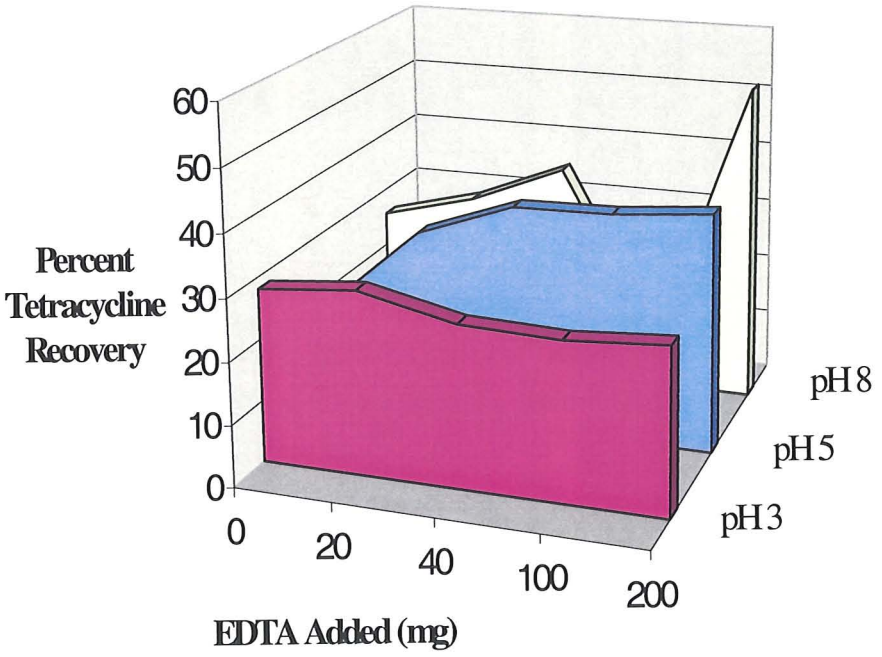


Figure 25. Percent recovery of spiked tetracycline from pond water with variations in EDTA and pH. HLB columns were used for tetracycline extraction.

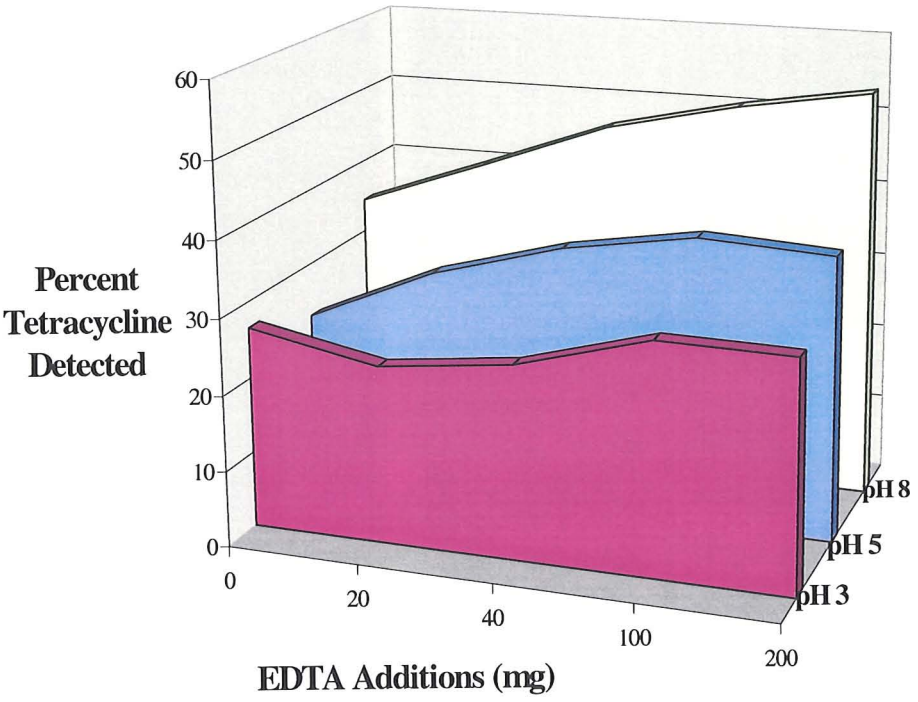


Figure 26. Direct injections into the Quattro Ultima of tetracycline fortified pond water, with variations in pH and EDTA.

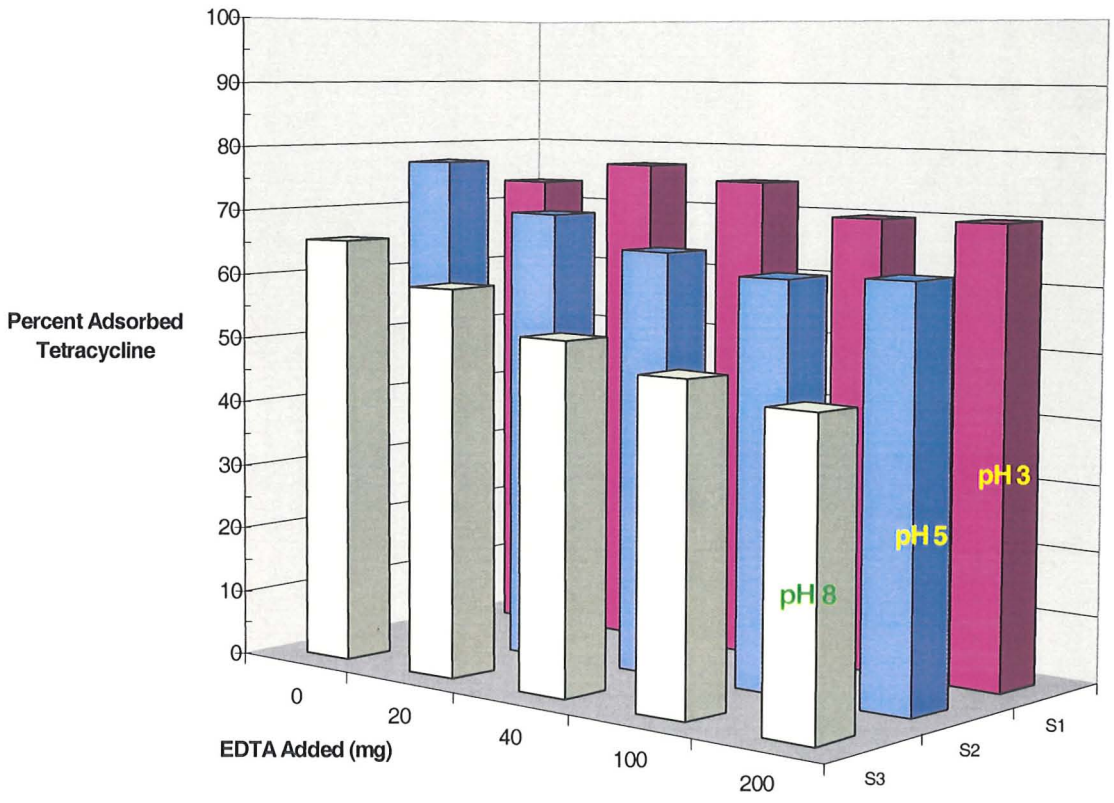


Figure 27. Increased adsorption of tetracycline with decreasing pH in pond water.

results in greater amounts of tetracycline in solution and less being bound to the matrix. Tetracycline binding, then, can be a reversible process and can fluctuate with changes in pH. It may also be that the tetracycline-metal complex becomes stronger in acidic conditions, stronger then the attraction of EDTA to metals which would have out-competed tetracycline in neutral pH pond water. Additionally, tetracycline is known to be stable in neutral solutions (The Merck Index 1983) but highly unstable under very acidic and very alkaline conditions (Oka *et al.* 2000, Halling-Sorensen *et al.* 2002). Tetracycline can reversibly epimerize to 4-epitetracycline, anhydro-tetracycline or iso-tetracycline (Peterson *et al.* 1993, Oka *et al.* 2000) under weakly acidic conditions but at pH < 3 the process is irreversible and tetracycline can become degraded. It is likely that increased binding to the matrix and metals, as well as the degradation of tetracycline at low pH, can account for the results of low extraction efficiencies from pond water with decreasing pH.

It was found, owing to the high selectivity and sensitivity of the Quattro Ultima, combined with the concentrations of tetracycline being used in the experiments, that at concentrations >10 ng ml^{-1} there was no difference in the concentrations of tetracycline detected between the HLB extraction and direct injections of pond water samples (Figure 25 and Figure 26). Therefore, in the photolysis and absorption experiments, water samples were directly injected into the Quattro Ultima without prior extraction or clean up.

4.3.2 Tetracycline Adsorption in Distilled, River and Pond Water

It was evident in the extraction experiments that a small portion of tetracycline was free (25%) and another portion (25%) was extractable by the addition of EDTA, but that a significant portion (≅ 50%) of tetracycline could not be extracted from pond

water. This non-extractable portion of tetracycline could either be bound to the matrix (dissolved organic carbon, proteins, ions) of the water or have become unstable and degraded, though the latter is unlikely since tetracycline is known to be stable in neutral and slightly alkaline solutions. In the tetracycline absorption study (Figures 28 and 29) there was a statistically significant difference ($P < 0.05$) in the concentration of tetracycline detected between distilled, river and pond water. Over the range of concentrations, from 500 $\mu g\ L^{-1}$ to 4000 $\mu g\ L^{-1}$, the spiked amount of tetracycline in distilled water was equal to the concentrations detected. In the river and pond water, approximately 50% and 80%, respectively, of the tetracycline was not detectable. At the pH ranges of the waters in this study (7.5 to 8.3; Table 8), tetracycline degradation due to pH is unlikely since in distilled water, with a pH of 7.9, 100% of the spiked amount was detected.

The nature of the water matrix, then, seems to play a significant role in the binding of tetracycline. The portion of tetracycline that is bound to divalent ions and metals such as Ca^{2+}, Mg^{2+} and Al^{3+} present in the water can largely be extracted by the addition of EDTA. However, tetracycline is known to bind to proteins and silanol (SiOH) groups (Oka *et al.* 2000) as well as to humic and organic acids (Sithole & Guy 1987a, Lindsey *et al.* 1998), all of which substances can be present in surface waters. In distilled water, which is free of ions and DOC (See Table 8) there was no loss of tetracycline to sorption. In the river water with a DOC concentration of 2.4 mg L^{-1}, when 4 mg L^{-1} tetracycline was added, 2.2 mg or about 55% could be detected. In pond water, a complex matrix with a DOC concentration of 50.4 mg L^{-1}, at a spiked concentration of 4.0 mg L^{-1}, only 1.03 mg (about 25 %) could be detected. Not only sorption but also particulate size and quantity in the water may play a role in the amount of tetracycline that is free in solution. Sithole and Guy (Sithole & Guy 1987a)

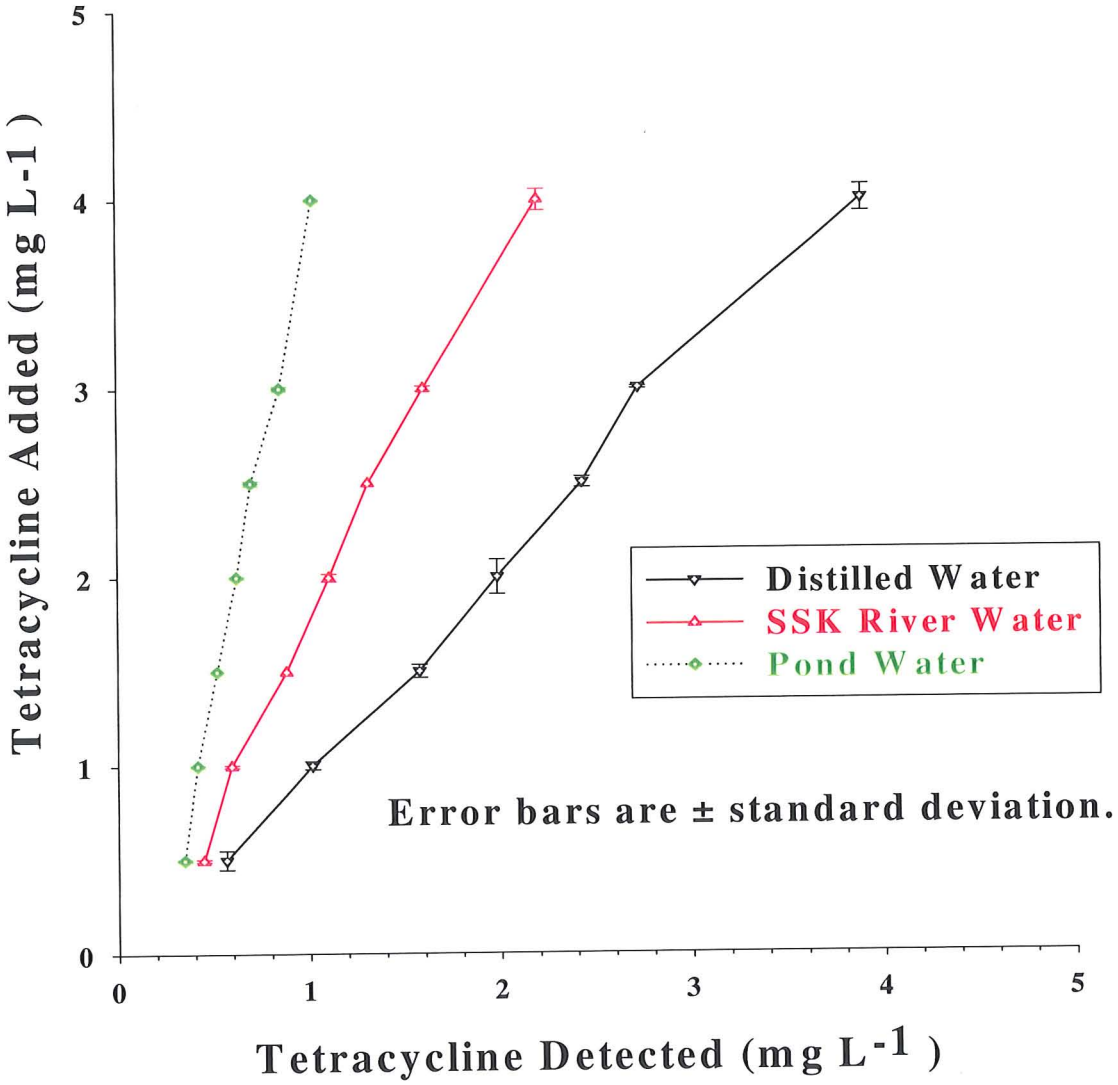


Figure 28. Tetracycline added versus free tetracycline detected in distilled, river and pond water.

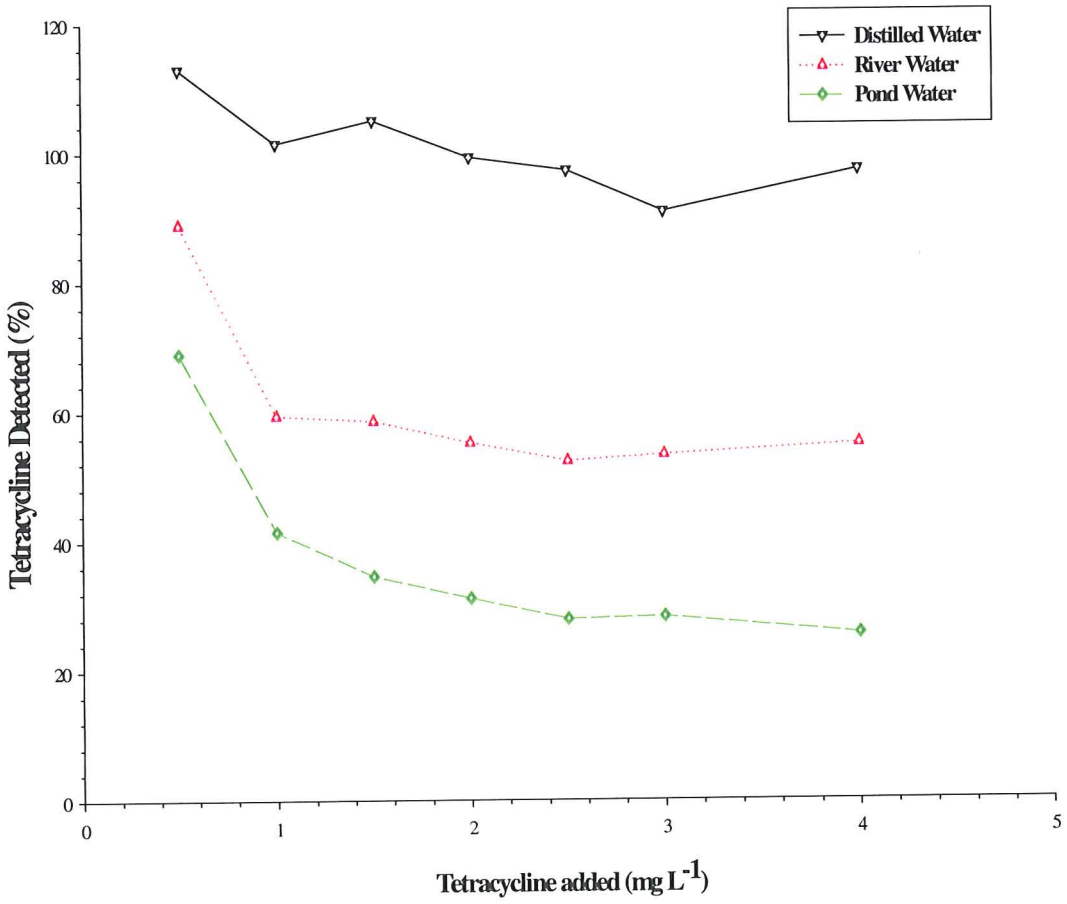


Figure 29. Tetracycline added versus tetracycline detected in distilled, river and pond water expressed in percent recovery.

state that in preliminary studies, 10 to 90% of tetracycline remained in solution if particulates were present at a level of 500 μg ml^{-1} of peat suspension. Tetracycline sorption and desorption to environmental matrices is evidently a complicated topic of research and warrants follow up studies in the future. However, in terms of microbial ecology, the sorption work done here is critical since conventional studies have generally ignored the importance of the free or active portion of antibiotics in natural waters versus the portion of antibiotic that is bound to the matrix and inactive (Flegler *et al.* 1974, DePaola *et al.* 1995, Lanzky & Halling-Sorensen 1997, Al Ahmad *et al.* 1999, Ingerslev *et al.* 2001, Halling-Sorensen *et al.* 2002). In the case of tetracycline, bound tetracycline is perceived to be inactive against microorganisms (Halling-Sorensen *et al.* 2002). Therefore, when tetracycline is spiked at, say, 4 mg L^{-1} into water, the portion of tetracycline that is active against microorganisms susceptible to tetracycline in the river water, based on the results from the adsorption study, is 2.2 mg L^{-1} while in pond water it would be 1.03 mg L^{-1}. Furthermore, in chemical degradation studies, adsorption of the chemical to the matrix is often not taken into account and the portion bound is included in the total degraded. For example, in the Kuhne *et al.* (2000) study of the stability of tetracycline in manure and Ringers solution, tetracycline concentrations were taken the day following tetracycline spiking and the bound portion of tetracycline to the matrix was included in the amount degraded.

4.3.3 Tetracycline Photolysis in the Laboratory and Natural Sunlight in Sterile and Non-sterile Distilled, River and Pond Water

The half lives and decay rates of tetracycline in all three waters in both the laboratory as well as in natural sunlight indicated that tetracycline undergoes pseudo first order degradation (Figures 30 and 31). In both the non-sterile and sterile laboratory

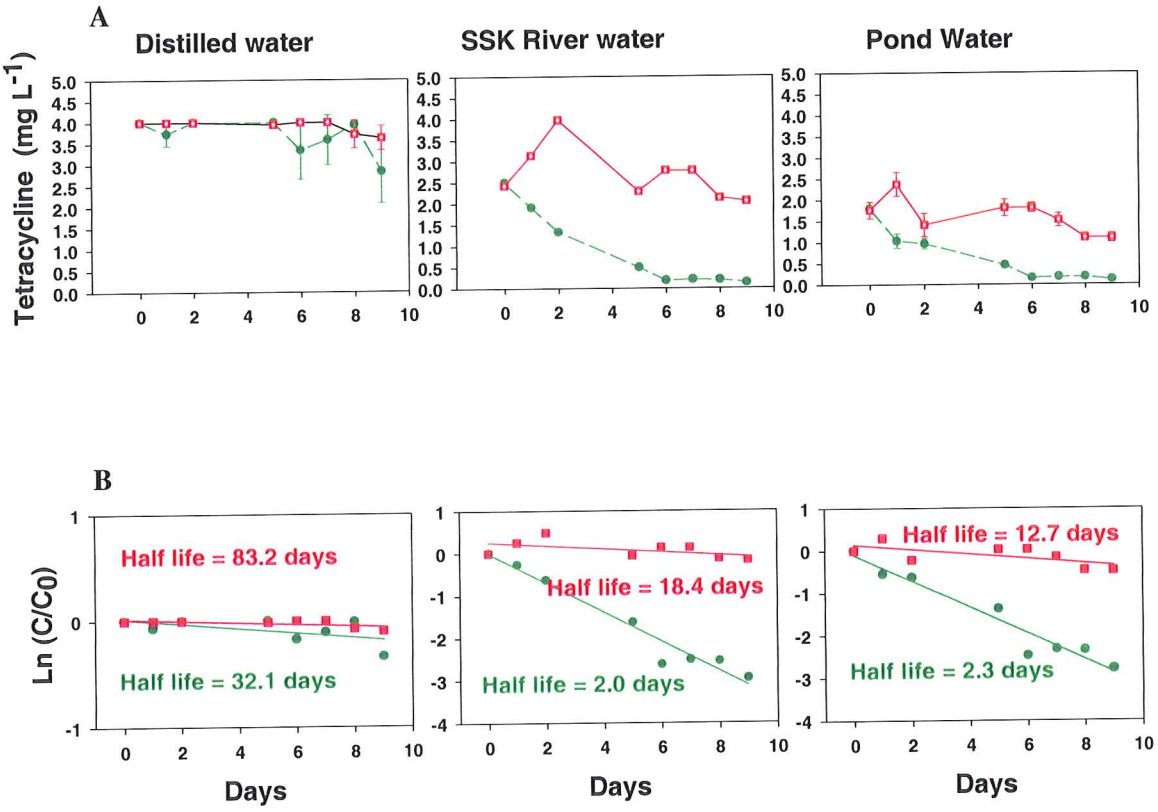


Figure 30. The effect of laboratory light on the change in concentration (mg L^{-1}) of tetracycline in non-sterile distilled, river and pond water over 9 days (A) while (B) is the natural log of C/C_O with regression line. Green is the L^+ treatment while red is L^-. Error bars are ± standard deviation.

