A STUDY OF HOLDING CONDITIONS, FEED RATION, AND ALGAL FOODS FOR THE CAPTIVE CARE OF FRESHWATER MUSSELS

by

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Dissertation submitted to the Faculty of the

Virginia Polytechnic Institute and State University

In partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

in

Biology

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May, 2000

Blacksburg, Virginia

Key Words: Freshwater Mussels, Captive Care, Feeding

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(ABSTRACT)

The use of glass racks and suspended pocket nets for holding freshwater mussels collected from the Ohio River and relocated to lined-ponds was studied over 3 years. Survival of mussels in ponds was 73 % after 1y, 44 % after 2 y, and 5 % after 3 y. The glycogen levels of mussels in ponds for 1y were significantly greater than that of mussels in ponds after 2.5 y and 3 y, indicating a chronic decline in body condition in mussels. Despite the presence of a diverse and dense assemblage of algae and organic detritus in the ponds, the stomachs examined at 3 y were empty and the bodies were emaciated.

In the laboratory, I determined the amount of algae cleared by the rainbow mussel, Villosa iris (Lea,1829) fed different algal rations, and estimated the algae concentration needed to maintain mussels in captivity. Filtration rate in the first feeding hour was highest in ration B (1.0 mg dry wt$\cdot L^{-1}$) and lowest in ration C (3.4 mg dry wt $\cdot L^{-1}$). After 1 h, filtration rates declined in ration B but increased in rations C and A. V. iris likely achieved gut satiation in the first hour using maximum filtration (712.5 mL$\cdot h^{-1} \cdot g^{-1}$) and then decreased filtration (259 mL$\cdot h^{-1} \cdot g^{-1}$) thereby regulating ingestion rate during the following 2 h. I estimate, therefore, that V. iris daily maintenance requirement for carbon is 8.2 mg C (1.2 x 10^9 cells of N. oleoabundans) or ca. 2.4% of dry body weight. Assimilation efficiencies (AE) and carbon budgets also were established for the rainbow mussel, Villosa iris (Lea 1829), using radio-labeled cultures of Neochloris oleoabundans

(Chantanachat and Bold 1962) at three cell concentrations. Approximately 70% of the ingested carbon was assimilated (assimilation efficiency) by V. iris fed 5 x 10^5 cells $\cdot mL^{-1}$ (3.4 mg dry weight $\cdot L^{-1}$). At 5 x 10^4 cells $\cdot mL^{-1}$ (0.34 mg dry weight $\cdot L^{-1}$), AE was 47.5 %. At 5 x 10^3 cells $\cdot mL^{-1}$ (0.034 mg dry weight $\cdot L^{-1}$), AE was 40%. V. iris had the greatest amount of energy available for growth, reproduction, and body condition in captivity at 3.4 mg dry weight $\cdot L^{-1}$.

The gross composition (protein, carbohydrate (CHO), and lipid) of four algae (Bracteacoccus grandis, Neochloris oleoabundans, and Scenedesmus quadricauda, and Phaeodactylum tricornutum) was examined at four different phases of growth. The CHO content (% algal dry wt) increased with growth phase (age of the algal culture) with the exception of B. grandis. N. oleoabundans and P. tricornutum contained the greatest CHO content (33.07$\pm$ 6.89 % and 39.37$\pm$ 3.07 %, respectively) at late stationary phase. The total lipid content increased with growth phase for N. oleoabundans and P. tricornutum. Lipid content of B. grandis decreased with age, and S. quadricauda showed no difference in lipid content (% algal dry wt) between growth phase. N. oleoabundans' lipid content (31.85 $\pm$ 9.4%) was greater than all other species. Generally, there was no effect of phase on the sterol content, with the exception of the sterol content of S. quadricauda increased with growth phase. The mean sterol content of the four algae ranged 1.0 % $\pm$ 0.4 to 1.8 $\pm$ 1.8 of the total lipid dry wt; maximum sterol % of lipid was 5 % for Scenedesmus and 4.4% for B. grandis. There was no effect of growth phase or species on the protein content (% of algal dry wt). The protein content ranged 60.6 $\pm$ 17.1 to 70.3 $\pm$ 9.5 % of algal dry wt.