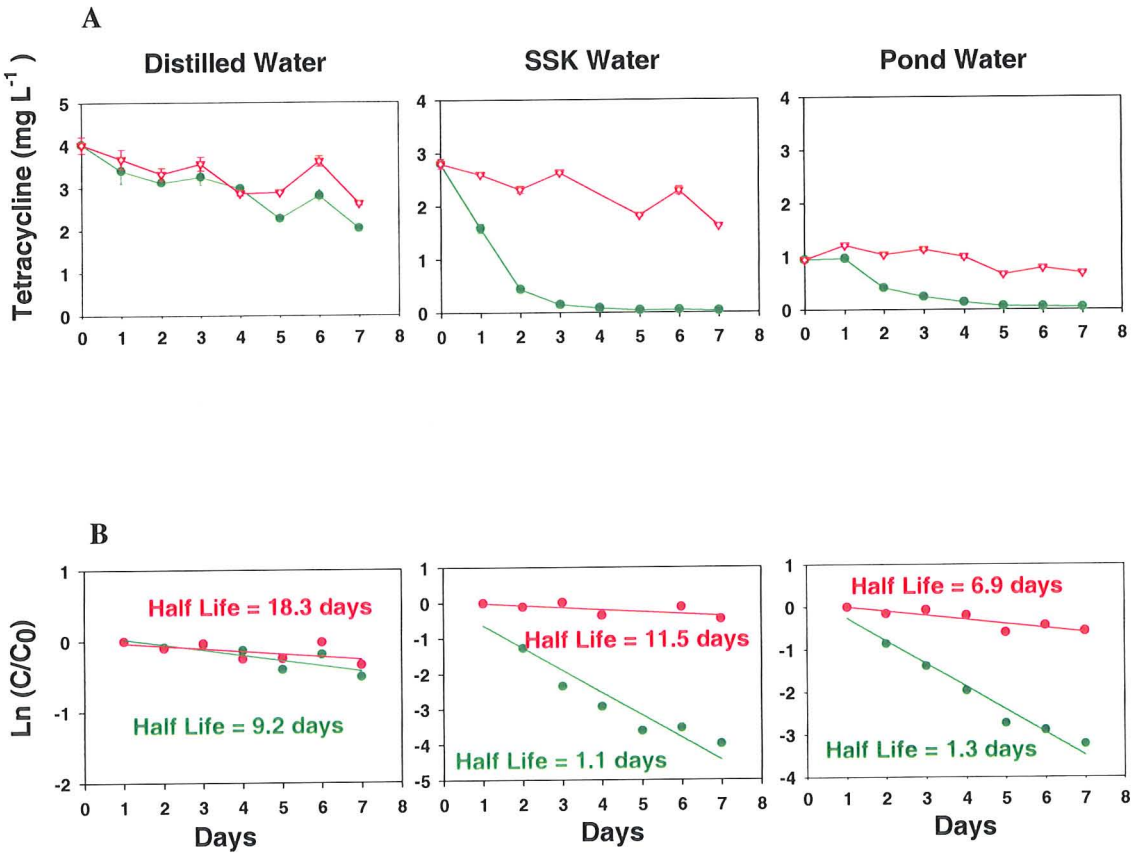


Figure 31 The effect of laboratory light on the change in concentration (mg L^{-1}) of tetracycline in sterile distilled, river and pond water over 7 days (A) while (B) is the natural log of C/C_0 with regression line. Green is the L^+ treatment while red is L^-. Error bars are ± standard deviation.

photolysis experiments there was a statistically significant difference between the L^+ and L^- treatments ($P < 0.05$) and between the different water treatments ($P < 0.05$). However, there was not a significant difference between the L^+ and L^- in non-sterile distilled water due to large variation in data. There was also a significant difference ($P < 0.05$) between the treatments in the sterile versus the treatments in the non-sterile experiments (e.g., non-sterile river L^+ versus sterile river water L^+). But the difference was not significant ($P > 0.05$) between the L^+ non-sterile distilled water treatment versus L^+ sterile distilled water due to large data variability.

The half-lives of tetracycline in the non-sterile waters were consistently shorter in L^+ treatments, which were 32, 2 and 3 days in distilled and river and pond water respectively, than in the L^- where they were 83, 18 and 13 days in distilled, river and pond water, respectively. Similarly, the half-life of tetracycline in sterile waters was consistently shorter in L^+ treatments where it was 9, 1 and 1 day for distilled, river and pond water, respectively, than in the L^- where it was 18, 11 and 7 days in distilled, river and pond water, respectively. This corroborates the findings of other researchers who have studied tetracycline degradation in sea water in that tetracycline is light sensitive and undergoes photolytic degradation (Oka *et al.* 1989, Peterson *et al.* 1993). Tetracycline half-lives in both the sterile and non-sterile treatments were significantly shorter in the river and pond water as compared to distilled water. This suggests that factors other than photolysis may play a role in tetracycline degradation. Microbial degradation, at least at the 4 mg L^{-1} tetracycline concentration in the present study, is unlikely since the half-lives in sterile waters were significantly shorter than the half-lives in non-sterile waters. It is possible, however, that the high tetracycline concentration in the laboratory photolysis experiments may have completely debilitated the microorganisms in the three waters and that microbial degradation may play a role

at a tetracycline concentration < 4 mg L^{-1}. Additionally, the use of ^{14}C labeled tetracycline is unlikely to determine if tetracycline is being microbially degraded unless the rate of degradation progresses more quickly than photolytic rates. This is because photolysis of tetracycline would result in ^{14}C metabolites being produced that may be consumed rather than the parent tetracycline molecule. Furthermore, to date there has been no description in the literature of bacteria or a consortium of bacteria that can utilize the parent tetracycline as a carbon source (Chopra & Roberts 2001). It is likely the complex matrix of river and pond water is responsible for the shorter half-lives observed in these waters relative to distilled water.

Solar radiation is not only attenuated by DOC compounds in wetland and river waters (Arts *et al.* 2000) but the radiation also helps to break down the DOC's into smaller constituents (Waiser 2001b). This breakdown of DOC's could lead to releases of energy plus the formation of $^{\cdot}OH$ and peroxide free radicals as well as excited singlet, doublet or triplet states of oxygen and DOC's. It is thought that DOC's, when irradiated with light radiation, besides producing radicals under certain conditions, do not become ionised (loss or gain of an electron) but rather become energized and unstable and this may then catalyse the removal of organics in DOC water compared to water with no DOC's. Gerecke *et al.* (Gerecke *et al.* 2001) studied the photolytic removal of the organic phenylurea herbicides in surface waters in the presence or absence of DOC's and found, similar to my tetracycline photolysis study, faster degradation rates in water containing DOC's. Gerecke *et al.* (2001) also varied the concentrations of DOC's from 0 to 4 mg L^{-1} in their experiments and found that the half-lives were linear and shorter with increasing DOC concentration in the water. To distinguish if the faster degradation rates observed were due to excited oxygen or excited DOC's, Gerecke *et al.* (2001) used D_2O and azide to quench O_2 and thus were

able to attribute the faster decay rates of phenylurea as likely to have been catalysed by energized DOC's. A recent study by Lam *et al.* (Lam *et al.* 2003) looked at the effects of varying concentrations of DOC's, nitrate and carbonates, both of the latter compounds are able to produce $^{\cdot}OH$ radicals, to photolytic decay rates, of pesticides such as atrazine and pharmaceuticals such as ciprofloxacin and clofibric acid. The pesticides and pharmaceuticals were irradiated, in water, in a photosimulator mimicking natural sunlight. Lam *et al.* (2003) found that reactions mediated by $^{\cdot}OH$ radicals were predominant in waters with high nitrate concentrations while in low nitrate waters DOC's played a more significant role in the photolytic removal of organic contaminants. Carbonates in the water matrix of the Lam study, conversely, led to longer half-lives of contaminants and this was attributed to the carbonates scavenging $^{\cdot}OH$ radicals from the water. They also found that under certain conditions, DOC's acted as a radiation filter and played a more important role in scavenging radicals rather than in radical production thereby leading to slower decay rates of contaminants studied. This latter observation may be the reason why in my study tetracycline breakdown was slower in pond water with $\cong$ 50 mg L^{-1} DOC's relative to river water with $\cong$ 3 mg L^{-1} DOC's. Depending on the concentration of the DOC's along with other ions in the water, the DOC's can both promote as well as slow the photolytic decay rates of organic contaminants. Lastly, tetracycline breakdown in the present study was slower in non-sterile waters relative to sterile waters and this may have been due to the macroscopic particles in the non-filtered water acting as a shield protecting tetracycline from the light. Thus, it can be seen that the removal or persistence of organic contaminants from surface waters is a complex process and will likely vary with varying concentrations of DOC's, ions and particulate matter present in the waters matrix.

The results from the laboratory tetracycline photolysis experiments compare favourably with that of other researchers, though comparisons are not entirely valid due to significant differences in the nature of apparatus, the matrixes of the water as well as radiation flux acting on the experiment. Nevertheless, Oka (1989), looking at the decomposition products of tetracycline in seawater when irradiated with UV light, found a 50% decrease in tetracycline concentration after 300 minutes of irradiation, which is considerably slower than the rates of tetracycline loss in the sunlight experiment presented here (Figure 32). Peterson *et al.* (1993), meanwhile, found a tetracycline half-life to be 120 h in seawater. As a general comparison to other antibiotics, Al-Ahmad *et al.* (1999) and Kummerer *et al.* (2000) both studied ciprofloxacin, a fluoroquinolone, bio-degradation in a mineral salts media over 40 days. Both studies found no decrease in the concentration of ciprofloxacin after 40 days and determined that ciprofloxacin was unlikely to biodegrade easily. Ingerslev *et al.* (2001), meanwhile, using 100 μm filtered lake water and working at 15°C in a laboratory under "diffuse light" conditions, determined the half-life of tylosin, an antibiotic used in animal therapy as well as a prophylactic, to be 40 days.

It is surprising, considering the importance of solar radiation in energizing the degradation of organic compounds in the environment, that of the few studies examining antibiotic degradation in surface waters and manure, no experiment has been conducted under natural sunlight conditions (Al Ahmad *et al.* 1999, Ingerslev & Halling-Sorensen B. 2000, Kuhne *et al.* 2000, Kummerer *et al.* 2000, Ingerslev *et al.* 2001), although Oka (1989) and Lam *et al.* (2003), in both of their studies, used a photosimulator mimicking natural solar radiation. In the present solar radiation experiment the degradation rates of tetracycline in the three different waters followed pseudo first order kinetics (Figure 32). There were statistically significant differences

between the UV^+ and UV^- light treatments ($P < 0.05$) with shorter half-lives in the UV^+ treatments, as well as among the three different waters ($P < 0.05$). Tetracycline half-lives in the UV^+ treatment were 26, 17 and 18 min in distilled, river and pond water compared to 39, 28 and 32 min in the UV^- treatment in distilled, river and pond water, respectively. The faster decay in the UV^+ treatment relative to UV^- shows that UV radiation played a significant role in catalyzing the removal of the parent tetracycline from all three different waters. As further evidence for the role of UV radiation, absorbance measurements of river and pond water spiked with tetracycline showed increased absorbance of radiation < 400 nm (Figure 33) relative to river and pond water without tetracycline. Tetracycline is known to absorb radiation in the UV portion of the spectrum with UV maxima occurring at 220, 268 and 355 nm in 0.1 N HCl (The Merck Index 1983). As in the laboratory experiments, degradation was faster in river and pond water compared with distilled water. This suggests the water matrix is a significant factor in affecting the rates of tetracycline decay, as discussed above. The half-lives of tetracycline in the sunlight were considerably shorter than the half-lives observed in the laboratory and can be accounted for by the greater intensity ($\cong$ 80:1) of the sunlight radiation versus that of fluorescent lighting in the laboratory.

The results from the present solar radiation study are similar to that of Lam *et al.* in that in their simulated solar radiation study the half-life of ciprofloxacin was 13 minutes in deionised water. The half-life of tetracycline in distilled water in my study was 26 minutes. It should be noted, however, that Lam *et al.* (2003) used deionised water and carefully controlled the concentrations of DOC, nitrate and carbonate and did not use natural surface waters. It is interesting to note that two previous researchers, Al-Ahmad *et al.* (1999) and Kummerer *et al.* (2000), found no decrease in the

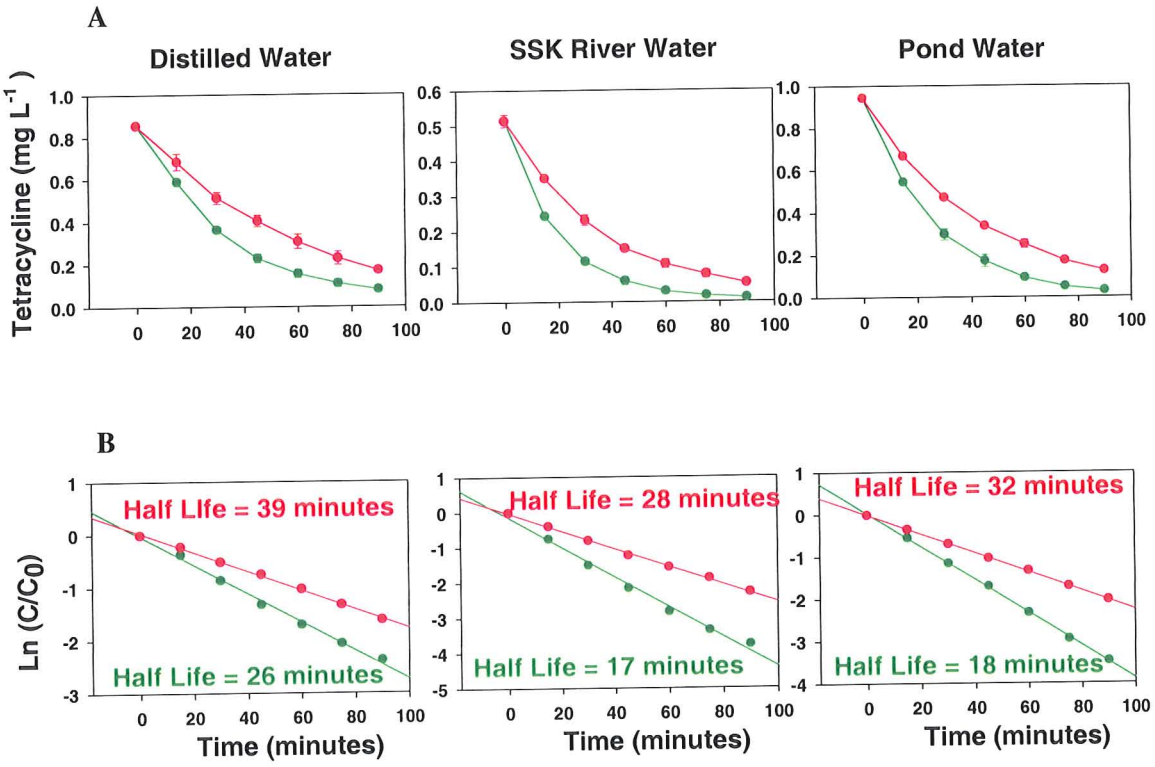


Figure 32 The effect of natural light on the change in concentration (mg L^{-1}) of tetracycline in sterile distilled, river and pond undergoing photolysis (A) while (B) is the natural log of C/C_O with regression line. Green is the UV^+ treatment while red is UV^-. Error bars are ± standard deviation.

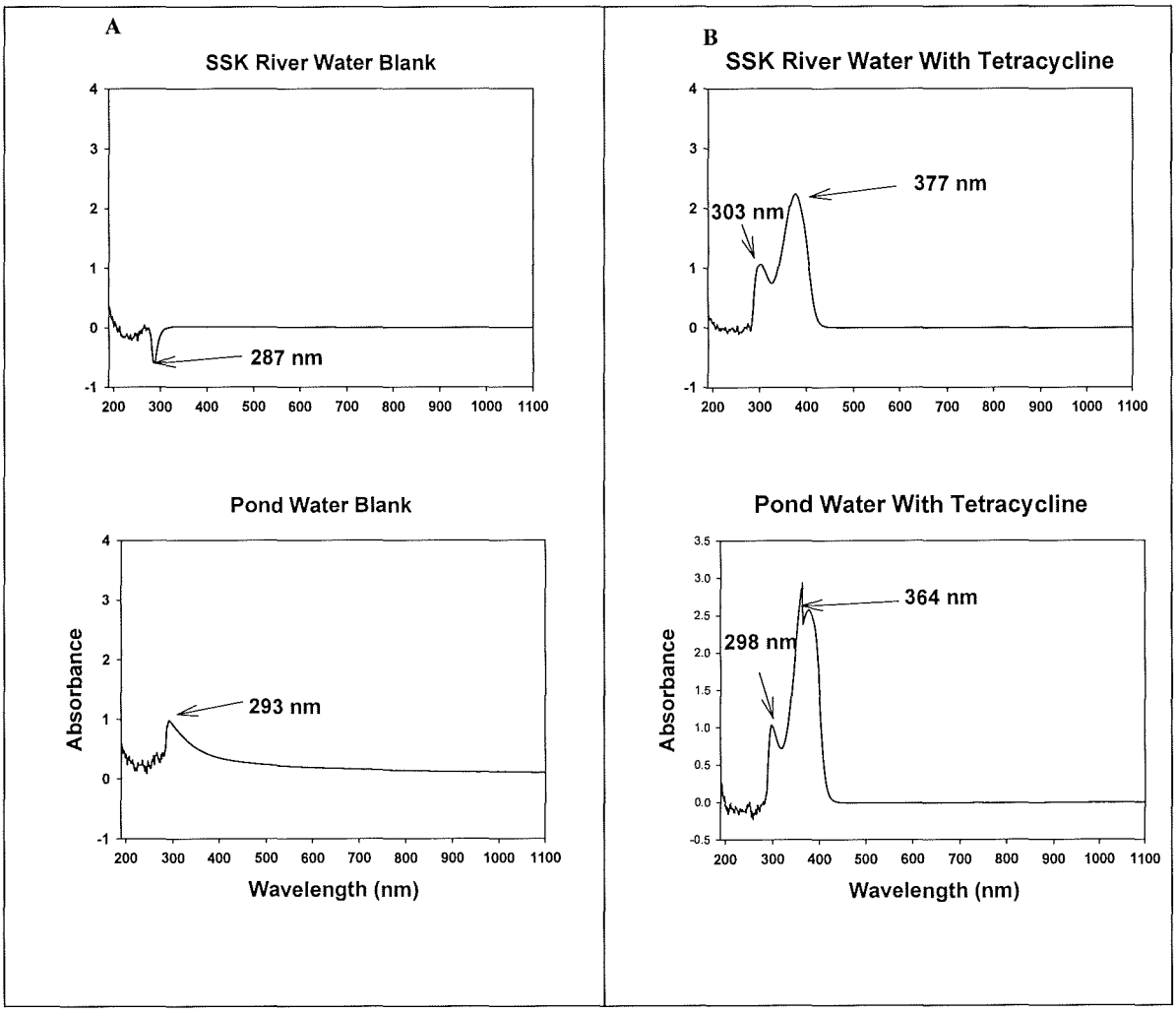


Figure 33. Absorbance measurements versus wavelength (nm) of river and pond water without tetracycline (A) while (B) are absorbance measurements of water with added tetracycline. Absorbance peaks are labeled.

concentration of ciprofloxacin over forty days but that ciprofloxacin was rapidly degraded in the Lam study. Additionally, Winkler *et al.* (Winckler *et al.* 2001) found no degradation of clofibric acid over 21 days in a study conducted in laboratory light conditions whereas Lam *et al.* (2003) found the half-life of clofibric acid to be 19 hours in their study. Both of these results point to the need to conduct experiments in as environmentally relevant a manner as possible as well as the difficulties in comparing results between studies.

In both the laboratory and solar radiation experiments, it was noted that a significant amount of tetracycline was bound to the matrix and seemed to be unaffected by solar radiation, thus the decay rates observed are only for the tetracycline in free form in the water. Though tetracycline is photosensitive, the formation of a tetracycline-matrix complex seems to result in a decrease or even cessation of tetracycline photolysis. Hamscher (2002) reported the persistence over two years of tetracycline in the soil of an agricultural field on which tetracycline laden manure had been sprayed. The direct occurrence of solar radiation on the surface of the soil did not degrade tetracycline. Similarly, in deep waters and in ones where sunlight is highly attenuated by DOC's, as occurs in prairie wetlands (Arts *et al.* 2000), the effects of light on tetracycline is likely to be considerably minimized.

In the background controls of both river and pond water in almost all of the experiments, trace levels of tetracycline were detected. This points to the essential need to run background controls of both water and soil samples in any experiment where antibiotics are being studied in the environment. Unfortunately, it has been almost universally assumed that antibiotics are anthropogenic in terrestrial or aquatic systems and do not occur in pristine environments. However, the detection of tetracycline should not be too surprising since originally tetracycline was isolated from the soil

bacteria *Streptomyces aureofaciens* and later from other *Streptomyces* species, *S. rimosus and S. viridofaciens* (Chopra & Roberts 2001). In fact, 70% of all antibiotics currently in therapeutic use are produced by actinomycetes, a group of bacteria commonly found in soil (Mincer *et al.* 2002) while a smaller proportion of antimicrobials, such as fluoroquinolones and sulfonamides, are synthetic and do not occur naturally (Nester *et al.* 1998). Antibiotics, including tetracycline, have been known to be naturally produced at 1000 to 10000 $\mu g\ L^{-1}$ concentrations in soils (Martin & Gottlieb 1952, Sithole & Guy 1987a, Sithole & Guy 1987b). The tetracycline detected is most likely to be naturally occurring, however it may also arise from sources such as livestock manure runoff and sewage effluents. It may be possible, using stable isotope signatures, to differentiate between antibiotics produced industrially by batch fermentation versus those that occur naturally in the environment. Naturally occurring antibiotics, like tetracycline, chloramphenicol and penicillin, are usually mass produced in fermentation vats by fungi or bacteria that are fed a consistent carbon source (Heggie 2001). The relatively consistent carbon source utilised by these microorganisms may result in specific C^{13}/C^{12} carbon ratios in the antibiotic molecule. Antibiotics that are naturally produced in the environment, however, may have a different C^{13}/C^{12} ratio because the carbon source utilized by the naturally occurring microorganisms may be distinct from that utilized by microorganisms in industry. It may be argued that bacteria and fungi consume varying fractions of C^{13} and C^{12} in the exponential growth stage as compared to stationary stage (Hall *et al.* 1999, Henn *et al.* 2002) and this may seem to lead to an inconsistent C^{13}/C^{12} carbon ratio. However, antibiotics are produced by microorganisms in the stationary growth phase (Nester *et al.* 1998) when a consistent C fraction is utilized and thus the stable carbon isotope signature of the antibiotic should remain consistent. Nevertheless, it is essential to run

background controls in any experiments dealing with antibiotics that may be naturally produced since not all antibiotics are anthropogenic to soil or aquatic environments.

4.4 Conclusions

Tetracycline extraction efficiencies from spiked distilled, river and pond water were significantly increased by the addition of EDTA. Low pH decreased the extraction efficiencies of tetracycline possibly by increasing the adsorption of tetracycline to the matrix and EDTA. It was also found that pre-extraction of tetracycline from aquatic matrices is unnecessary, when using the LC/MS/MS, if the tetracycline concentrations >10 $\mu g\ L^{-1}$. Additionally, the result from the adsorption study indicated that a significant portion of tetracycline is bound to the matrix in the water and, though present, was not extracted by pH and EDTA adjustments. Light, in combination with the matrix of the water, plays a significant role in the breakdown of tetracycline while microbial degradation is unlikely. In natural sunlight, UV radiation plays a significant role in catalyzing the removal of the parent form of tetracycline from different waters. However, it is noted that a significant amount of tetracycline is bound to the matrix. Thus the decay rates observed are only for the tetracycline in free form. Bound tetracycline seems to be unaffected by light radiation and therefore tetracycline is likely to persist in aquatic environments. The effects of light on tetracycline may be considerably minimized in deep waters and in shallow waters with high DOC's where sunlight is highly attenuated. It is evident from the work conducted here that the absorptive and photolytic behaviour of tetracycline in different waters is extremely complex and requires further study. The nature of the absorptive and photolytic behaviour of tetracycline should be carefully considered in studies measuring the

effects of tetracycline on microbial populations and microbial processes such as nitrification and decomposition.

CHAPTER 5

MICROBIAL COLONIZATION OF *SCIRPUS* UPON WETTING, IN THE PRESENCE AND ABSENCE OF TETRACYCLINE, IN RIVER AND POND WATER

5.1 INTRODUCTION

Microorganisms are central to the existence of life on earth. The process of decomposition, mediated by both fungi and bacteria, is an essential component of the carbon cycle because otherwise carbon would forever be locked in dead organic matter. Decomposition is simply the movement of carbon and other nutrients from dead organic matter to living fungal and bacterial communities that in turn are used as sources of carbon and nutrients by organisms at the same or higher trophic levels. Anthropogenic pollutants damaging to either bacteria or fungi may therefore seriously damage the flow of carbon and thus could seriously disrupt ecosystem function and structure.

The influence on aquatic bacterial communities by anthropogenic organic pollutants such as pesticides have, and continue to be, extensively studied (Wolfaardt *et al.* 1995, Lawrence *et al.* 1998). There are many studies that have looked at the rise and decline in bacterial resistance in the presence of antibiotics (DePaola *et al.* 1995, Goni-Urriza *et al.* 2000, Mazel & Davis 2003, Sengelov *et al.* 2003) but the effects of antibiotics, until recently not considered to be ecologically damaging despite over one million tonnes of antibiotics released into the biosphere annually (Mazel & Davis 2003), on terrestrial or aquatic microbial processes such as soil nitrification and decomposition, do not exist (Halling-Sorensen *et al.* 1998, Daughton & Ternes 1999). These inflows of antibiotics into aquatic systems by runoff from fields sprayed with manure (Hamscher *et al.* 2002) or from urban effluent (Halling-Sorensen *et al.* 1998,

Heberer 2002) may have profound consequences to microorganisms and carbon cycling since the very nature of antibiotics is to limit microbial growth.

There have been a number of studies that have studied trends in fungal and bacterial colonization of plant litter once a plant collapses and enters the aquatic environment (Baldy *et al.* 1995, Suberkropp & Weyers 1996, Kuehn *et al.* 2000, Baldy *et al.* 2002, Hieber & Gessner 2002) utilizing DAPI, a staining dye which fluoresces when it binds to nuclear DNA or ^{3}H-leucine incorporation to estimate bacterial biomass and production, respectively, or ergosterol and [1-^{14}C] acetate to estimate fungal biomass and production, respectively. These studies have shown an initial decline, after submersion, in both bacterial and fungal biomass followed by an increase in fungal and bacterial biomass a considerable time after submersion. The initial decrease in microbial biomass has been attributed to the unsuitability of the aquatic environment to terrestrial microbes and the later surge in microbial activity as being due to a re-colonization of litter by aquatic microbial assemblages. However, there have not been any studies that have precisely followed the microbial process that occurs immediately after dry plant litter falls into water.

In natural aquatic systems sessile (attached) bacteria vastly outnumber planktonic (suspended) bacteria (Costerton *et al.* 1995, Dunne Jr. 2002). Suspended bacteria are generally thought to be more susceptible to antimicrobial compounds compared to sessile bacteria (Ceri *et al.* 1999, Wolfaardt *et al.* 1999, Aaron *et al.* 2002). Bacteria may attach to a surface by way of extracellular polymeric substance (EPS) and a community of a single or many strains of bacterium attached to a surface are often enclosed in the EPS and constitute a biofilm (Costerton *et al.* 1995, Wolfaardt *et al.* 1999, Dunne Jr. 2002). A biofilm is thought to afford considerable advantages, such as

aiding in the transfer of genetic material, protection against enzymes and antimicrobial substances, to its bacterial constituents (Wolfaardt *et al.* 1999). A biofilm, though, can also be a liability since it is a concentrated and rich source of carbon and nutrients to grazers that can feed on it. In nature, however, a biofilm is rarely composed of only bacterial cells and a biofilm is likely to be comprised, depending on the surface to which it is attached, of communities of different bacteria, fungi, algae, viruses and other microbial organisms.

Confocal laser microscopy (CLSM), in conjunction with flourscent dyes, has been utilised to study biofilms on a variety of surfaces such as plastics, metals and soils (Caldwell *et al.* 1992, Wolfaardt *et al.* 1995, Lawrence *et al.* 1997, Lawrence *et al.* 1998). Some of the advantages of using CLSM are that (1) the biofilm can be scanned non-destructively, (2) besides scanning the surface, optical sections, along the z-axis, of the interior of a biofilm can also be visualized, (3) the same treatment can be consecutively scanned, (4) there are no requirements for extensive sample preparations, extraction and instrumental analysis and lastly, (5) the scanned images can be saved in a computer and analysed for further study. Laser microscopy, in conjunction with molecular probes, therefore, is an attractive and heretofore untried method that can help to visually follow the pattern of colonization once a plant enters the aquatic environment from a standing position.

In decaying aquatic litter fungi have been found to account from 63% to 99% of the total microbial biomass and production (Newell *et al.* 1995, Suberkropp & Weyers 1996, Kominkova *et al.* 2000, Kuehn *et al.* 2000, Hieber & Gessner 2002, Verma *et al.* 2003). The variability in fungal dominance may be due to the nature and carbon content

of the plant substrate (Gessner & Chauvet 1994, Kominkova *et al.* 2000, Gulis 2001), nature of the aquatic system as well as seasonal variances or because of bacterial competition and inhibition (Flegle *et al.* 1974, Gulis & Stephanovich 1999, Moller *et al.* 1999, Gulis & Suberkropp 2003). Some of the methods previously tried to determine the proportions of fungal and bacterial activity in litter have included the adding of selective inhibitors such as antibiotics and antifungals and measuring the resulting effect on bacterial and fungal biomass or respiration (Flegle *et al.* 1974, Padgett 1993). Though these studies have not always been successful, they have been able to provide indirect evidence of fungal inhibition of bacteria and bacterial inhibition of fungi in decaying litter in aquatic environments.

No study has examined in detail the pattern of fungal and bacterial colonization that occurs once a plant dislodges and enters an aquatic environment. Additionally, there have been no previous studies that have utilized CLSM to study aquatic litter decomposition. The colonizing behaviour of fungi and bacteria on the hardstem bulrush *Scirpus lacustris* once it begins to decay in river and wetland water were experimentally studied. After a plant dislodges and falls into the aquatic environment, anthropogenic pollutants may disrupt microbial colonization of the plant. There have been no previous studies that have examined the effects of antibiotics on an integral environmental process such as decomposition. Therefore the effects of 500 and 4000 ug L^{-1} tetracycline on the fungal and bacterial constituents colonizing the surface of *Scirpus* stems decaying in tetracycline spiked river and pond water were experimentally investigated. Lastly, the effect of tetracycline on ^{3}H-leucine incorporation into the protein of planktonic bacterial communities was measured.

5.2 Materials and Methods

5.2.1 Water and *Scirpus* Collection

River water was obtained from the South Saskatchewan River, Saskatoon, Saskatchewan, Canada (106° 38'W, 52° 07'N) and pond water was obtained from Pond 1 situated at the St. Denis National Wildlife Area (See Figure 2b, Chapter 2). Water from these sites was collected in 10 and 20 L plastic containers, stored at room temperature and allowed to acclimate to laboratory conditions before being used in experiments. Standing, dry, dead *Scirpus* stems from the previous year's (2001) stand were collected from the periphery of Pond 50 and brought to the laboratory where they were kept at room temperature prior to being used in experiments.

5.2.2 Effects of Tetracycline on Protein Production in Planktonic Bacteria

The effects of 0, 1, 10, 100, 1000 and 4000 μg L^{-1} tetracycline on bacterial protein production in river and pond water planktonic bacteria was evaluated by measuring the relative incorporation of L-[4,5-^{3}H]leucine (Amersham Biotech, Montreal, Canada), specific activity of 161 Ci $mmol^{-1}$, into protein according to Wicks and Robarts (Wicks & Robarts 1988). Stock ^{3}H-leucine was diluted by 10X with sterile distilled water and 50 μl of the diluted solution was pipetted into a sterile 10 ml Vacutainer glass tube (Becton Dickinson, N.J, U.S.A.) that held 1 ml of 1.2 μm filtered river or pond water. The tube was gently swirled and incubated for 30 minutes at room temperature (21 ± 3°C). To stop the incubation, 60 μl of ice-cold trichloroacetic acid (TCA) was pipetted into the tube, the contents swirled and the tube was allowed to stand for 15 minutes. The content of the incubation tube was filtered through a 25 mm diameter, 0.2 μm pore size Sartorius (Edgewood, N.Y.) cellulose nitrate filter. The incubation tube was rinsed

three times with 1 ml of 5% ice-cold TCA and finally with 2 ml of 80% ethanol. Unincorporated leucine was washed from the filter with 2 ml of fresh 5% ice-cold TCA. The filter was placed in a scintillation vial and 5 ml of Filter Count (Canberra Packard, Canada) was added. To completely dissolve the filter the scintillation vial was vortexed for five to ten mins. The numbers of disintegrations per minute (DPM) in each vial was determined with a Packard scintillation counter by using an external standard. The experiment was done in triplicate and all of the containers, pipettes and filters were sterilized by autoclaving. Two killed controls (addition of formalin to a final concentration of 4%) were included in both experiments. The mean DPM in the killed controls was subtracted from the mean DPM in the treatments.

5.2.3 The effect of tetracycline on microbial colonization on the surface of *Scirpus* decaying in river and pond water.

The experimental apparatus (Figure 34) consisted of a 1 or 2 L stock container, silicon tubing (2 mm inner diameter (Fischer Scientific, Canada)) and a glass tube (10 mm diameter). A Watson Marlow (Cornwall, U.K.) peristaltic 16 channel pump was used to deliver the river and pond water from the stock containers to the glass tubes that held the *Scirpus* stems. The flasks and tubing were sterilized by autoclaving prior to being used in the experiments.

Experiments were conducted to study the microbial colonization behaviour on standing dead *Scirpus* stems immediately after the stems were submerged in pond and river water in the presence or absence of tetracycline. Twelve *Scirpus* stems $\cong$ 6 mm in diameter were selected and cut to 14 cm lengths.

Figure 34. Photograph of apparatus used to determine the colonizing behaviour, over 33 days, of bacteria and fungi on the surface of previously dry *Scirpus* stems after they were introduced to natural and tetracycline-spiked river and pond water. Note the *Scirpus* stems in glass tubes in the foreground.

The first experiment, with river water, consisted of three duplicate treatments: the control treatment was river water without amendments while the second and third treatments were river water spiked with tetracycline at 500 and 4000 μg L^{-1}, respectively. The second experiment was the same as the first except that Pond 1 water was used. In both experiments waters was run through the experimental apparatus for three days prior to the introduction of stems so as to allow tetracycline, in the tetracycline treatments, to bind to any active sites in the experimental apparatus. The flow rate of the water was 6 ml h^{-1} for the 33 days the experiments were run. Every 24 hours the flasks were emptied and fresh water introduced. Tetracycline was filtered through 0.2 μm pore-size filters prior to being pipetted into the flasks, water added and the contents swirled to ensure a uniform distribution of tetracycline.

5.2.4 Confocal Laser Scanning Microscopy (CLSM)

CLSM was utilized for the examination of biofilms, in the presence or absence of tetracycline, to measure changes in the microbial biofilm colonizing the surface of *Scirpus* stems in pond and river water. Prior to scanning with the CLSM, decaying *Scirpus* stems were removed from the glass tubes and placed in an observation apparatus (Figure 35). Water from the same treatment as the stem under observation was used to partially fill the observation chamber in order to keep the stem and biofilm hydrated and active. The biofilm on the stem was stained with SYTO-9 (Excitation 488 nm, emission 500 nm), of the Live-Dead staining kit of Molecular Probes (Eugene, Oregon) that stains the nucleic acid of all viable and non-viable cells. A working solution of SYTO-9 was prepared by pipetting 2 μl of the stain into 1 ml of sterile distilled water and this working solution was pipetted directly onto a randomly selected portion of the stem. Because SYTO-9 is photosensitive it was applied under subdued light but as a further precaution the observation apparatus was covered with aluminium

foil until the stems were scanned. Fifteen minutes were allowed for SYTO-9 to penetrate the biofilm and stain the cells. After allowing the stain to set, the observation apparatus was filled with water from the same treatment as the stem under observation so as to immerse the stem. Laser scans of the biofilms were carried out using an MRC 1024 CLSM (Biorad, Hemel Hempstead, U.K.) attached to a Microphot SA Microscope (Nikon, Tokyo, Japan) (Figure 35). For observation a 40x 0.55 NA (Nikon, Tokyo, Japan) water immersion lens was used.

The SYTO-9 stained part of the biofilm was randomly scanned from the top of the biofilm, closest to the microscope lens, to the surface of the *Scirpus* stem at 5 μm intervals. This single scan represented one z-series, which would be comprised, if the biofilm were 100 μm thick, of a stack of 21 images. Ten z-series were collected of the biofilm per stem on each sampling day, which was on Days 0 (prior to submersion), 7, 10, 15, 24 and 33. Image analysis was performed in Scion Image Beta 4.02 (Maryland, U.S.A). Microbial biomass was determined by measuring microbial cell area in every image by a semi-automated macro developed by Dr. Darren Korber (University of Saskatchewan, Canada). [The macro computes the % of an image that is covered with microbial cells. In the present work, in all treatments, this percent cell area covered was multiplied by 1000. Therefore, regardless of the units used, the treatments are comparable against each other. The biomass is given in μm^2 for convenience.] Biofilm thickness was calculated by multiplying the 5 μm intervals by the number of images, less one, in a z-series while fungal hyphae lengths were determined by manually measuring the length of living hyphae in each image.

Sigma Stat 2.03 (SPSS Inc.) was used to perform all statistical analysis. Parameter means ± standard error are given below.

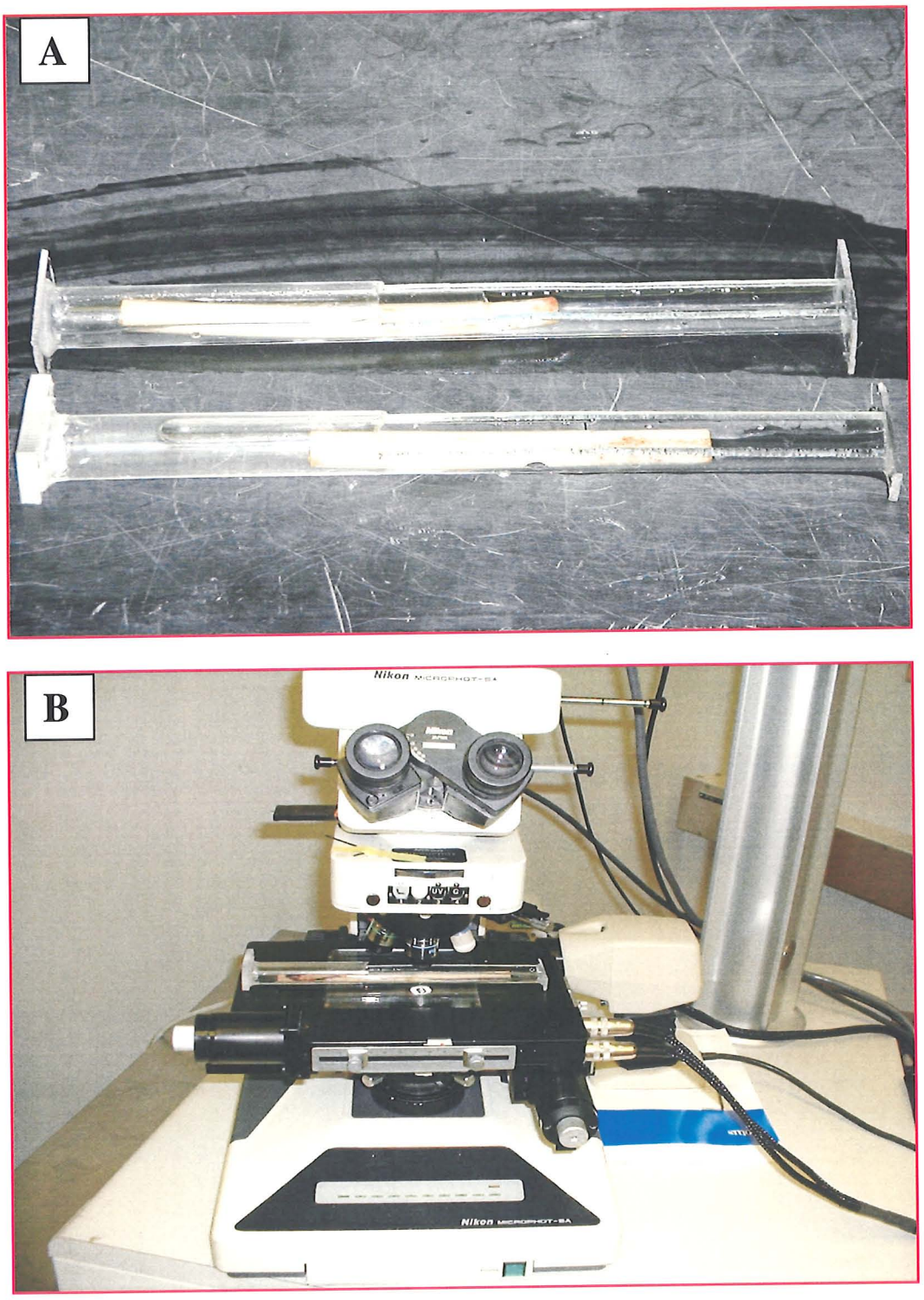


Figure 35. Photograph of two observation chambers used to hold *Scirpus* stems (A). (B) is the observation apparatus with *Scirpus* stem ready for scanning with the CLSM. Note the presence of water in observation chamber (A) to keep the stem and biofilm hydrated and active.

5.3 Results

5.3.1. Effects of Tetracycline on Protein Production in Planktonic Bacteria

There was a significant difference ($P < 0.05$) between the amount of ^{3}H-leucine that was incorporated into bacterial protein between the control and the 10, 100, 1000 and 4000 μg L^{-1} tetracycline treatments but not between the control and 1 μg L^{-1} treatment ($P = 0.13$) in the experiment conducted using river water (Figure 36). With pond water (Figure 37) the results were quite different. There was a statistically significant difference ($P < 0.02$) between ^{3}H-leucine incorporation in the control and the 4000 μg L^{-1} treatment but not between the control and other treatments ($P > 0.05$). Bacterial productivities, as indicated by the DPM's recorded, was much greater in the pond water.

5.3.2 Effects of wetting and tetracycline on biofilm colonization on the surface of *Scirpus*

There were almost no living bacterial cells and no living fungal cells observed on the *Scirpus* stems prior to wetting (Figure 38). In all of the river water treatments, in the absence or presence of tetracycline, microbial biomass increased rapidly after submersion of the stems and peaked on Day 10 and rapidly declined thereafter (Figures 39 and 40). However, tetracycline had a marked effect on microbial biomass as the total biomass measured over 33 days declined with increasing tetracycline concentration. On Day 10, microbial biomass in the control treatment was ≅ 5 times higher than in both the 500 μg L^{-1} and 4000 μg L^{-1}tetracycline treatments. These variances in microbial biomass on Day 10 were significantly different ($P < 0.05$) between all treatments but not between the 500 and 4000 μg L^{-1} ($P = 0.4$) treatments.

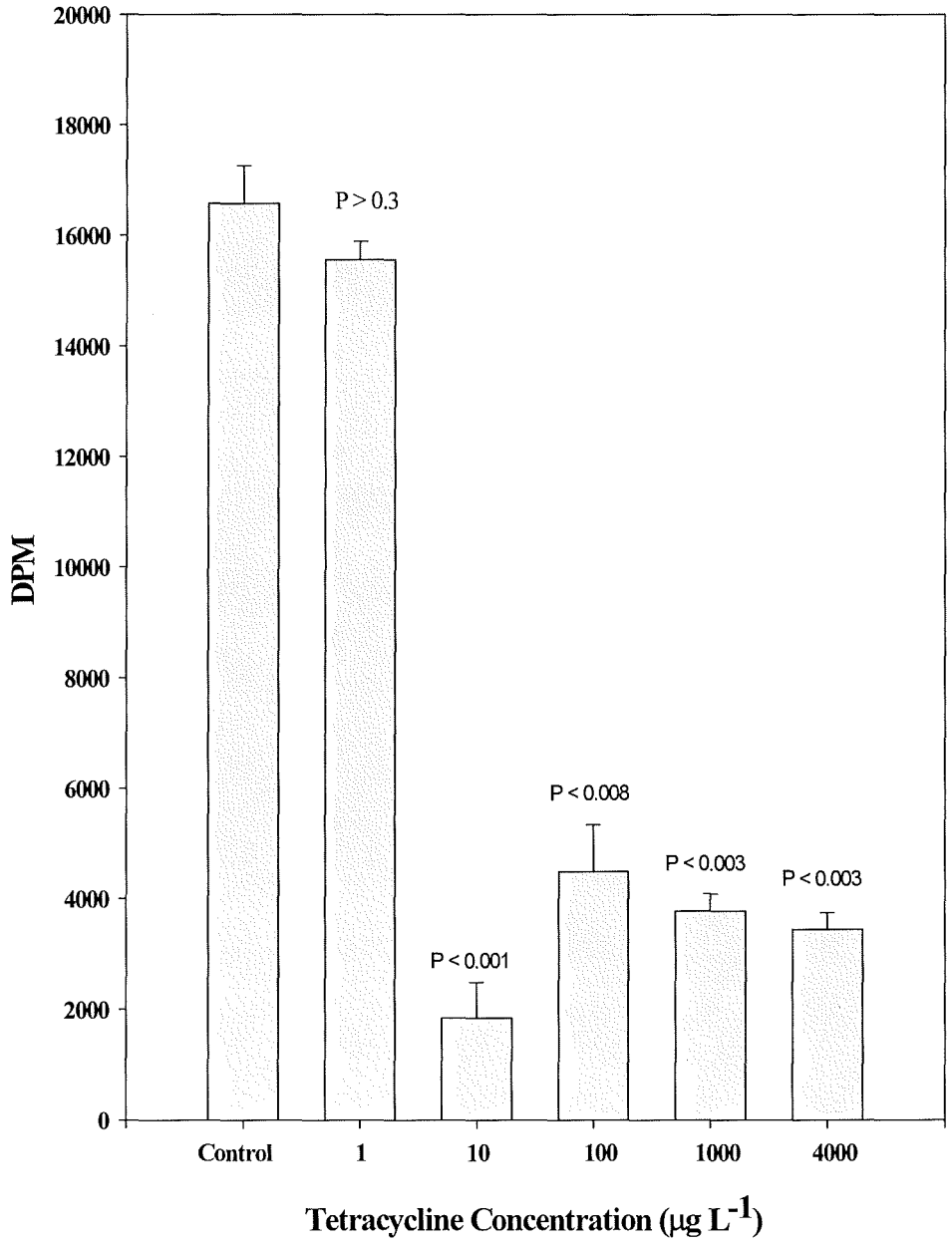


Figure 36. Effects of 1, 10, 100, 1000 and 4000 μg L^{-1} of tetracycline on ^{3}H-leucine incorporation into the protein of bacteria present in river water. Treatments that were significantly different ($P < 0.05$) from the control are indicated.