ACKNOWLEDGEMENTS

I would like to thank my committee members for their guidance, support, and constructive but gentle criticism of my research. I especially want to thank my advisor, Dr. Bruce C. Parker, for providing me financial support, space, and independence to conduct the research I wanted. Thank you Christine Parker for the many wonderful meals, conversation, and loving encouragement. I also wish to express my deepest gratitude to Dr. Richard J. Neves who provided me with endless insight into the management of natural resources, who never doubted my commitment to my work, and who always said exactly the right thing when times seemed quite difficult, and of course, thanks for his generous financial support.

I would like to thank Mathew A. Patterson for his help, incredible patience, and conversations on ecology and music. I am especially grateful to Dr. Daniel A. Kreeger, Academy of Natural Sciences, who generously provided his expertise on the design and analysis of my feeding studies, and without whom I could not have conducted this work. I thank Dr. Roger Newell, "Duke of Bivalve Biology", Horn Point Environmental Lab.

Finally, I want to thank Dr. Ann M. Kilkelly for teaching me how to tap dance my way through graduate school, for opening her heart and home to me, and giving me the opportunity to be an artist. I never knew life could be so wonderful, and FUN!!! To the girls, Carol Sue, Suzanna, Alicia, Nina, Deb, Gail, Anga, Samantha, Natalie, Michelle, Allison, Eileen, Rio, and Karen. And my deepest thanks to Carol Crawford-Smith, and UJIMA. Last, but not least. THANKS TO MOM AND DAD, and my fabulous sister and brother for their love and emergency loans.

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INTRODUCTION

The Unionidae is the largest family of freshwater pearly mussels, world-wide in distribution, with the greatest diversity (nearly 300 species) of freshwater mussels in the continental United States (Ortmann 1911, Baker 1928, Banarescu 1990). Within the last fifty years, significant declines in freshwater mussel populations have occurred due to river channelization and dredging, impoundment, and water pollution (Bogan 1993). More recently, the non-indigenous zebra mussel (Dreissena polymorpha) has invaded the Mississippi and Laurentian drainage systems threatening many vulnerable species with possible extinction (Herbert et al. 1991, Ricciardi et al. 1998). Unionids can often comprise a significant proportion of the benthic biomass (Negus 1966, Kryger and Riisgard 1988, Strayer et al. 1994), and by feeding at the base of the food chain on microscopic particles and depositing organic by-products to the benthos, they serve as important trophic agents in the functional ecology of the system. In addition, because of their unusual life history traits, unionids do not recover rapidly once populations have been depleted (McMahon 1991). In addition, the harvest and exportation of shell material for the cultured pearl industry has been valued annually at $40 - $50 million. Thus, the gradual decline of freshwater mussel populations throughout North America will significantly affect the ecosystem dynamics in some river systems and will affect the economic welfare of many commercial shellers.

To preserve the ecological and economic values of freshwater mussels to society, propagation of mussels for stock enhancement and preservation of endangered species has become a priority in the United States. One suggestion for perpetuating populations

of unionids heavily infested by zebra mussels has been to transfer some of these species to temporary refugia (free of zebra mussels) and determine whether controlled propagation is a feasible alternative to sustain long-term survival of freshwater mussels (Cope and Waller 1995). If captive animals can serve as broodstock, then a source of glochidia for induced host-fish infestations and juvenile culture will be available for stock enhancement of natural populations and for re-establishing endangered species.

My research goals were to determine the feasibility of holding freshwater mussels, salvaged from zebra mussel-infested waters, in pond environments, and to determine key feeding requirements of freshwater mussels in order to develop an appropriate feeding regime for their care in captivity. There are five chapters in this thesis; each chapter reports and discusses the results of specific objectives. Chapter 1 is a review of water quality criteria suggested for the captive care of freshwater organisms, along with a review of stress physiology in bivalves with which researchers must be concerned when transporting and holding unionid mussels. My objectives in this review were: 1) to develop a protocol for the collection and quarantine of unionids that prevented the spread of zebra mussels into uninfested refugia; 2) to construct a facility that housed up to 10,000 unionids; 3) to conduct a literature review of water quality parameters and make recommendations for holding unionids in captivity; and 4) to develop on-site algae cultures for feeding unionids.

In Chapter 2, my primary goal was to determine the feasibility of maintaining riverine mussels in 0.25 ha lined-ponds. My specific research objectives were to

compare survival, condition, and reproductive success among different species of unionids held in different containers and stocked at two different densities in ponds.