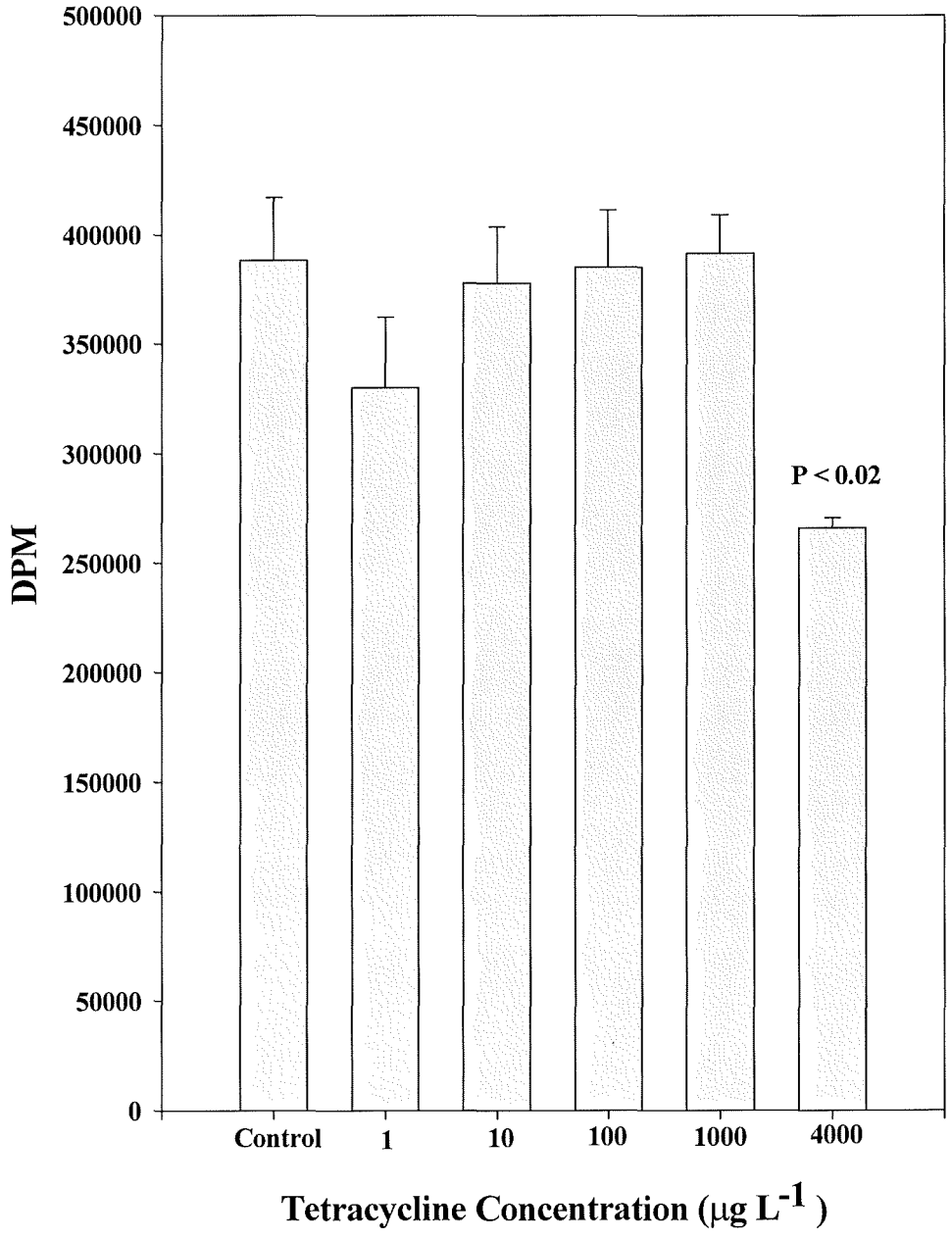


Figure 37. Effects of 1, 10, 100, 1000 and 4000 μg L^{-1} of tetracycline on ^{3}H-leucine incorporation into the protein of bacteria present in pond water. Treatments that were significantly different ($P < 0.05$) from the control are indicated.

Microbial biomass in all three river treatment biofilms showed a trend, on and after Day 7, of being concentrated in the middle portions of the biofilm and becoming sparse towards the surface nearest the stem and the top surface nearest the microscope lens (Figure 41 and Figure 42).

In terms of biofilm thickness, however, the trend was the reverse of microbial biomass with the thickest biofilm being observed in the 4000 $\mu g\ L^{-1}$ treatment and the thinnest in the control and 500 $\mu g\ L^{-1}$ treatments (Figure 43). There was a rapid increase in biofilm thickness in all three treatments by Day 10 and this was followed by a slight decline in thickness to Day 25 after which the biofilms again increased in depth. The peaks in biofilm thickness in the control, 500 and 4000 $\mu g\ L^{-1}$ treatments occurred on Days 7, 10 and 33, respectively, and seemed to become delayed with increasing tetracycline concentration. The mean biofilm thickness, from Day 7 to 33, was 49 μm, 38 μm and 87 μm for the control, 500 and 4000 $\mu g\ L^{-1}$ treatments, respectively. At the greatest separation of points, Day 33, the differences in mean biofilm thickness was statistically significant ($P < 0.05$) between the control and 4000 $\mu g\ L^{-1}$ and between the 500 and 4000 $\mu g\ L^{-1}$ treatments but not between the control and 500 $\mu g\ L^{-1}$ treatment ($P = 0.4$).

With respect to fungal biomass, there was not a significant difference ($P > 0.05$) in fungal biomass between any of the treatments due to variances in the data (Figure 44). However, the total fungal hyphae length measured from Day 7 to Day 33 in the control, 500 and 4000 $\mu g\ L^{-1}$ treatments were 12902 μm, 11602 μm and 34596 μm,

Figure 38. CLSM scan of the *Scirpus* stem surface prior to being wetted showing a network of dead fungal hyphae and bacterial cells along with sheets of the surface layer of *Scirpus*.

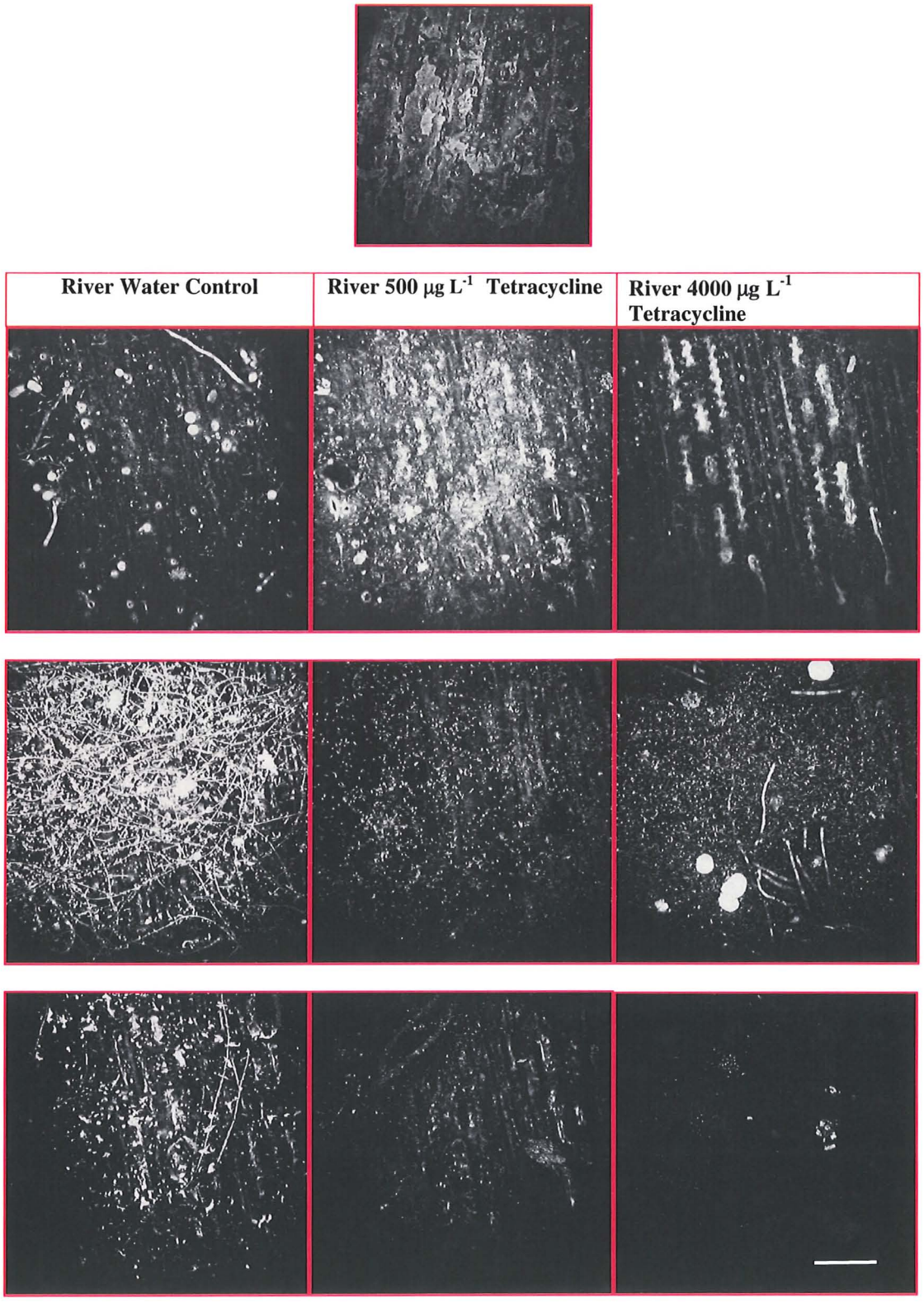


Figure 39. Collage of CLSM scans of biofilms growing on surface of *Scirpus* stems decaying in river water and river water amended with 500 and 4000 μg L^{-1} tetracycline. Scan in Row 1 is at time 0, before wetting, while scans in rows 2, 3 and 4 were made on days 7, 10 and 15, respectively. Treatments indicated by columns titles. Scale bar is ≅ 50 μm in length.

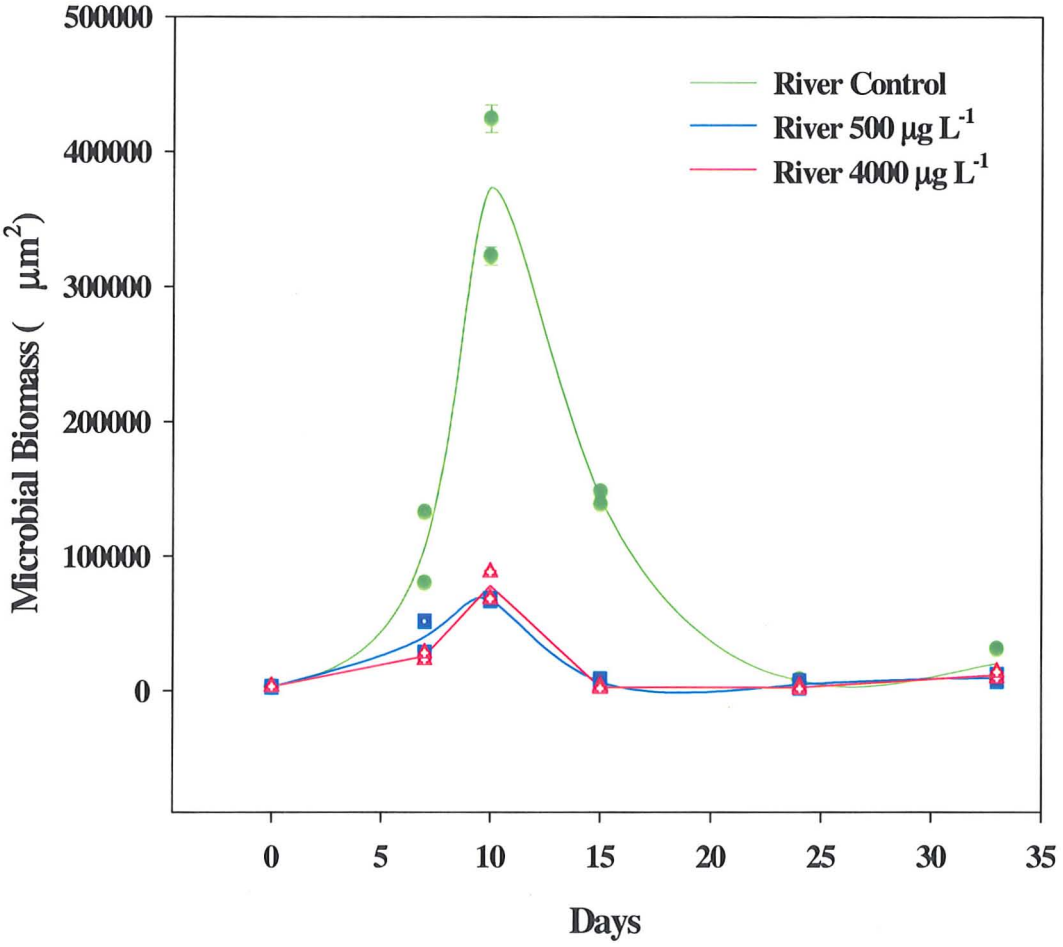


Figure 40. Changes in microbial biomass, as measured by laser microscopy over 33 days on the surface of *Scirpus* stems decaying in river water and in river water amended with 500 and 4000 µg L^{-1} tetracycline. The points are the treatment (N=2) means ± standard error while the solid line is the average of the two treatments.

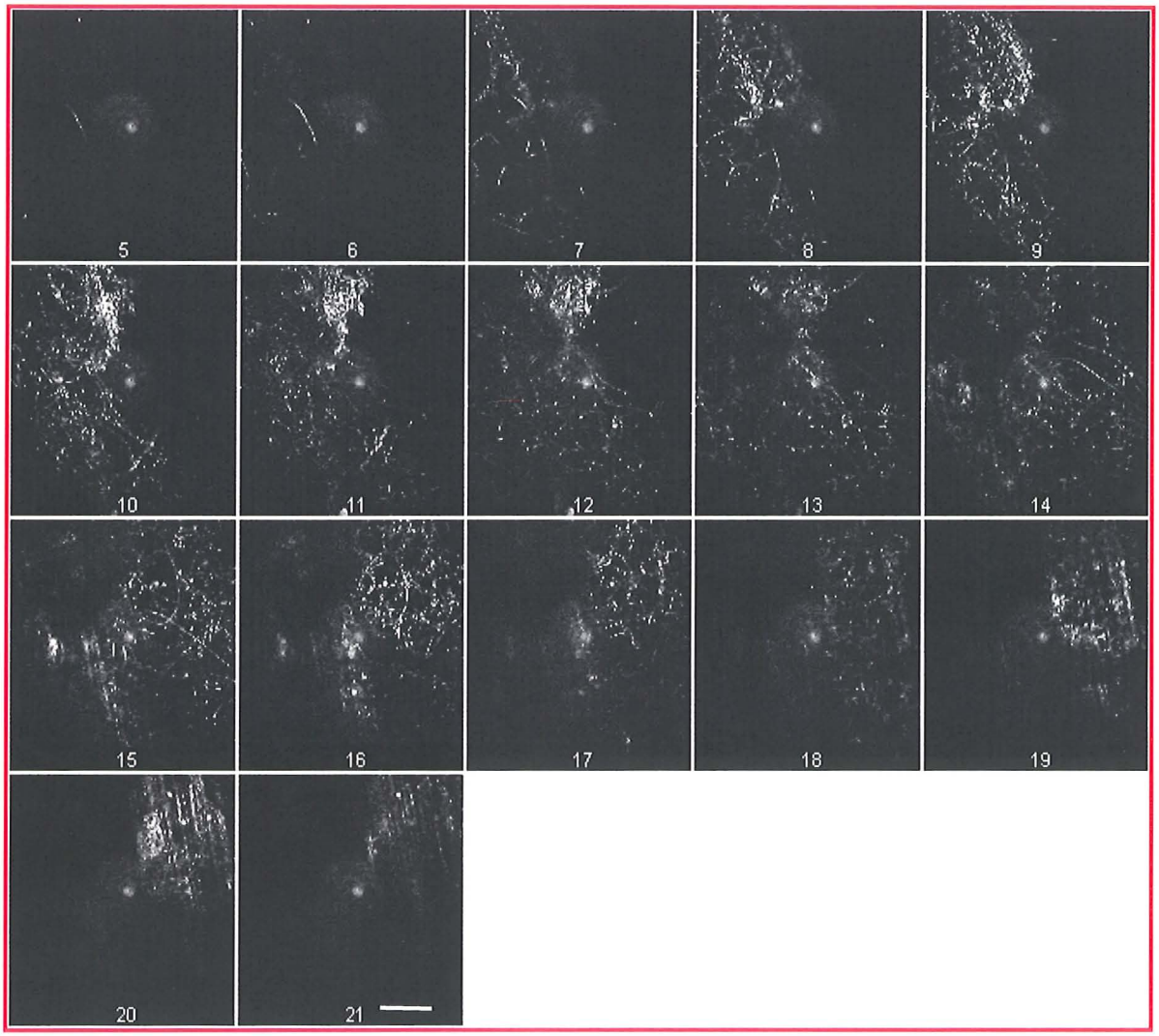


Figure 41. Montage of one z-series indicating the predominance of microbial biomass in images 8-17 while the top surface of the biofilm (images 5-7) and that closest to the *Scirpus* stem (images 18-21) contain relatively little microbial biomass. Images 19-21 show the relatively uncolonized portions of the *Scirpus* stem. The z-series was taken 7 days after the submersion of *Scirpus* in river water. The distance between each image is 5 μm and the total thickness of the biofilm is 80 μm. Scale bar is $\cong$ 50 µm in length.

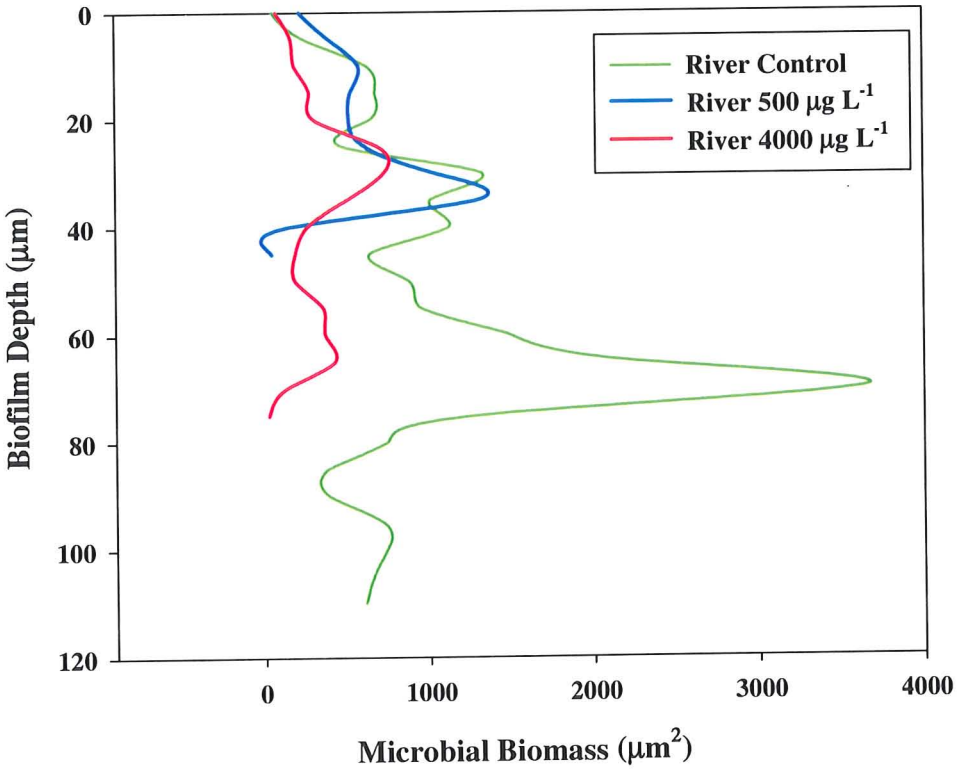


Figure 42. Graphical representations of microbial biomass in one z-series of a 7 day old biofilm growing on the surface of a *Scirpus* stem decaying in river water and in river water spiked with 500 and 4000 µg L^{-1} tetracycline. Note the significant portion of biomass occurring in the center of the biofilms while the top of the biofilm (0) and that closest to the *Scirpus* stem contain less microbial biomass. Biofilm depth in the control, 500 and 4000 µg L^{-1} treatments is ≅ 115, 80 and 45 µm, respectively.

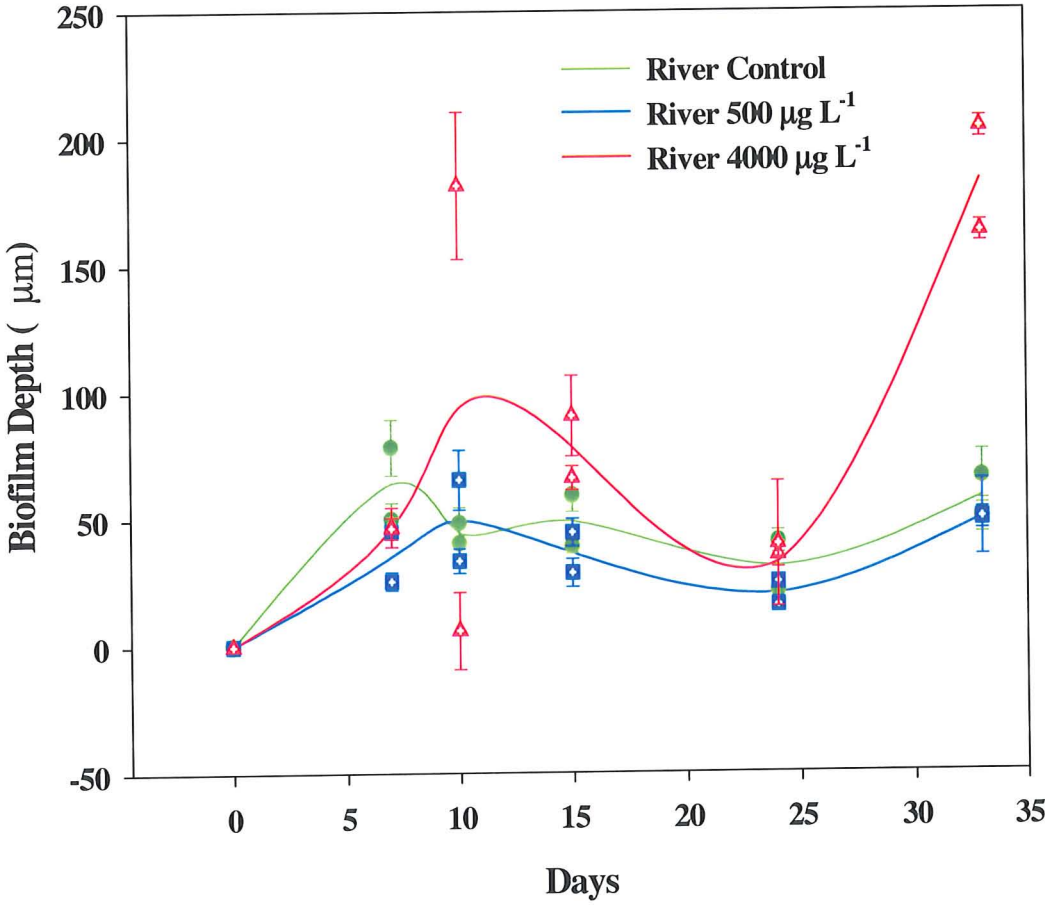


Figure 43. Changes in biofilm thickness, as measured by laser microscopy over 33 days, on the surface of *Scirpus* stems decaying in river water and in river water amended with 500 and 4000 µg L^{-1} tetracycline. The points are the two treatment means ± standard error while the solid line is the average of the two treatments.

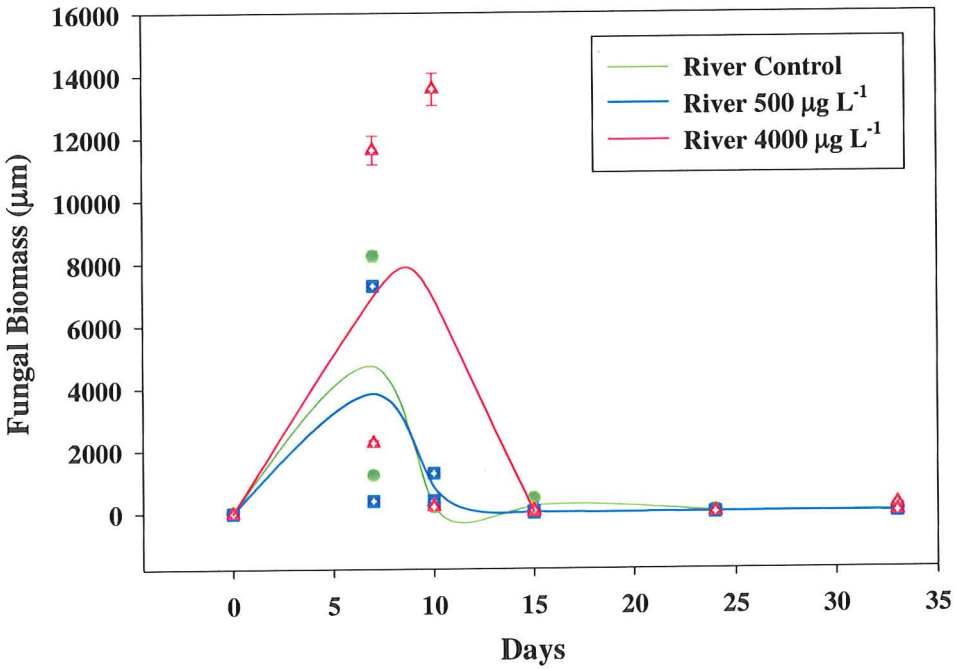


Figure 44. Changes in fungal biomass, as measured by laser microscopy over 33 days, in the biofilm growing on the surface of *Scirpus* stems decomposing in river water and in river water amended with 500 and 4000 µg L^{-1} tetracycline. The points are the two treatment means ± standard error while the solid line is the average of the two treatments.

respectively, with the higher tetracycline concentration seeming to stimulate fungal biomass. The peak in fungal biomass occurred on Day 7 in all three treatments.

In the experiments conducted with pond water a trend of decreasing biomass with increasing tetracycline concentration, similar to the river water experiment, was apparent (Figure 45 and Figure 46). Mean microbial biomass, over 33 days, was ≅ 44% lower in the 500 μg L^{-1} treatment and ≅ 65% lower in the 4000 μg L^{-1} treatment compared to the control. The increase in biomass following wetting of stems in the control treatment was rapid and peaked on Day 10 and quickly declined thereafter (Figures 46). In the 500 μg L^{-1} treatment the biomass also increased rapidly and peaked on Day 7 (Figure 46) while in the 4000 μg L^{-1} treatment the peak occurred on Day 24 and was considerably delayed when compared to the control and 500 μg L^{-1} treatments. However, due to large variances in data there was not a statistically significant difference ($P > 0.05$) between any of the treatments on any of the sampling days.

As was the case in the river water biofilms, the greatest amount of microbial biomass was generally contained in the middle portions of the biofilm with the peripheries containing much less microbial mass (Figure 47 and Figure 48). In terms of biofilm thickness the trend was similar to that seen in the river water experiment with the thickest biofilm being observed in the 4000 μg L^{-1} tetracycline treatment (Figure 49). The peak in biofilm thickness occurred on Day 7 in the control and 500 μg L^{-1} treatments with the 4000 μg L^{-1} treatment showing a broad peak between Days 10 and 24. The mean biofilm thickness from Day 7 to 33 was 86 μm, 59 μm and 93 μm for the control, 500 and 4000 μg L^{-1} treatments, respectively. There was no statistically significant difference ($P > 0.05$) in biofilm thickness between any of the treatments due to the large variances in the data.

The total fungal hyphae lengths measured from Day 7 to 33 in the control, 500 and 4000 μg L^{-1} treatments were 874 μm, 2942 μm and 5257 μm, respectively, with the highest tetracycline concentration seeming to stimulate fungal biomass, similar to the experiment with river water. The peak in fungal biomass occurred on Day 7 in the 4000 μg L^{-1} treatment and on Day 10 in the control and 500 μg L^{-1} treatments (Figure 50). However, due to variability in the data there were no statistically significant differences ($P < 0.05$) between any of the treatments.

Comparing the river and pond water experiments, overall there was no statistical difference between the river and pond water treatments in microbial biomass, biofilm thickness and fungal hyphae due to large variances in data. However, the total biomass in all treatments over 33 days of the experiment shows that biomass in the control pond water treatment was approximately twice the biomass in the control river water treatment. The microbial biomass in the 500 and 4000 μg L^{-1} pond water treatments was about 5 and 4 times greater, respectively, than the biomass in the same treatments in the river water experiment. Mean biofilm thickness tended to be higher in the pond water treatments relative to the river water treatments and at maximum separation of the data, Day 24, and these differences were significantly different ($P < 0.05$). Sampling variability did not result in statistical differences between the river and pond water experiments in terms of fungal hyphae. However, striking differences were seen when hyphae lengths were summed over the course of the experiment. There was approximately 15, 4 and 7 times more hyphae produced in the river water control, 500 and 4000 μg L^{-1} treatments, respectively, compared to the same pond water treatments. Therefore, total biomass and biofilm thickness tended to be greater in the pond water treatments but fungal biomass in the river system was greater than for the pond water.

In both experiments it was noted that initially after wetting, bacterial and fungi heavily colonized the outer covering of the *Scirpus* stems. This outer layer had a tendency to curl outward and away (Figure 51) from the surface of the stem and perhaps this is why there was a propensity for microbial biomass to accumulate slightly above the surface of the stem. However, it was apparent (Figures 41, 45 and 50) that once the outer layer had curled up or been utilized as a carbon source, the newly exposed surface beneath went largely uncolonized by both bacterial and fungal communities. Furthermore, bacteria were observed near or on the surface of living fungal hyphae but it could not be determined if the two were symbiotically associated or if the bacteria just happened to be on or near the living hyphae. Lastly, it is noted that in the control treatments in both experiments the fungal hyphae, after Day 7, had turned brown and had become encrusted with bacteria (Figure 52). Figure 53 shows that SYTO-9 stains the bacteria and fungi similarly and, as discussed below, it is not possible to distinguish, in terms of cell area in an image, between the two without prolonged image analysis. Figure 54 is a longitudinal section of a *Scirpus* stem with fungal hyphae embedded between the *Scirpus* plant cells indicating, as further discussed below, that microbial activity occurs inside of the stem simultaneously with activity on the surface but which is impossible to measure by CLSM without destroying the stem.

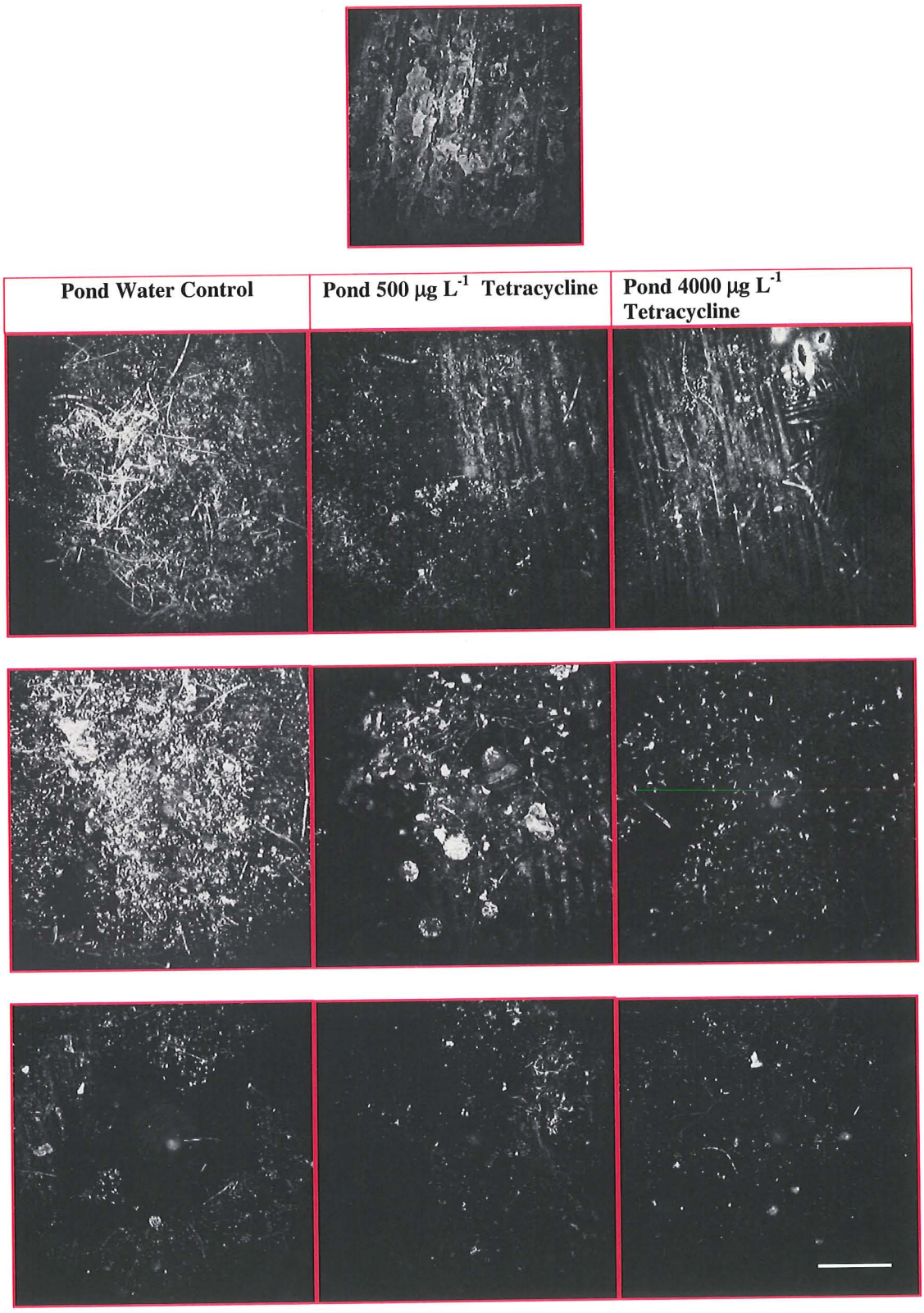


Figure 45. Collage of CLSM scans of biofilm growing on surface of *Scirpus* stems decaying in pond water and pond water amended with 500 and 4000 µg L^{-1} tetracycline. The scan in Row 1 is at time 0 while scans in rows 2, 3 and 4 were made on days 7, 10 and 15, respectively. Treatments are indicated by column titles. Scale bar is ≅ 50 µm in length.

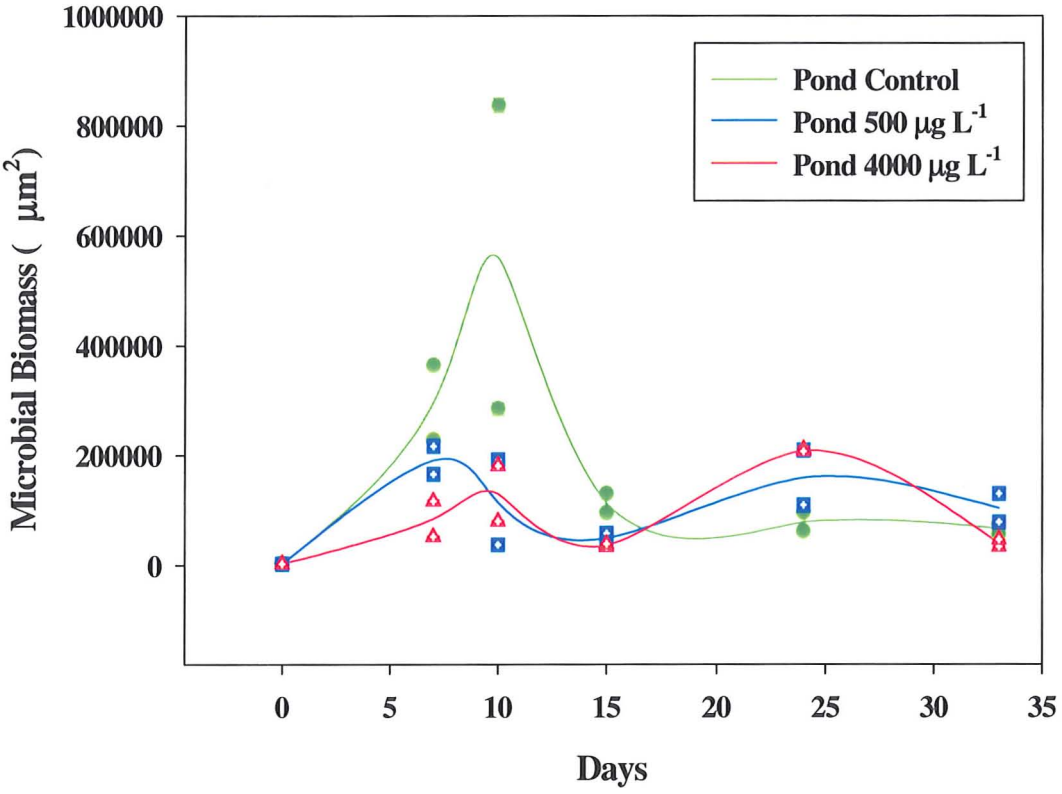


Figure 46. Changes in microbial biomass, as measured by laser microscopy, over 33 days on the surface of *Scirpus* stems decaying in pond water and in pond water amended with 500 and 4000 µg L^{-1} tetracycline. The points are the two treatment means ± standard error while the solid line is the average of the two treatments.

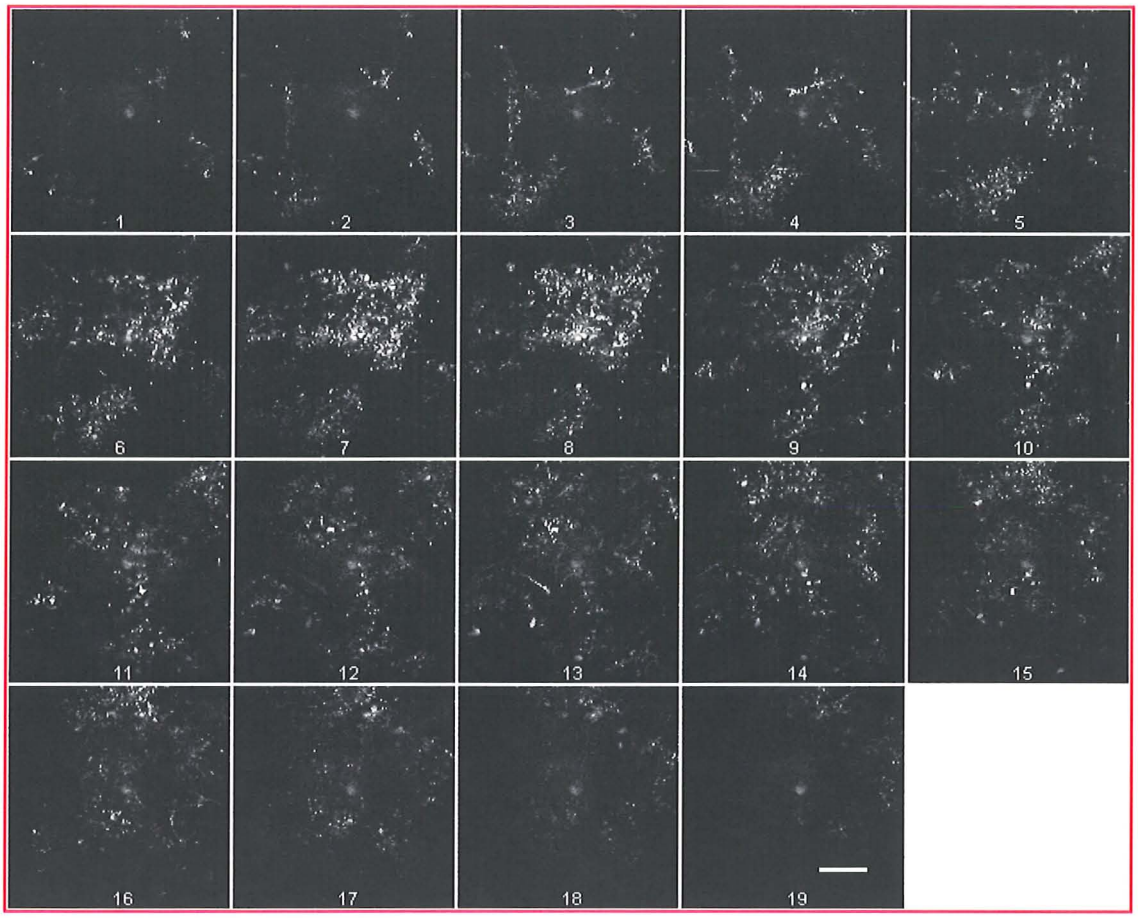


Figure 47. Montage of one z-series indicating the predominance of microbial biomass in images 6-15 while the top surface of the biofilm (images 1-5) and that closest to the *Scirpus* stem (images 16-19) contain relatively little microbial biomass. This z-series was taken 7 days after the submersion of *Scirpus* in natural pond water. The distance between each image is 5 μm and the total thickness of the biofilm is 90 μm. Scale bar is $\cong$ 50 μm in length.

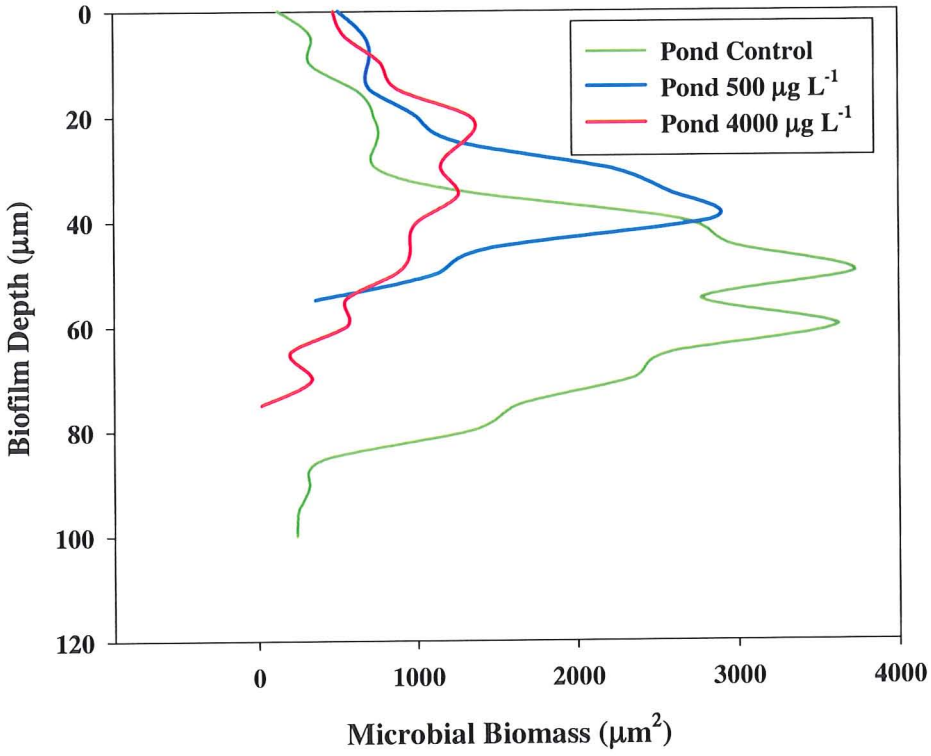


Figure 48. Graphical representations of microbial biomass in one z-series scan of a 7 day old biofilm growing on the surface of a *Scirpus* stem decaying in pond water and in pond water spiked with 500 and 4000 µg L^{-1} tetracycline. Note the significant portion of biomass occurring in the center of the biofilms while the top of the biofilms (0) and that closest to the *Scirpus* stem contain less microbial biomass. Biofilm depth in the control, 500 and 4000 µg L^{-1} treatments is ≅ 100, 55 and 80 µm, respectively.