The success of conservation programs are currently limited in large part by our poor understanding of the feeding physiology and nutritional needs of freshwater mussels More specifically, filtration rates and the physical and biological factors affecting filter-feeding behavior must be identified to successfully culture mussels in captivity. A dearth of information on the filtration rates of marine bivalves has been published, with few studies on freshwater mussel filtration rates (Paterson 1984, Kryger and Riisgard 1988, Tankersley and Dimock 1993, McCall et al. 1995, Roper and Hickey 1995, Silverman et al. 1995, Vanderploeg et al. 1995). In Chapter 3, I examined the filtration rates of rainbow mussels (Villosa iris Lea, 1829) and determined the amount of algae cleared by mussels batch-fed a single concentration of algae, and filtration rates of rainbow mussels fed different concentrations of algae with the concentration replenished every hour for 3 h. This study was conducted to estimate the optimal algal cell concentration for feeding rainbow mussels in captivity.

No published information is available on digestive processes such as gut retention time, assimilation efficiency, and nutrient utilization by freshwater mussels. Bivalve energy stores will decline without proper feeding, and decreased energy reserves in adult bivalves negatively affected growth rates and developing offspring (Bayne and Thompson 1970, Gabbot and Walker 1971, Bayne 1972, Helm et al. 1973, Bayne et al. 1975, Patterson et al. 1997). Ultimately, the best food resources for maintaining mussel condition in captivity should be digested and assimilated with high efficiency while

providing the necessary nutrients for growth and survival. Detritus is a major food source of mussels in natural systems (Negus 1966, Strayer et al. 1994). In hatchery settings, however, the consistent production of algae, which has been nutritionally characterized, would be more practical than producing a detritus food source for the large-scale feeding of captive freshwater mussels. Radio-labeled algae cultures have been widely used to study carbon assimilation in bivalves including Corbicula fluminea, (Lauritsen 1986), Crassostrea virginica,(Newell and Langdon 1986, Crosby et al. 1990), Mytilus edulis (Kreeger et al. 1996, Wang and Fisher 1996), Mytilus trossulus (Kreeger and Langdon 1994), Ostrea edulis (Allen 1962), Argopecten irradians concentricus (Peirson 1983) and Geukensia demissa (Kreeger et al. 1988, Kreeger et al. 1990, Kreeger and Newell 1996). Many of these studies indicated that the efficiency of carbon assimilation is highly dependent on the concentration of food. In Chapter 4, I fed the rainbow mussel unialgal cultures of radio-labeled Neochloris oleoabundans at three cell concentrations and determined 1) gut passage and gut retention time, 2) the amount of carbon ingested at each cell concentration, 3) the proportion of the ingested carbon assimilated at each cell concentration, and 4) which cell concentration maximized total assimilation.

Microalgae play an important role in mariculture as food for molluscs, some crustaceans, and fish. The successful growth and development of cultured animals reared on microalgae is dependent on the proportion and availability of the biochemical constituents in the algae. Different researchers claim either total carbohydrate or protein to be more important depending on the animal species and life stage (Langton et al. 1977,

Flaak and Epifanio 1978, Webb and Chu 1983, Wikfors et al. 1984). Others report that lipids are more important for larvae as an energy source for developing organelles and metamorphosis followed by protein and then carbohydrates (Chu and Dupuy 1981, Wikfors et al. 1984, Enright et al. 1986, Napolitano et al. 1990). Adult bivalves, on the other hand, direct protein toward tissue growth and maintenance, and utilize carbohydrate as an energy source either for immediate respiration or long-term storage (Gallager and Mann 1982). Lipids are important to adults for maintaining cellular function, synthesis of hormones, and the regulation of processes by prostaglandins (Castell 1970, Pike 1971). Lipids also are very important during gametogenesis, especially in females to provide an energy source for subsequent embryo development (Pollero et al. 1983). Carbohydrates are accumulated by adult bivalves during the fall as fuel for over-wintering and subsequent gametogenesis during winter (Gabbott 1976). In Chapter 5, I examined the gross biochemical composition of 4 freshwater algae from the classes Chlorophyceae (3 species) and Bacillariophyceae (1 species). The gross composition (protein, carbohydrate, and lipid) as well as the fatty acid and sterol composition of these algae was examined at four different phases of growth. Based on the nutritional assays, I recommend various algae as diets for mussels, and recommended at what growth phase the algae are likely to be most beneficial to adult and juvenile mussels.