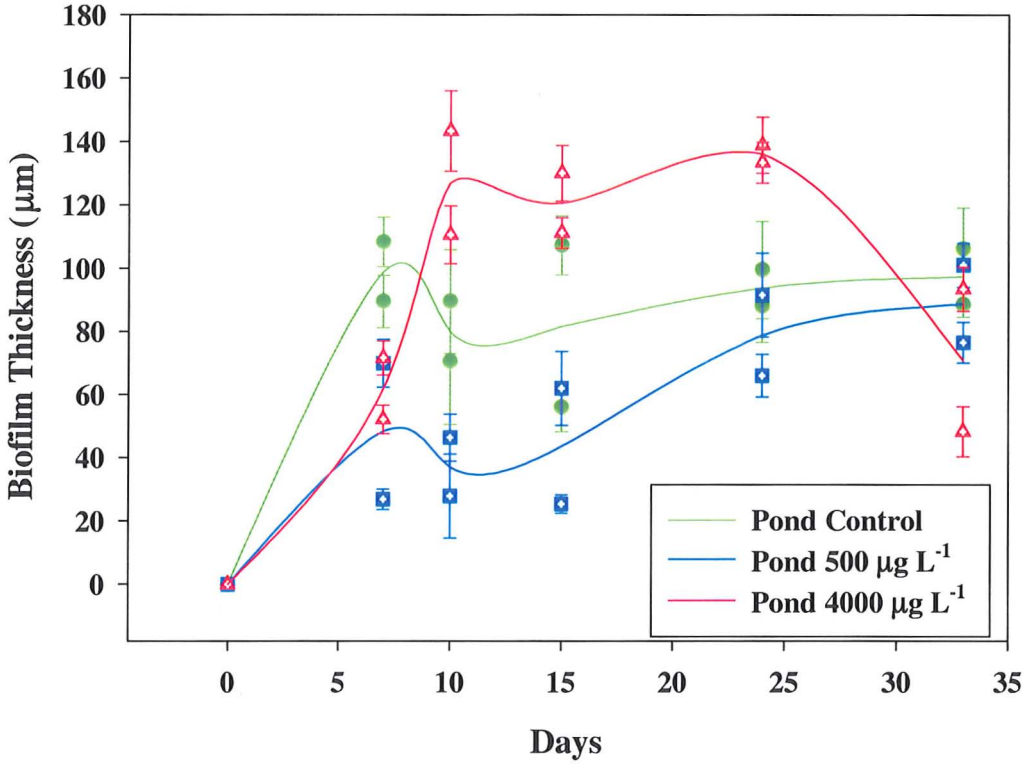


Figure 49. Changes in biofilm thickness, as measured by laser microscopy over 33 days, on the surface of *Scirpus* stems decaying in pond water and in pond water amended with 500 and 4000 µg L^{-1} tetracycline. The points are the two treatment means ± standard error while the solid line is the average of the two treatments.

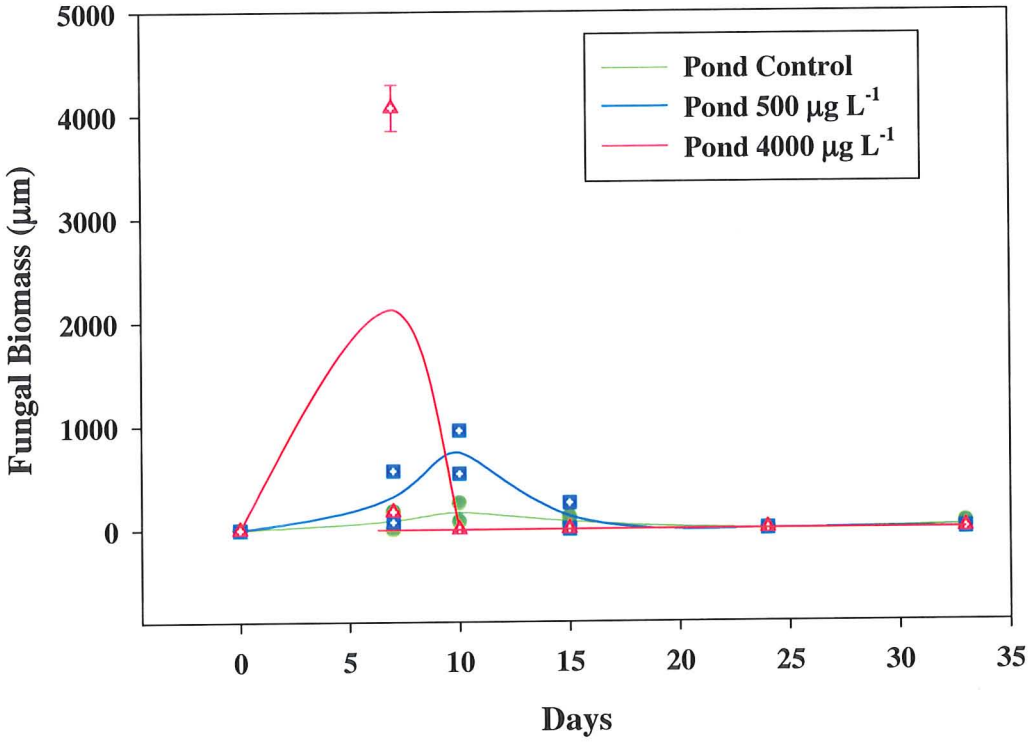


Figure 50. Changes in fungal biomass, as measured by laser microscopy over 33 days, in the biofilm growing on the surface of *Scirpus* stems decomposing in pond water and in pond water amended with 500 and 4000 µg L^{-1} tetracycline. The points are the two treatment means ± standard error while the solid line is the average of the two treatments.

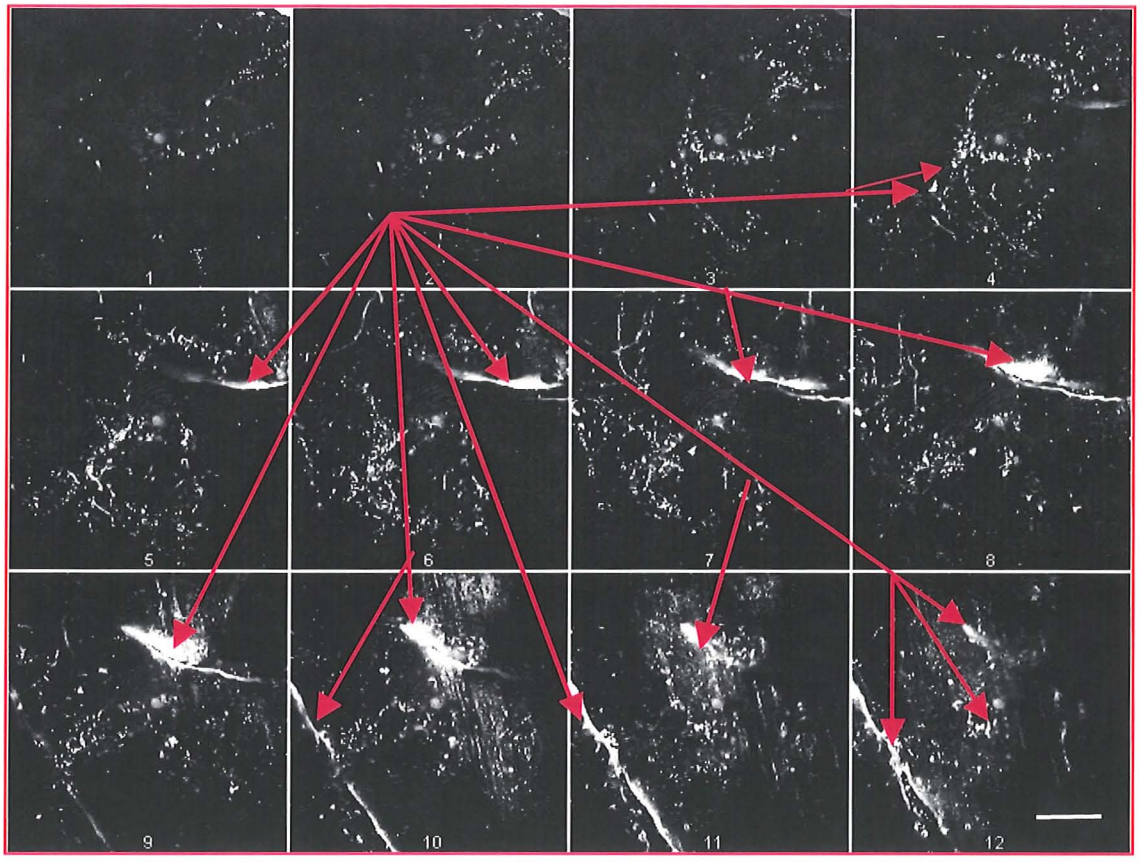


Figure 51. Montage of one z-series indicating intense microbial activity around numerous, (The red arrows point to shards of the outer *Scirpus* surface and not all of the many pieces are marked for clarity) large and small portions of the outer layer of *Scirpus* stems. The scan was made on Day 10 and is from the control river water treatment. Scale bar is ≅ 50 μm in length.

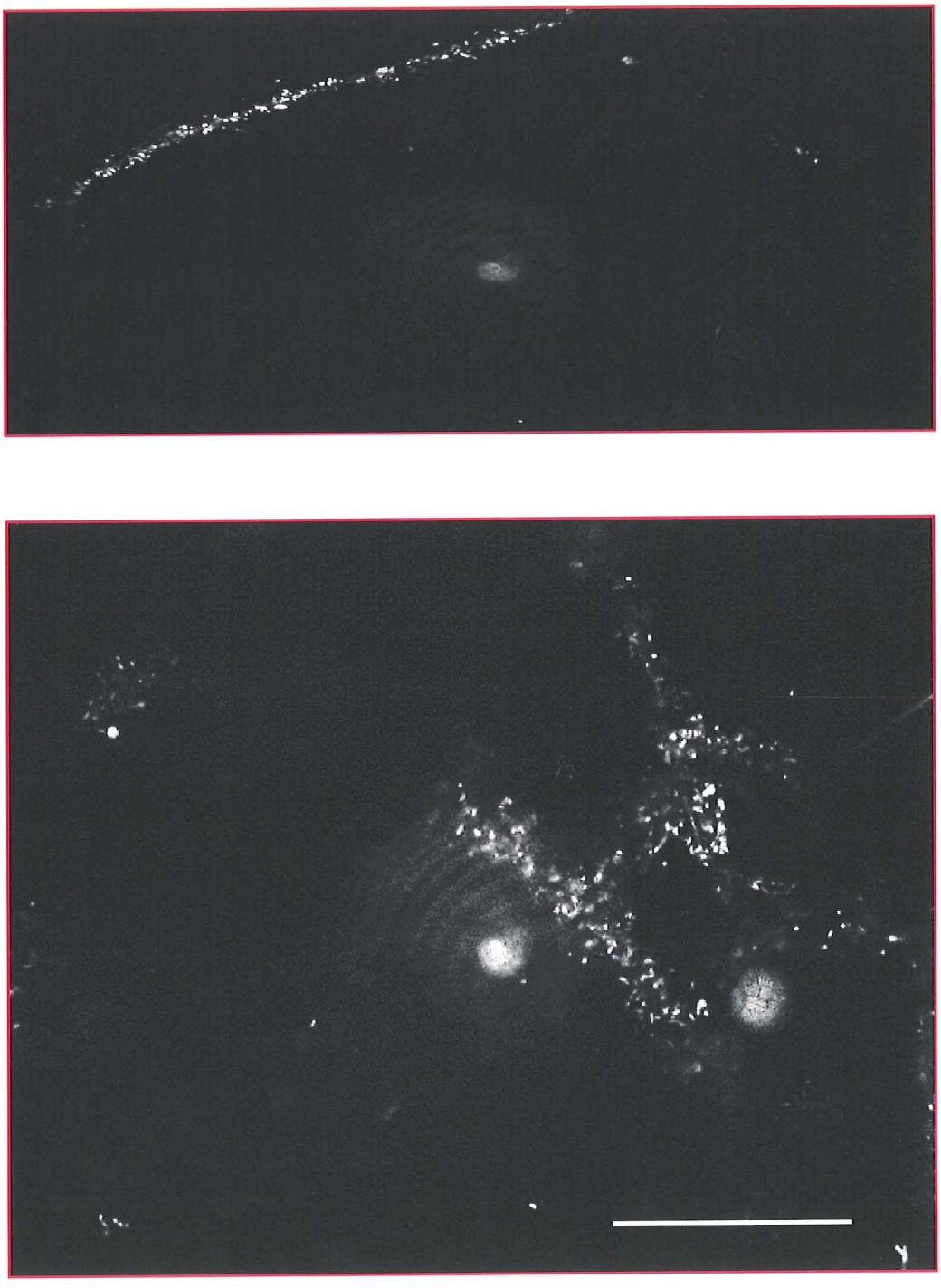


Figure 52. Two images showing the surface of dead fungal hyphae colonized by bacteria. The top image is from pond Control Day 10 while the bottom image is from pond Control Day 33. Scale bar is ≅ 50 μm in length.

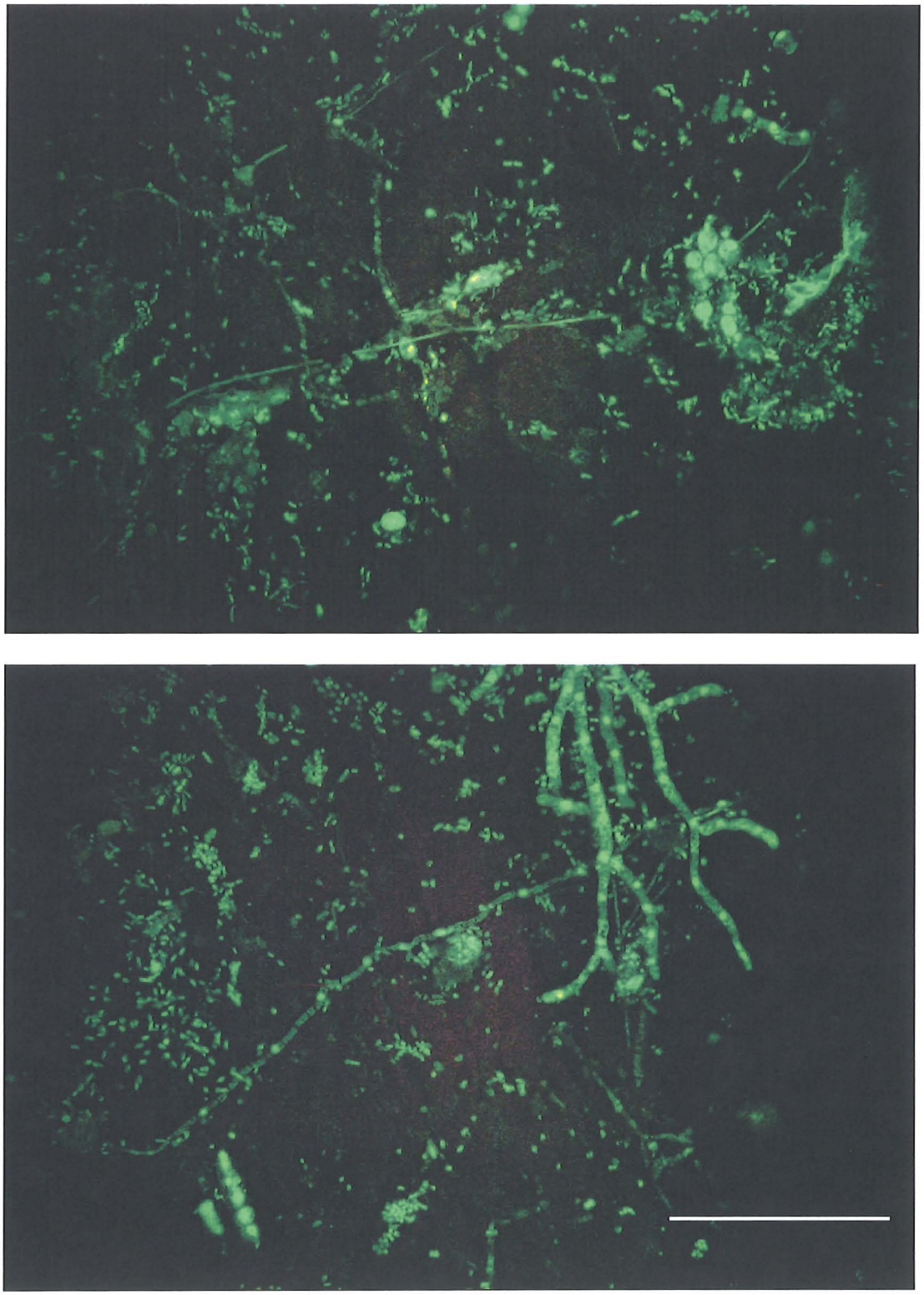


Figure 53. Fourteen day old biofilms, stained with SYTO-9, on the surface of *Scirpus* decaying in pond water with fungal and bacterial cells fluorescing equally. Scale bar is ≅ 50 μm in length.

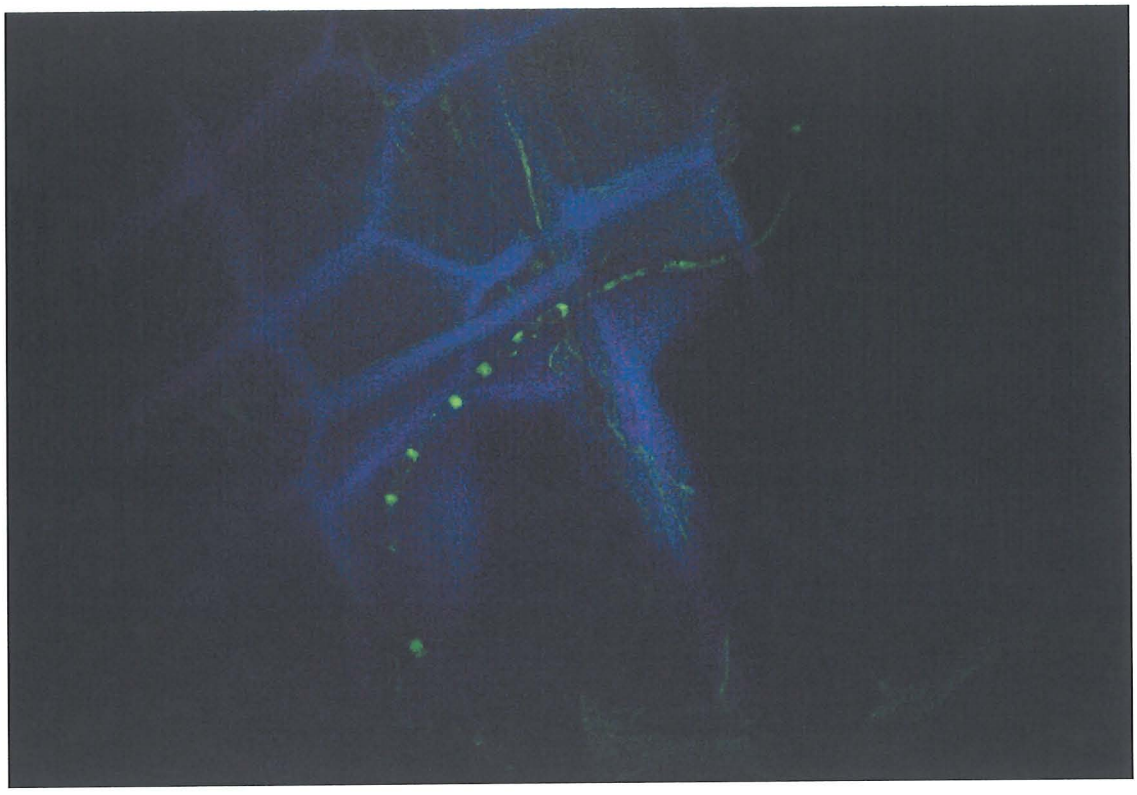


Figure 54. CLSM scan showing a fungal hypha, fluorescing green with SYTO-9, penetrating the cell walls, blue, of a *Scirpus* stem. The *Scirpus* stem was sectioned longitudinally and scanned fourteen days after being allowed to decay in pond water.

5.4 Discussion

5.4.1 Effects of Tetracycline on Protein Production in Planktonic Bacteria

^{3}H-leucine incorporation into bacterial protein was significantly reduced at a tetracycline concentration $\geq$ 10 $\mu g\ L^{-1}$ in the experiment carried out in river water. In the same experiment carried out in pond water a significant reduction from the control in ^{3}H-leucine incorporation occurred at a tetracycline concentration of 4000 $\mu g\ L^{-1}$. The reduction in leucine incorporation into protein with the addition of tetracycline in both the river and pond water experiments was not unexpected because tetracycline is known, and used, to inhibit protein synthesis in susceptible bacteria (Nester *et al.* 1998, Brodersen *et al.* 2000, Chopra & Roberts 2001, Halling-Sorensen *et al.* 2002). Tetracycline binds to the bacterial 70s ribosome and this binding prevents the association of the ribosome with aminoacyl-tRNA thereby hindering the production of protein (Brodersen *et al.* 2000, Chopra & Roberts 2001). The molecular mechanisms of the tetracycline-ribosome association, which are still under considerable study, are outside the scope of the present work (Schnappinger & Hillen 1996, Brodersen *et al.* 2000, Chopra & Roberts 2001).

However, the work done by biochemists on the mechanisms of how tetracycline traverses the bacterial membrane, in both Gram-positive and Gram-negative bacteria, from the external environment to the interior of the cell, brings to question a generally held belief about bioavailability of tetracycline. It has been assumed that chelation negates the biological activity of tetracycline against susceptible bacteria (Peterson *et al.* 1993, Halling-Sorensen *et al.* 2002). In fact, chelation to magnesium is probably necessary for tetracycline to pass through the cell membrane of Gram-negative bacteria while the neutral form of tetracycline is likely necessary to pass across the cell membrane of Gram-positive bacteria (Chopra & Roberts 2001). Once tetracycline is

inside the bacterial cytoplasm it seems that a magnesium-tetracycline complex binds to the ribosome thus interfering with protein synthesis (Chopra 2001). The bioavailability of tetracycline to bacteria in aquatic environments, then, is dependent on the type of metal ions tetracycline is bound to and chelation does not necessarily result in decreased tetracycline efficacy. Even if we were to study the effects of tetracycline on a singular strain of bacterium in river or pond water, the ratio of free and magnesium bound tetracycline, as well as the pH, as discussed in Chapter 4, in a given aquatic environment would likely markedly effect the activity of tetracycline against that one bacterial strain.

These differences in the impact of tetracycline against bacteria, caused by the matrix of the waters, was apparent in the river and pond water experiments where ^{3}H-leucine incorporation into bacterial protein in the river water experiment was significantly reduced, as compared to the control, at $\geq$ 10 $\mu g\ L^{-1}$ of tetracycline whereas a significant negative effect in the pond water experiment was seen only at 4000 $\mu g\ L^{-1}$ tetracycline relative to the pond water control. Why was such a high concentration of tetracycline required to inhibit protein syntheses in bacteria in pond water relative to bacteria in the river water? This can partially be explained by the tetracycline saturation work conducted in the previous chapter. It was noted that when tetracycline was spiked at the same concentration in both river and pond water, say 4000 $\mu g\ L^{-1}$, approximately 50% of the tetracycline remained free in the river water while only 25% was free in the pond water. Therefore, when tetracycline was spiked at 4000 $\mu g\ L^{-1}$in both waters the proportion of free tetracycline active against susceptible bacteria in the river water would have been 2000 $\mu g\ L^{-1}$ while in pond water it would have been 1000 $\mu g\ L^{-1}$. However, even taking this into account does not explain that significant suppression of

^{3}H-leucine incorporation into protein occurred only at 4000 μg L^{-1} in pond water whereas a significant effect was seen at ≥ 10 μg L^{-1} in river water. There may be other explanations as to why such a higher concentration of tetracycline was needed to significantly suppress leucine uptake by bacteria in pond water.

First, in order for equal suppression of ^{3}H-leucine incorporation into the protein of planktonic bacteria to have taken place in both waters at a similar concentration of free tetracycline, we would have to assume the bacterial populations in both waters to be equally susceptible to tetracycline. Not all bacteria are susceptible to tetracycline and there are various mechanisms whereby bacteria are able to resist the actions of tetracycline even at high concentrations. These mechanisms include efflux pumps in the bacterial cell membrane that can export tetracycline out of the cell, ribosomal protection proteins whereby the bacterial ribosome is protected even in the presence of tetracycline, and found in one bacterium, enzymatic inactivation of tetracycline (Chopra & Roberts 2001). There may have been a greater number of resistant bacterial strains in the pond water relative to the river water resulting in greater resistance to tetracycline and therefore more leucine incorporation at higher tetracycline concentrations in the pond water relative to the river system. Secondly, the free tetracycline in both waters would have been effective only against Gram-positive bacteria since, as mentioned above, the neutral form of tetracycline is likely required to cross over the Gram-positive bacterial cell membrane. Therefore, if a greater proportion of bacteria in the pond water were Gram-positive there would have been less of an effect seen on leucine incorporation into protein even at a higher concentration of tetracycline since there is significantly less free tetracycline in the pond water relative to river water. This is highly unlikely, though, since marine systems are thought to predominantly consist of Gram-negative bacteria (Sieburth 1979). Thirdly, the

tetracycline bound to magnesium would have been effective against Gram-negative bacteria. If less magnesium were present in the pond water relative to river water, the effect on leucine incorporation in Gram-negative bacteria would have been lessened in the pond water system. This too is very unlikely because prairie wetlands are know to contain magnesium in very high concentrations (Waiser 2001b). Additionally, the effectiveness of antibiotics is often related to the phase of growth the bacteria are in with bacteria in the log phase of growth being more susceptible to antibiotics then bacteria in the stationary phase (Stewart 2002). Differences in the growth phases of bacteria in each system could also account for disparities in bacterial sensitivities to tetracycline between the river and pond water experiments. The opposite of reasons indicated above would explain why such a low concentration of tetracycline was required to suppress ^{3}H-leucine incorporation into bacterial protein in river water. But, as can be appreciated, prairie wetland systems, like other aquatic systems, are extremely complex not only with regards to the chemical matrix, but also in terms of the bacterial populations. Consequently, considerable furthur work is required to elucidate the reasons as to why a higher concentration of tetracycline was required to suppress protein synthesis in pond water bacteria and why such a low concentration of tetracycline was required to reduce protein synthesis in planktonic bacteria in river water.

There has been no other research, to the author's knowledge, conducted where ^{3}H-leucine incorporation into protein has been used to measure planktonic bacterial sensitivity to antibiotics in natural environmental waters. Researchers have generally used the minimum inhibitory concentration (MIC) of antibiotics in either media broth or agar plates to test for bacterial susceptibility and resistance (Al Ahmad *et al.* 1999, Ceri *et al.* 1999, Aaron *et al.* 2002, Sengelov *et al.* 2003). As an example, Sengelov *et*

al. (2003) used 8000 μg L^{-1} tetracycline in leurtina broth agar plates to differentiate between resistance and susceptibility to tetracycline of bacterial populations in agricultural soil to which manure laden with antibiotics was applied. Ceri *et al.* (1999), meanwhile, studying the susceptibilities to antibiotics of planktonic versus biofilm growth forms of three pure bacterial strains, used media broth and measured the turbidity of the broth to estimate bacterial growth, or lack of, under variable concentrations of different antibiotics. Additionally, considering that the matrix can reduce the efficacy of tetracycline, as demonstrated in the previous chapter, it is likely that the concentration of tetracycline active against the bacteria in both of the above studies was likely greatly reduced because of tetracycline binding to the media and agar. Even so, the present leucine incorporation into protein experiment indicates that bacteria were very sensitive to tetracycline at a concentration ≥ 10 μg L^{-1} and that even such a low concentration, significantly below the 8000 μg L^{-1} MIC of the above study, may dramatically alter planktonic bacterial function. Additionally, the work in two differing waters shows that the very complex waters matrix of aquatic systems will considerably influence the activity of tetracycline against planktonic bacteria.

5.4.2 Microbial Dynamics on the Surface of *Scirpus* Stems Following Wetting In the Presence of Absence of Tetracycline

CLSM scans of the surface of *Scirpus* stems stained with SYTO-9 were made prior to wetting at time 0. These scans revealed a surface essentially devoid of living (almost no cells that fluoresced green) bacterial cells and fungi. However, in a few scans remnants of a complex network of fungal hyphae, together with a few dead or dormant bacterial cells and shards of the outer covering of the stem, were clearly visible. This network of fungal hyphae was not ubiquitous over the whole surface of the

stem. This suggests that when the stems were standing at Pond 50 either they were freshly colonized by fungi, and therefore the fungi did not have the time to colonize the entire surface, or that fungal growth may have been intermittent with growth in favorable conditions of moisture and temperature and dormant under unfavorable environmental conditions. The stems were microscopically examined using SYTO-9 a few hours after wetting and there were newly formed fungal hyphae fluorescing green. This observation corroborates previous work (Verma *et al.* 2003) where it was found that fungi likely grow very rapidly after wetting and under favorable conditions but remain dormant under less suitable conditions. Kuehn *et al.* (1998) had also noted considerable evolution of CO_2 5 minutes after dry litter of *Juncus effusus* had been wetted. Based on the present microscopy work it appears likely that germination and growth of fungi on the surface of *Scirpus* may begin within minutes of wetting. Considering that the colonization of possibly dead fungal hyphae by bacteria in the present study occurred in less then 10 days (further discussed below), it is not surprising that fungi respond to wetting and moisture so quickly.

In the present study, submergence of *Scirpus* stems in both river and pond water, regardless of the presence or absence of tetracycline, resulted in a dramatic increase in microbial biomass, including fungal biomass, both of which peaked on around days 7-10 of the experiments and quickly subsided thereafter. In a few studies where microbial dynamics (fungal and bacterial) after litter submergence was measured, some researchers have observed an increase in both bacterial and fungal biomass (Suberkropp & Weyers 1996, Baldy *et al.* 2002, Hieber & Gessner 2002) while one study observed an overall decrease (Kuehn *et al.* 2000). In two studies bacterial biomass increased but fungal biomass decreased (Denward *et al.* 1999, Kominkova *et al.* 2000). A close look at the above studies shows that the length of

experiments varied considerably with the Suberkropp & Weyers (1996) study being the shortest, lasting 14 days, while the Kuehn et al. study was the longest at 270 days. Additionally, the objective of these studies was not to study microbial colonization of dry litter immediately after wetting, as in the present study, and therefore the attribution of increases and decreases in fungal and bacterial biomass was over a prolonged period. However, in all of these studies there was an increase in both fungal and bacterial biomass immediately after wetting.

In the study conducted by Kuehn *et al.* (2000), which ran for 270 days, both bacterial and fungal biomass decreased from Day 0 to Day 191 after submergence of *Juncus effusus* litter. Microbial biomass then increased from Day 190 to Day 270. This initial decrease in microbial biomass was attributed to the unsuitability of the aquatic habitat to terrestrial microbial species and the later increase in biomass to the colonization of the litter by aquatic fungi. However, in this study bacterial biomass, measured by DAPI, was first measured 10 days after submergence of litter and there was a decline in bacterial biomass thereafter. Assuming little or no bacterial biomass prior to wetting of the *Juncus effusus* litter it is likely that the bacterial biomass measured by Kuehn *et al.* (2000) on Day 10 was greater, if not near the peak, than it was before litter submergence. Fungal biomass, estimated by ergosterol, meanwhile, was measured a day after litter submergence and while there was an overall decline from Day 1 to Day 191, fungal biomass in fact increased from the first measurement on Day 1 to the next measurement made on Day 10. Similarly, Denward *et al.* (1999), studying the effects of solar radiation on bacterial and fungal constituents on *Phragmites australis* leaves decaying in water, observed an overall decline in fungal biomass from the initial measurement of ergosterol on Day 0 to Day 60. However, similar to the Kuehn *et al.* study there was in fact an increase in fungal biomass from

Day 0 to Day 3 and it was after Day 3 that fungal biomass declined. In the study of Kominkova *et al.* (2000) there was a decline in fungal biomass, as measured by ergosterol, in submerged *Phragmites australis* leaves from Day 0 to around Day 85. However, fungal biomass was higher on Day 20 then on Day 0 (unfortunately fungal biomass was not measured prior to Day 20). In all three of these studies, though there was an overall decline in microbial biomass, both fungal and bacterial biomass increased immediately after dry litter was submerged, in a similar manner to the present study, and then both declined.

In a study conducted by Hieber and Gessner (2002), approximately 15 g each of alder and willow leaves were enclosed in separate nylon bags and these bags were then submerged in a stream in order to study the relative proportions of bacterial, fungal and invertebrate litter shredders involved in leaf litter decay. There was a rapid increase in microbial biomass after litter submergence and the biomass peaked 4 weeks after the start of the experiment in both litter types. The peak in microbial activity was considerably later than that observed in the present study of *Scirpus* and was possibly due to the fact that the leaves formed a large clump which could have been colonized more slowly by microbes compared to the surface of the *Scirpus* stem. In the Kominkova *et al.* (2000) study microbial dynamics on submerged leaves of *Phragmites australis* were followed. Microbial biomass peaked around Day 17-20, though no measurements of biomass were made between Day 0 and Day 17-20 and the peak in microbial biomass could have occurred earlier. Microbial biomass on poplar leaves in the Suberkropp and Weyers (1996) study reached a peak on Day 14 after submersion. In a study conducted by Verma *et al.* (2003, See also Chapter 3), one in which live *Scirpus* stems were cut into 2 cm pieces and allowed to decay in a pond under conditions of exposure or protection from UV radiation, they found that fungal

biomass, assayed by ergosterol, increased immediately and peaked 2 weeks after submergence but declined thereafter under both radiation exposures.

Researchers have not previously looked closely at the microbial assemblages associated with the initial stages of submergence. Because of this, the decline in microbial biomass, specifically fungal biomass, have been attributed to the unsuitability or dying of terrestrial fungi in the aquatic environment (Kominkova *et al.* 2000, Kuehn *et al.* 2000). All these studies, including Verma *et al.* (2003), noted increases and decreases in microbial biomass but due to the limitations of the methods used they could do little but speculate as to the reasons for this behavior. However, the present work using laser microscopy reveals that what is more likely to be a cause of the rapid increase in microbial biomass in submerged litter is likely nothing more than increased moisture and the presence of a suitable carbon substrate able to be exploited for microbial growth and reproduction and the subsequent decrease in microbial biomass as being due to the exhaustion of the carbon substrate.

In the present study it was observed that upon wetting the outer covering, made up of the cuticle on the outside plus the deeper epidermal and cortex cells, of the *Scirpus* stem was first utilized as a carbon source. Subsequently, once this disappeared, a more refractive portion of the stem was probably left which went largely uncolonized and was shunned as a carbon source by both fungi and bacteria. Fungi and bacteria very likely also colonized the interior of the stem but it was not possible to determine this without sacrificing the stems. In studies of plant decomposition in terrestrial systems, such as forest floors, the nutrient makeup of plant material has been loosely classified into easily decomposable, such as proteins and carbohydrates, compounds such as lignin, cellulose, waxes and fats that are more recalcitrant, and chemicals like tannins and phenols that have a mineralization time from 100 to 1000 years (Walse *et al.* 1998).

The outer cuticle of plants, the extremely thin waxy layer that is one or two cell layers thick, is thought to be a poor source of nutrients for some fungi but not for epiphytic fungi that predominantly colonize the surface of plant litter (Osono & Takeda 2000). Though scientists speak of carbon sources that are labile and refractive, it should be kept in mind that lability and refractivity is based on the microbial constituent present. As an example, if a fungus does not have the enzymes necessary to degrade lignins but does have the capacity to use tannins, the lignin is a refractive carbon source to this fungus while tannin is labile. In the same way, the un-colonized surface of the stem may have been labile but the fungi and bacteria present lacked the enzymes necessary to exploit that part of the *Scirpus* stem as a carbon source and for this reason did not colonize it. Nevertheless, the above studies are based on terrestrial decomposing litter where there is often a scarcity of moisture but still do provide useful information with respect to interpretation of aquatic litter decomposition.

There is some corroborating evidence from a number of studies that the increases and decreases in microbial biomass in decaying litter over time may be due to the changing nature of the substrate's carbon profile as it becomes consumed. It has been conjectured previously (Verma *et al.* 2003) that over a season *Scirpus* may be preferentially colonized by differing fungal species as the carbon substrate profile of the stem changed because not all fungi had the enzymatic capacity to degrade all of the stem's carbon. There is support for the idea of fungi preferring certain litter types but there does not appear to be, thus far, of sequential colonization of plant material based on changing carbon profiles as the litter is consumed. As mentioned in Chapter 3, Osono (2002) noted distinct phyllosphere fungal profiles of *Fagus crenata* leaves when the leaves were attached to the trees, when the leaves were on the ground and a subsequent period after the leaves had lain on the ground. However, these patterns in

colonization may have been due to changes in season and not necessarily due to changes in carbon substrate profile of the leaves.

Some studies have shown that differing litter types (bark, wood, leaves) from the same plant were colonized by different fungal species and in one study, conducted in a Spanish stream, a fungal strain of *Lunulospora curvula*, when given the choice between eucalyptus and alder leaves preferred eucalyptus leaves (Chauvet *et al.* 1997). In this study 146 litter samples from 92 different waterways in Belarus were pooled and analysed according to fungal taxa and substrate type. The results indicated that selective populations of fungi colonized and preferred specific substrates or litter types. In a study conducted by Osono & Takeda (2000), 26 endophytic and epiphytic strains of fungi from beech leaves were isolated. These fungal strains were then exposed to beech leaves and the changes in lignin, carbohydrates and polyphenols of the beech leaves were then measured after 8 weeks of incubation. Osono & Takeda found that the greatest mass loss in leaves and lignin, which is predominantly inside of the plant cell walls, occurred in preparations of endophytic, or internal fungal colonizers while the least mass loss occurred in preparations with the epiphytic strains. They also found differences between the endophytic strains with some strains unable to utilize lignin but able to utilize pectin and sugars. Therefore, it is plausible that the increase and the subsequent decrease in microbial biomass in all studies, regardless of the aquatic or terrestrial nature of the microbes, are due to the carbon profile of the litter used. This idea would suggest that when litter is first colonized by microbes only the plant portions labile to the colonizing microbes is stripped away first and this may be followed by a lag period before the other portions of the stem are colonized and utilized as carbon sources.

It remains to be studied if the fungi and bacteria that colonized the *Scirpus* stem, once the stem breaks and enters the aquatic environment, are terrestrial in origin or are primarily residents of the aquatic environment, or a combination of both terrestrial and aquatic species. As mentioned, there were some fungal and bacterial spores, likely terrestrial in origin, on the dry *Scirpus* prior to immersion and these could have germinated once moistened. Though one can easily imagine that fungal and bacterial spores from waters could easily make their way into the air by aerosilization and then fall back onto land, and therefore onto plants, through rain. In a survey for ergosterol in a variety of environmental matrices (Verma *et al.* 2002) no ergosterol was detected in pond water but fungal spores and conidia in the water may have become deposited on *Scirpus* stems and germinated thereafter in the present study.

There is some indirect evidence for the terrestrial-aquatic hypothesis provided in the long-term room temperature experiment in Chapter 3 where the *Scirpus* stem moisture at the beginning of the experiment was 6% and by Day 12 had reached 82%. It could be that with prolonged submersion the moisture content in the stem increased and this made the stems less of a hospitable habitat for the microbial assemblages if they were primarily of terrestrial origin. Additionally, also seen in Chapter 3, the ideal plant moisture content for fungal production in the aerial stem portions seemed to be in 15-22% range but the ideal range for fungi in the submerged plant portions was in the 45-50% range. However, the present work did not differentiate between aquatic and terrestrial microbes but measured that a decrease in microbial biomass coincided with the loss of the outer surface layer of the stem. The divide between terrestrial and aquatic species is unlikely to be distinct, especially in wetlands where there is a transition of both habitats, but has been alluded to by other researchers (Kuehn *et al.* 2000) and has also been treated previously in Chapter 3 and by Verma *et al.* (2003).

Considerable work remains to be done in researching the dynamics and ratios, if any, of terrestrial and aquatic fungal associations as well as the intricate fundamentals of microbial colonization that occur on a dead plant when it falls into water from a standing position.

One of the reasons for the large variations in microbial biomass and fungal colonization seen in the present study could have possibly been due to the fact that when *Scirpus* was standing on the periphery of Pond 50, favourable moisture and temperature conditions would have led to rapid microbial growth and sequestration of carbon from the outer surface of the stem. Additionally, weathering may also have stripped the outer layer away in many places on the stem. The colonization and the weathering would have resulted in a patchwork of the outer layer on the stem with some areas being devoid of the layer while in other areas the outer layer would be intact. This is corroborated by the microscopy work done prior to submersion of the stems where considerable areas were observed to have no outer covering on the stem. When the *Scirpus* stem was submersed for the present study, the stems would have been selectively colonized on the areas with the outer layer being colonized first while other areas of the stem with no surface layer being un-colonized resulting in large deviations in microbial biomass. This problem of large deviations probably could be minimized if green stems were allowed to dry and then placed in water to decay since the outer covering would largely be intact and contiguous.

The microbial dynamics in all of the above studies, then, are similar to the results of microbial biomass on *Scirpus* stems, though at first look some of the studies seem to be at odds with the present study. This similarity in results obtained using different methods, i.e., bacterial biomass by DAPI and fungal biomass by ergosterol and laser microscopy, provides evidence that laser microscopy is a powerful tool in

measuring the microbial dynamics associated with submerged litter. Because of the complexities in identifying hyphomycetes growing on and within decaying aquatic litter, the use of nucleic acid specific, thus hopefully species and strain specific fluorescent probes, in conjunction with CLSM promises to illuminate the precise roles played by fungi in aquatic and other ecosystems (Baschien *et al.* 2001, Lorang *et al.* 2001, McArthur *et al.* 2001, Nikolcheva *et al.* 2003).

While there have been a number of studies measuring the effects of antibiotics on pure bacterial strains and bacteria that have already formed a biofilm (Korber *et al.* 1994, Coquet *et al.* 1998, Aaron *et al.* 2002, Walters III *et al.* 2003), there have been no previous studies conducted to measure the effects of antibiotics on microbes colonizing natural organic material or how a microbial process, such as decomposition, can be disrupted by antibiotics. The results from the present study show that even when bacteria were challenged by a very high concentration of tetracycline, despite a decrease in microbial biomass with increasing tetracycline concentration, a biofilm was still able to be established on and near the surface of *Scirpus* stems and that the process of decomposition, though considerably stunted by tetracycline, still continued. Total microbial biomass in the biofilms was adversely affected by the presence of tetracycline in both river and pond waters and this may have been largely due to the fact that the antibiotic was introduced prior to the formation of a biofilm. Therefore, many of the reasons for decreasing biomass with increasing tetracycline concentration are the same as were cited previously for the decrease in leucine label incorporation into protein by planktonic bacteria. Namely, the susceptibility of bacteria to tetracycline that then resulted in a lower total microbial biomass compared to the control treatments. A result similar to that of biofilm formation in the presence of an antibiotic has been observed in medical research. Uropathogenic *Escherichia coli* were introduced to

urinary catheters which had been previously incubated with MIC and sub-MIC concentrations of ciprofloxacin (Reid *et al.* 1993) so that the ciprofloxacin formed a thin film on the surface of the catheters. The *E. coli*, though there was a significant reduction in the formation of a biofilm, still was able to colonize the catheters. In the present study, not only was a biofilm able to establish itself on or near the stems in the presence of tetracycline, but also there was a surprising increase in EPS formation in 4000 $\mu g\ L^{-1}$ treatments in both the river and pond water experiments. To the best of the authors' knowledge, there has previously been no observation of a stimulation of EPS when bacteria have been challenged with an antibiotic (Professor G.M Wolfaardt, Environmental Microbiology, University of Stellenbosch, South Africa and Dr. J.R. Lawrence, Research Scientist, NWRI Saskatoon, Canada, personal communications).

EPS is thought to be important and beneficial to bacteria for many reasons. It may aid in the attachment of bacteria to surfaces, promote genetic transfer of material between bacteria in a biofilm, act as a nutrient reserve and be a stabilizing influence in fluctuating environments (Wolfaardt *et al.* 1999). Bacteria tend to produce more EPS when they are attached to surfaces then when they are sessile (Wolfaardt *et al.* 1999). EPS is also known to be very important in protecting the microbes against enzymes and antimicrobial compounds and this aspect of biofilms has been considerably studied (Korber *et al.* 1994, Coquet *et al.* 1998, Wolfaardt *et al.* 1999, Mah & O'Toole 2001, Aaron *et al.* 2002, Olson *et al.* 2002, Stewart 2002, Stone *et al.* 2002, Mazel & Davis 2003, Walters III *et al.* 2003). In these and other studies it has been noted that a greater concentration of antibiotics was required to be effective against bacteria that had formed a biofilm versus the same strain of bacteria that grew planktonically. As an example, Ceri *et al.* (1999) studied the resistance of planktonic versus biofilm growth forms of *E. coli* American Type Culture Collection (ATCC) 25922, *Pseudomonas*

aeruginosa ATCC 27853 and *Staphylococcus aureus* ATCC 29213100 to a suite of individual antibiotics such as ampicillin, ciprofloxacin and vancomycin. They found that the concentration of a given antibiotic required to be equally effective against the biofilm bacteria was 100 to 1000 times greater when compared to the concentration required against the same strain of bacteria growing planktonically. Similarly, Aaron *et al.* (2002) used single and combinations of antibiotics such as ciprofloxacin, tobramycin and piperacillin against planktonic and biofilm forms of *Pseudomonas aeruginosa* isolated from the sputa of adults with cystic fibrosis. They found that the biofilm bacteria were considerably less susceptible to single antibiotics and also to two or three drug combinations of antibiotics than the planktonic form of bacteria (Aaron *et al.* 2002).

It has been speculated that biofilms are more resistant to antibiotics and antimicrobial substances due to the EPS acting as a molecular filter blocking out toxic substances from the external environment (Dunne Jr. 2002). Once the EPS was formed in the present study, and the bacteria became encapsulated in the EPS polymer, the EPS may have acted like a barrier removing or reducing the direct influence of tetracycline against the bacteria producing the EPS polymer. Scientists have theorized that other mechanisms, such as the environment within the biofilm being anaerobic leading to inhibition of certain antimicrobials, or that growth rates of organisms within the biofilm may be slower resulting in minimum efficacy, may also be responsible for the reduced impact of antimicrobial agents against biofilm bacteria. Additionally, it has been suggested that when bacterial cells attach to a surface a change in phenotype occurs and some of these changes might induce some genes to become active so as to offer additional protection against antibiotics and antimicrobial substances (Mah & O'Toole 2001). The exact reasons of why there was an increase in EPS production, or if a single

or several species of bacteria or fungi produced the EPS, in the present study are unknown. However, an intriguing and plausible speculation is that EPS production was stimulated by the presence of a toxic or highly inhibitive concentration of tetracycline and was therefore a protective mechanism against tetracycline or its metabolites.