CHAPTER ONE

A PROTOCOL FOR THE SALVAGE AND QUARANTINE OF UNIONID MUSSELS FROM ZEBRA MUSSEL-INFESTED WATERS

ABSTRACT

In 1995, a quarantine facility was assembled on Middle Island, of the USFWS Ohio River Islands National Wildlife Refuge, that could hold several thousand unionids salvaged from the zebra mussel-infested Ohio River. The facility was supplied with well water and equipped with fourteen, 500 L tanks and aerated by a 0.5 hp regenerative blower. Twenty-seven hundred unionids of 6 species were collected in 1995, scrubbed to remove zebra mussels, wrapped in wet burlap, and transported 1-3 h in ice-cooled containers to the quarantine facility. Unionids were quarantined for a minimum of 30 d, reinspected for zebra mussels, and then relocated to pond refugia if uninfested. . Unionids were fed 10 L of a dense algal suspension 3 times weekly. Ninety-seven percent of unionids survived the summer 30 d quarantine. Suggestions for salvage and quarantine of unionids include: collect unionids when glycogen reserves are high and during cool months when metabolism is low, keep unionids cool during handling and transport, check for zebra mussels every 7 d to shorten the quarantine period to < 30 d, and feed unionids at least twice daily to maintain their condition.

INTRODUCTION

Nearly 70 mussel species (Unionidae) are at risk of extinction in the U. S., and another 12 species support a declining commercial harvest of shells for the cultured pearl industry in Asia (Williams et al. 1993). Habitat degradation; toxicological effects of chemical pollution from municipal, agricultural, and industrial effluents; and the destruction of mussel beds are responsible for the decline in unionid populations. In addition, invasion of the zebra mussel (Dreissena polymorpha) has the potential to wipe out many unionid populations (Neves 1993). For example, the lower Ohio River and its native unionid fauna have become heavily infested with zebra mussels to the extent that several states fear the eventual loss of many unionid beds and likely entire populations from the river (Chaffee 1993).

Extreme longevity, an unusual reproductive cycle, high juvenile mortality, and a sedentary lifestyle make unionids highly susceptible to perturbations (McMahon 1991). Because of these life history traits, unionids do not rapidly recover once populations are depleted. One suggestion for perpetuating populations of unionids heavily infested by zebra mussels has been to transfer some of these species to temporary refugia (free of zebra mussels) and determine whether controlled propagation is a feasible alternative to sustain long-term survival of species (Cope and Waller 1995). If captive animals can serve as broodstock, then a source of glochidia for induced host-fish infestations and juvenile culture will be available for stock enhancement of natural populations and for re-establishing endangered species. The immediate threat posed by the zebra mussel in the Ohio River led biologists at Virginia Tech, United States Geological Survey (USGS),

United States Fish and Wildlife Service (USFWS), Ohio Biological Survey (OBS), and West Virginia Department of Natural Resources (WVDNR) to establish a management priority of salvaging Ohio River unionids. A quarantine facility, therefore, was assembled in 1995 on Middle Island, Ohio River Islands National Wildlife Refuge (ORINWR) in St Mary's, West Virginia. Salvaged unionids from the zebra mussel-infested Ohio River were quarantined at this facility prior to being relocated to pond refugia. The long-term goal of this research was to determine the feasibility of using ponds as refugia for maintaining broodstock and for preserving species from possible extinction. The objectives of this paper were: 1) to develop a protocol for the collection and quarantine of unionids that prevented the spread of zebra mussels into uninfested refugia, 2) to construct a facility that housed up to 10,000 unionids, 3) to conduct a literature review of water quality parameters and make recommendations for holding unionids in captivity, and 4) to develop on-site algae cultures for feeding unionids. Information on the effect of nutritive stress (starvation) on unionid energy reserves and maintaining condition of unionids in quarantine facilities was recently provided by Patterson et al (1997) and Patterson (1998). I provide suggestions for improving each phase of the salvage and quarantine process, and recommendations for the salvage and care of quarantined unionids.

PROTOCOL FOR QUARANTINE

Acquisition and Handling of Unionids. During July of 1995, September, and October, 1995, a total of 2693 mussels of six species (630 Amblema plicata plicata, 731

Quadrula pustulosa pustulosa, 600 Elliptio crassidens, 481 Pleurobema cordatum, 137 Obliquaria reflexa, and 114 Potamilus alatus) were collected from the Ohio River. These unionids were targeted because they were big-river species occurring in zebra mussel-infested waters, and they could be collected in large numbers for experimental tests designed to determine the feasibility of using ponds as refugia for unionids threatened by the invading zebra mussel. Information recorded at the collection sites included method of collection (scuba or brail), river mile, water temperature, substrate type, and depth.