Total fungal biomass, from Day 7 to Day 33, in the river water was about 3 times higher in the 4000 $\mu g\ L^{-1}$ than the control while total fungal biomass was about 6 times greater in the 4000 $\mu g\ L^{-1}$ pond treatment compared to total fungal biomass in the pond control. This indicates that fungi were likely not directly inhibited by tetracycline but why did fungal biomass increase with increasing tetracycline concentration? There are two plausible reasons that may account for the increase in fungal biomass. First, the 70s ribosome, which tetracycline targets, is also present in the mitochondria of eukaryotes (Nester *et al.* 1998) and there is the possibility that microscopic mycovores like amoebae and mites (Newell 1996, Newell & Porter 2000), may have been negatively affected by tetracycline resulting in an increase in fungal biomass. However, neither of these was observed. Secondly, the increases in fungal biomass at the 4000 $\mu g\ L^{-1}$ concentration in both water experiments, despite diminishing microbial biomass (most of the decrease in microbial biomass was therefore bacterial biomass), suggests that bacteria may have had an antagonistic relationship with fungi and were able to restrict fungal dominance in the control and 500 $\mu g\ L^{-1}$ treatments.

While there have been no direct studies that have measured the influence of antibiotics on microbial colonization and plant decomposition, there have been attempts by researchers to determine the relative magnitude of roles played by fungi and bacteria, by using selective inhibitors, in litter decomposing in aquatic ecosystems. In these studies antibiotics have been used to suppress bacteria and nystatin or cycloheximide to suppress fungi in order to estimate fungal and bacterial contributions

to litter decay (Flegler *et al.* 1974, Padgett 1993, Velvis 1997). [Nystatin and cycloheximide are produced by the soil bacteria *Streptomyces noursei* and *Streptomyces griseus*, respectively. Nystatin binds to ergosterol and this nystatin-ergosterol complex disrupts the natural integrity of the fungal cell membrane leading to fungal death.] The use of selective inhibitors by these researchers has provided indirect evidence of largely antagonistic interactions between bacteria and fungi when the two compete for carbon substrates. In a study conducted by Flegler *et al.* (1974), to determine the role played by fungi in decomposing organic detritus, lake water and lake sediment containing plant litter was treated with the antifungals cycloheximide and nystatin. In another treatment, to inhibit bacteria, both penicillin and streptomycin were added to the water and sediment. In both treatments biochemical oxygen demand (BOD) was measured while direct counting and the pour-plate method were used to enumerate bacteria. Both of these parameters were determined prior to the introduction of inhibitory chemicals and 5 days after the experiment was commenced. No fungal hyphae length measurements or fungal isolations were conducted. Flegler *et al.* (1974) found that when antibiotics were added there was a decrease in both BOD and bacterial biomass while in the antifungal treatment there was an increase in BOD and bacterial biomass. The researchers concluded that in their system fungi inhibited bacterial growth.

In some recent work the interactions between fungi and bacteria on decaying litter in the aquatic environment were examined but instead of using selective inhibitors the researchers used other methods to research any inhibitory properties between fungi and fungi as well as between fungi and bacteria (Gulis & Stephanovich 1999, Moller *et al.* 1999, Gulis & Suberkropp 2003, Mille-Lindblom & Tranvik 2003). Gulis and Stephanovich (1999) isolated 29 species of aquatic hyphomycetes from Belarus

watercourses, grew the isolates in liquid media and then examined the filtrate of each isolate for antibacterial as well as antifungal properties. They found that the filtrate from 15 species inhibited bacterial growth while the filtrate from four inhibited fungal growth. In a study similar to the preceding, a single strain of fungus and a bacterium was isolated from leaves decaying in a stream and microcosms of sterile, decaying leaf litter were inoculated with only the fungal strain, only the bacterial strain or the bacterium and fungi in combination(Gulis & Suberkropp 2003). Microbial biomass, leaf mass loss and several other parameters were used to determine the resultant activity of fungi and bacteria in the different microcosm preparations. Gulis and Suberkropp found that fungal biomass and production were relatively unaffected by the presence of bacteria but they did find that bacterial production was slightly lower in the presence of fungi than in the absence of fungi. Overall, however, they determined that antagonistic behaviour between fungi and bacteria in their study to be mild.

The opposite was the case in a microcosm study conducted on beech leaves by Moller *et al.* (1999). These researchers isolated, from a forest floor, a conglomeration of bacteria plus a single strain of fungus that was dominant in this habitat. The bacterial and fungal isolates were then introduced to microcosms of sterile beech leaves either by themselves or in combination with each other. The highest mineralization rate of the leaves was seen in the microcosm that contained only the fungal isolate. In the treatment where the bacteria and fungi were combined, the mineralization rate was decreased by 50% thus strongly indicating the inhibition of fungi by bacteria. Mille-Lindblom and Tranvik (2003) also used microcosms containing sterile, homogenized *Phragmites australis* litter to study antagonistic or synergistic interactions between fungi and bacteria. The microcosms were inoculated with either bacteria or fungi or the two in combination and allowed to incubate for approximately 80 days. At the end of

the experiment the accumulated fungal biomass in the treatment where the fungi was alone was 2400 μg C g^{-1} dry weight litter but the fungal biomass was 200 μg C g^{-1} dry weight litter in the fungi + bacteria treatment. Accumulated biomass for bacteria also was higher when the bacteria were alone with 60 μg C g^{-1} dry weight versus 33 μg C g^{-1} dry weight in the bacteria + fungi treatment. In this experiment, then, antagonism was mutual with both microbial groups inhibiting each other. All of the above studies have shown some form of antagonism between bacteria and fungi in plant decay systems. There has thus far been only one study conducted on decaying litter where a synergistic or mutually beneficial association has been observed where both bacteria and fungi were more productive in the presence of each other than when alone (Bengtsson 1992).

In the *Scirpus* experiments fungal biomass was similar in the control and 500 μg L^{-1} treatments in river water which suggests that the bacteria or mycovores which were inhibiting the fungi were present in both the control and 500 μg L^{-1} treatments but was/were not present, or unable to exert any control, in the 4000 μg L^{-1} tetracycline treatments. Additionally, comparing the river and pond water experiments against each other, there was about 3 times more fungal biomass in the 4000 μg L^{-1} tetracycline river treatment versus the river control while there was approximately 6 times more fungal biomass in the 4000 μg L^{-1} tetracycline pond water treatment compared to the pond control suggesting that perhaps fungi were more inhibited by bacteria in the pond water system then in the river water system. The dominance of fungi in different aquatic and litter decay systems, which have ranged from 63% to 99% of total microbial biomass (Hieber & Gessner 2002, Verma *et al.* 2003), may be partially due to the inhibition produced by bacteria in those respective systems with bacteria playing a greater role in decomposition in the litter system where fungal biomass was lower. Fungi are thought to dominate organic decay processes in terrestrial systems such as forests and

agricultural soils but even in these systems there is considerable antagonistic activity between bacteria and fungi in some soils, likely due to inhibitory chemicals produced by some or many bacteria, inhibiting fungal germination and growth (de Boer *et al.* 2003). In some terrestrial systems, though, there have been reports of bacteria dominating litter breakdown (Velvis 1997). It is interesting to note that the majority of antibiotics, and many antifungal compounds such as cyclohexamide and nystatin, are derived from actinomycetes, a group of filamentous bacteria that contribute significantly to the turnover of carbon substrates such as lignocellulose and hemicellulose in sediment and soil systems (Mincer *et al.* 2002).

Unfortunately, because SYTO-9 stained bacteria and fungi equally (Figure 53), the proportionate parts played by fungi and bacteria in the present *Scirpus* study were unable to be estimated. However, the indications are, based on scanned images as well as the increase in fungal hyphae with decreasing bacterial biomass that fungi, while they may still be dominant, are highly unlikely to be in the 90% range of microbial biomass. The fungal production measurements made in Chapter 3, which as was stated were in the lower ranges of figures reported by other researchers, may be due to the facts that bacteria play a more significant role and also may be exerting an inhibiting influence on fungi in prairie wetland systems compared to other systems. More work is required and also stains that stain bacteria differently from fungi so that the relative ratios of biomass can be measured utilizing confocal laser microscopy. However, even then the ratios that are likely to emerge will be relative because much of the microbial biomass, such as the bacterial polymer, which can partially be estimated by the use of lectin stains, and fungal enzymes that are extruded onto the substrate, will unable to be largely accounted for due to limitations in staining dyes at the present time. Additionally, the longitudinal section of a *Scirpus* stem in Figure 54 demonstrates that

considerable fungal activity occurs within the stem and this too will remain unaccounted for using CLSM because of the opaque nature of the stem.

Total microbial biomass in the pond water system was approximately twice the total microbial biomass in the river system over the course of the experiments. This can be accounted for by the fact that the pond water system is much more enriched in dissolved organic carbon (DOC) and particulate organic matter (POM) compared to the river system (Waiser 2001a). The total dissolved organic carbon (DOC) in the river water (Table 8 in the previous chapter), was 3.3 mg L^{-1} while in pond water it was 48.8 mg L^{-1}. The DOC and POM would have provided an additional carbon source, over and above the carbon source provided by the decaying stem, thus resulting in the significantly more microbial productivities in the pond system compared to the river system. Additionally, the river water may have been limited in inorganic nutrients such as nitrogen and phosphorous, both of which are not limiting in Pond 50 water (Waiser 2001a), thus resulting in lower productivities on the stems decaying in river water. Prairie wetlands, such as Pond 50, are known to be amongst the most biologically productive systems in the world and part of the reason seem to be the very high DOC and POM present in these systems relative to other aquatic systems (Waiser 2001a).

Lastly, it was observed that bacteria were present on and near living fungal hyphae but it could not be determined if the bacteria had just happened to rest on the surface of fungal hyphae or if there was some symbiotic relationship between the bacteria and hyphae. This type of association, bacteria on or near both living and dead fungal hyphae, has been observed previously in decaying aquatic litter (Gulis & Suberkropp 2003) but it appears that no attempt thus far has been made to look in detail at the reasons for this behaviour. However, as speculated by Gulis and Suberkropp (2003), the connection may be due to bacteria utilizing breakdown products of plant

litter carbon that may be present near the hyphae. Bacteria have been known to colonize living and dead fungal hyphae in terrestrial soil decay systems (Brock 1966). While in a considerable part of this chapter we have discussed competitive tendencies between fungi and bacteria, there is no reason to assume that this competition occurs between all fungi and bacteria. There may well be strains of both bacteria and fungi that are resistant to the inhibitory compounds, antibiotics or antifungals, produced by either fungi or bacteria and there may well be mutually beneficial associations, associations that are intimate or based simply on the creation of a microhabitat by fungi, say, that is conducive to bacterial growth. After Day 10 in the present study, many of the fungal hyphae had turned brown and increasingly were colonized by bacteria, as is apparent in Figure 52. The fungal hyphae appeared to be dead, based on the fact that they did not take up the SYTO-9 stain. It is likely that the bacteria were using the hyphae as a carbon source and that the fungi had succeeded in playing its role in utilizing the nutrients from the stem, refractive to other organisms, into a source for its own growth and sustenance and it, in death, became a more labile and available nutrient source to other trophic levels.

5.3 Conclusions

Tetracycline significantly inhibited protein production, as measured by ^{3}H-leucine incorporation, in planktonic bacteria at concentrations > 10 $\mu g\ L^{-1}$ in the river water experiment but at 4000 $\mu g\ L^{-1}$ in the pond water experiment. The differences in inhibition between the two waters may be due to variations in the water matrix as well as differences in bacterial populations. Submersion of dry *Scirpus* stems in river and pond water, with or without the presence of tetracycline, resulted in dramatic increases in bacterial and fungal biomass in the form of a biofilm on the surface of the *Scirpus*

stem which peaked about 10 days after immersion in all treatments followed by a decline in biomass. The increase in microbial biomass seemed to coincide with an increase in moisture and the colonization of the external surface of the *Scirpus* stem and the decrease with the exhaustion of this substrate. The stem may have been internally colonized by fungi and bacteria and used as an energy source but this was not measured in the present study. The presence of tetracycline resulted in significant decreases in total microbial biomass in the two experiments. However, despite the presence of tetracycline bacteria and fungi were able to attach and form a biofilm on or near the *Scirpus* stem and continue the process of decomposition. The high concentration of tetracycline may have induced EPS formation so as to limit the toxicity of tetracycline. Fungal biomass was considerably higher at 4000 $\mu g\ L^{-1}$ compared to the control treatments, as bacterial biomass decreased, suggesting that in normal conditions bacteria exerted an inhibitory influence on fungi. Total microbial biomass on the stems was greater in the pond water experiment compared to the river water experiment, possibly because of the higher DOC and POM in the pond water, but fungal biomass was greater in the river system compared to the wetland system indicating that fungal dominance may vary in different water ecosystems. Lastly, laser microscopy is an appropriate tool to non-destructively measure the dynamics of microbial colonization on decaying litter. As such, since this is the first study in which laser microscopy has been used to study litter decay, there is considerable scope for future work and researchers.

CHAPTER 6 Conclusion and Recommendations

Environmental and anthropogenic factors influencing the microbial decomposition of the macrophyte *Scirpus lacustris* stems were studied from when the stems were standing to when they fell and were decaying in water. A suitable procedure for the extraction of ergosterol from *Scirpus* stems was first developed. Fungal production and biomass associated with standing dead *Scirpus* stems was measured in both the aerial and submerged stem parts over a season. Various temperature experiments were conducted in the laboratory, while one study was conducted in Pond 50 under various solar radiation schemes, in order to asses the impacts of temperature and solar radiation on fungal production and biomass associated with *Scirpus* decaying in Pond 50 water. The adsorptive, microbial and photolytic behaviour of tetracycline in distilled, river and wetland water was investigated prior to studying the impact of this antibiotic on microbes. The effect of tetracycline on planktonic bacteria was studied. Lastly, microbial colonization dynamics on the surface of *Scirpus* stems immediately following submergence of *Scirpus* in river and pond water, with or without the presence of tetracycline, were followed over 33 days by laser microscopy. These studies have resulted in considerable, and hitherto unknown, knowledge about the process of *Scirpus* decomposition in the wetlands of the northern prairies as well as the behaviour of tetracycline in prairie aquatic systems and its effects on environmental microbes.

Fungal decomposition on standing dead *Scirpus* stems, below or above the water, begins early in the spring despite low temperatures and seemingly low levels of moisture (15-22%) in the aerial stem parts. This implies that fungal production and biomass can be sustained even when moisture levels are low. Fungal biomass and production varied over the course of the season in both the aerial and submerged litter

parts of *Scirpus*. Part of the reason for these variances seemed to be due to fluctuations in temperature. Experiments conducted in the laboratory confirmed that temperature does have an impact on fungal production. It is likely, based on the short term temperature experiment, that fungal production will vary, on the same substrate, between the day and night on the prairies since temperature can fluctuate considerably in these shallow wetlands. (The temperature varied between 12°C and 25°C on the day the experiment to measure the influence of solar radiation on *Scirpus* decaying in pond water was commenced.) It is suggested, therefore, that an experiment should be conducted where fungal production is measured over a diel cycle to study if fungal productivities fluctuate in that time period. In the long term, temperature will also have a significant effect on biomass and production in the prairies with, all other factors being the same, a colder year likely to result in less fungal production and therefore slower plant decomposition. However, this difference cannot be extrapolated to cover different regions of the earth to indicate that fungal production will be lower in the arctic and higher in more temperate areas since the plant substrates, as well as the particular fungi utilizing the litter will likely be different and specialized to their respective habitats. The variances in fungal production and biomass that were measured may also be due to fungal succession on standing litter over the course of the season. Fungal successional patterns, if any, may be based on changes in temperature and radiation fluxes, or perhaps to the changing carbon profile of the stem as the stem is progressively stripped of its nutrients. A study measuring changes in fungal populations with respect to the three mentioned variables may lead to a further understanding of litter decomposition not only in prairies wetlands but also for other aquatic ecosystems.

Litter decomposition in the aerial portions of *Scirpus* stems is likely intermittent with fungal growth proceeding only during favourable conditions of temperature and

moisture. Not only the seasonal variances noted in Chapter 3 and discussed above bear this out but the germination of hyphae within a few hours of wetting *Scirpus*, plus the network of dead fungal hyphae observed microscopically on some parts of the stems surface and not others, also point to intermittent fungal growth. The optimum growth conditions for fungi occurred, in the aerial stem parts, when the moisture content in the plants was in the range of 15-22% and temperatures $\geq$ 15°C. However, prolonged wetness is likely to be deleterious to fungal production, as was noted in Chapter 3, at least in the aerial stem parts while the reverse is likely to be the case for the submerged plant portions. In other ecosystems, such as rainforests, the reverse is likely to be true with maximum productivities occurring with cessation of wetness, though one can never be sure since the biota in those loci is likely to be highly adapted and may prefer, as in the fungi in submerged *Scirpus* stems in the present study, a higher moisture content. Nevertheless, the annual fungal production was similar in the stem portions below and above the air/water or air/ground interfaces. However, because stems need to be wetted in order to measure productivities with the acetate to ergosterol method, the production measurements made in the aerial stem parts are only potential production estimates and the real productivities may be considerably lower than the productivities on submerged litter. Even so, it can be seen that considerable portions of the carbon and nutrients of *Scirpus* are utilized by fungi while it is standing and prior to abscission and submergence into the aquatic environment. The introduction of *Scirpus* in Pond 50, and this and other macrophytes in general in other Prairie wetlands, are the principal input of carbon and nutrients into these wetlands and are therefore the foundation of the food web in these ecosystems (van der Valk & Davis 1978, Murkin 1989).

By the time *Scirpus* dislodges and falls into an aquatic environment the surface of the stem is likely to look like a patchwork quilt with some areas devoid of the outer

covering while in other areas this will be intact. Upon entering the aquatic environment microbes will immediately, within hours, begin colonizing the parts of the stem where a suitable substrate, like the outer covering of the stem, is still available. It is not known if the microbes that colonize the litter once it falls into the water are already present on the surface of the stem, or introduced from the water or are a combination of the two. Nevertheless, the intense microbial activity brought about by an increase in moisture (assuming the litter is dry when it falls into the water) and the availability of a suitable substrate, will peak 7-15 days after immersion and will quickly subside thereafter. These peaks in production were observed in the experiment where *Scirpus* was allowed to decay in pond water under variable radiation schemes as well as in the *Scirpus* submersion experiments conducted in the laboratory and where laser microscopy was used to measure microbial activity. Solar radiation is unlikely to affect microbial colonization on the stems of macrophytes in Prairie wetlands, at least in Pond 50 and other systems where the radiation is considerably attenuated by the high DOC concentrations. Therefore, any impacts on microbes are likely to be minimal. Additionally, fungi are highly unlikely to dominate microbial biomass and production during the decomposition of macrophytes and, based on the increases in fungal biomass when bacteria were selectively inhibited in the tetracycline experiments, competition for substrate between fungi and bacteria very likely occurs on decaying *Scirpus* stems in Pond 50. The aforementioned is a good reason, along with the fact that the *Scirpus* stem is probably a more refractive substrate compared to other macrophytes, for the lower fungal biomass and production values in the present study compared to that reported for other systems. Prairie wetlands have not been exploited as sources of antibiotic and antifungal compounds so the discovery and isolation of chemicals that inhibit microbial life within these systems may be of economic benefit.

The decline in microbial activity in the present study coincided with the consumption of the outer covering of *Scirpus* but there may be other reasons for this decline and further experimentation is required before this question can be answered. Nevertheless, the scenarios documented here following litter submersion should in large measure hold true for other ecosystems where dry, dead plant litter falls into water and is colonized by microbes.

Prior to undertaking any work where the effects of a chemical against microbial populations or microbial processes are undertaken, some knowledge of the behaviours of the chemical under study is essential. Therefore, with this in mind, the absorptive and photolytic characteristics of tetracycline in distilled, river and pond water were conducted. The result from the adsorption study indicated that approximately 50% of the tetracycline was bound to the matrix of the river water while approximately 80% was bound to the matrix in the pond water. The portion of tetracycline that became bound could be freed by either changing the pH of the water or by the addition of a chelator such as EDTA. The environmental implication of this finding is that if tetracycline is introduced to an aquatic system, by runoff, sewage or disposal, depending on the matrix and pH of the water, the amounts of tetracycline that are bound and that are free in solution will vary as they did between the river and pond waters. More importantly, changes in the pH of the water will also change the bound to free distribution of tetracycline in different waters so that tetracycline that was bound and unavailable may become free and active if the pH is altered. The availability and effects of tetracycline on planktonic bacteria in the water will also differ, as demonstrated by the leucine incorporation experiment in river water where a very small amount of tetracycline caused a significant change in label uptake by bacteria whereas a significantly greater amount was required to produce an affect in the pond water

bacteria. These variances are probably due to the water's matrix as well as the microbial populations present. However, there is considerable work that needs to be done not only on the adsorptive and desorptive properties of tetracycline but also how populations of bacteria are affected by tetracycline under variable concentrations of ions, pH and DOC.

While tetracycline is known to break down in the presence of light, and as further demonstrated by the experiments conducted in the laboratory as well as in natural sunlight, in contrast microbial degradation is unlikely to be significant in natural aquatic systems. However, as was observed in the leucine and adsorption experiments, the matrix of the water will significantly impact the photolytic decay rates of tetracycline with some matrices hindering while other matrices promoting tetracycline photolysis. In the experiment where UV radiation was blocked, tetracycline breakdown was slower so that in waters where solar radiation is highly attenuated, as in Pond 50, or where the water is deep, the free tetracycline is unlikely to breakdown readily. Additionally, it was seen that tetracycline that was bound to the matrix was unaffected by light. The implication for the environment is that tetracycline, similar to terrestrial environments, is likely to persist in aquatic systems. Moreover, it should not be assumed that antibiotics are foreign to ecosystems since, as has been consistently pointed out in this dissertation, many of the antibiotics, as well as antifungal compounds, are naturally produced by organisms in the environment. Also, the author is not aware of studies conducted in pristine ecosystems where the natural, background levels of antibiotics have been established. The use of stable isotopes may aid in elucidating the origins of antibiotics being detected in the environment. It is evident from the experiments conducted that considerable more work is necessary before the

highly complex behaviour of tetracycline in different aquatic ecosystems can begin to be properly understood.

It can be said of tetracycline, however, that a very small amount, 10 $\mu g\ L^{-1}$, significantly inhibited protein production in planktonic bacteria and that there was a dramatic reduction in total microbial biomass on the surface of *Scirpus* stems decaying in pond and river water. However, despite the presence of tetracycline, bacteria and fungi were able to attach and form a biofilm on the *Scirpus* stem and continue the process of decomposition. Whether the rate of *Scirpus* decomposition was altered is not known and mass loss studies, in the presence of tetracycline, must be conducted in order to answer this question. Still, it can be said that tetracycline will alter microbial population structure but, as was seen, microorganisms are extremely versatile and adaptive and one cannot deduce or infer if microbial processes will be significantly changed. It is speculated that in normal circumstances, since the addition of tetracycline resulted in lower bacterial biomass but an increase in fungal biomass, that bacteria in both the river and pond may have exerted an inhibitory influence on fungi. The high concentration of tetracycline, however, seemed to have induced EPS formation. The reason or reasons for the stimulation of EPS production is/are not known but it is speculated that the production was to limit the toxicity of tetracycline. Since some microorganisms naturally produce antibiotics it should not be surprising that other microorganisms have evolved counter-measures to limit the effects of this inhibition and the formation of EPS may be one of those defensive mechanisms.

Much has been achieved and learned during the course of this study and has resulted in an increased understanding and new insights into the environmental and anthropogenic factors that may impact microbial life, decomposition processes and carbon cycling in the vast inland area of central North America.

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