Unionids were collected using SCUBA at river miles 170, 176, 238, and 292.5 in 1995 (zebra mussel density ranged from 0 to 200/m^2). All unionids were examined for zebra mussels. They were hand-scrubbed on site with plastic-bristled brushes, 3-M scrubbing pads, and scraped of any attached zebra mussels and byssal threads. Shell-damaged unionids (broken hinges, crevices in the ventral margin) could contain veligers or juveniles that escaped initial inspection. Special care was taken with these specimens, and broken-shelled individuals were discarded to reduce the likelihood of infestation in the quarantine facility. Unionids were kept in large mesh-bags in a shaded area of the river to keep specimens cool until they could be cleaned, tagged, and measured. They were transferred to 20 L buckets after being scrubbed and tagged. Water temperature was monitored, and buckets of unionids were frequently iced to keep temperatures less than 28°C, similar to July river temperatures. The water also was renewed to replenish dissolved oxygen. Unionids were tagged (Hallprint Pty. Ltd., South Australia, Australia) in order to follow survival and condition of individuals over time. A small area of shell

was scrubbed with a 3-M pad, and acetone was applied by cotton swab over this area to prepare a dry surface for the glue to set. A very small drop of "super or crazy" glue was placed on the shell. The tag was applied with some pressure for 2 min to ensure the glue had set. The unionid remained in air (8 min) until the glue had dried. Total emersion time during the tagging process was approximately 20 min. Zebra mussel-free water was not available at our remote collecting sites. Scrubbed unionids were returned to a shaded area of the river (in mesh bags) until transported to the quarantine site. Placing unionids in the river was a convenient holding method prior to transport, but could allow reinfestation by zebra mussel veligers present in the river, depending on the time of year. If possible, scrubbed unionids should be held and transported in fish trucks with aerated, clean (zebra mussel-free) water to avoid re-infestation of unionids, and to reduce stress (see Transportation). In autumn, unionids were scrubbed on site; water temperature in the buckets was below 20°C, similar to the river. Scrubbed unionids were held in the river until transfer, and then tagged while in quarantine.

Transportation. The physiological effects of transportation to the laboratory and subsequent acclimation to aquaria or other holding facilities are relatively unknown in freshwater bivalves. It is known that in unionids experiencing respiratory or metabolic acidosis as the result of emersion or certain environmental pollutants, $CaCO_3$ reserves are dissolved to buffer protons, and calcium concentration in the haemolymph increases (Byrne and McMahon 1991). The effect of emersion, anoxia, and hypoxia differs among bivalves (Dietz 1974, Chen 1998). Many unionids may experience short periods of

emersion due to receding waters in drought conditions or post-flood conditions. These species may be better adapted to certain environmental stresses. Deitz (1974) reported that handling of Ligumia subrostrata altered the ionic concentrations in unionid body fluids, indicating stress. However, he also showed that L. subrostrata survived over 40 d in moist air but survived only 5-7 d in anoxic water. Analyses of the body fluids showed that the high body fluid solute concentration was due to water loss and not a build-up of metabolic products. Pekkarinen and Souranta (1995) found that Anodonta anatina, typically a lacustrine species, experienced stress from 15-20 min waiting time in a bucket of river water in which they were partially emersed before transport to the laboratory. Further storage and transport in river water resulted in increased glucose and calcium concentrations in the body fluids (haemolymph and extrapallial). Calcium levels decreased to near normal levels after 2 wk, but were still elevated after 2 mo acclimation in the laboratory. The authors suggested that the degree of normalization in calcium levels depended on the season and stage of reproductive cycle. Englund and Pynnonen (1996) showed that haemolymph calcium concentrations in A. anatina transported for 5 h without water under summer temperatures (20°C) increased more than in unionids transported in water at 20°C and unionids transported at 1°C with ice (cold but moist air). Unionids transferred in moist air with ice were assumed to have lowered their metabolism, which reduced the effect of transportation stress. After 17 d in the laboratory, these calcium levels were at or below normal, and unionids acclimated in sediment recovered from the transfer faster than those without sediment.

Hypoglycemia is indicative of stress in fish (Heath 1995), but the exact mechanisms leading to hypoglycemia in unionids are not completely understood (Chen 1998). Most physiologists assume the increase in glucose results from the mobilization of glycogen to meet maintenance energy requirements. The ability to tolerate hypoxia and the anaerobic metabolic capacity of bivalves is related to their glycogen levels (Hochachka 1982). The condition (amount of glycogen) of the unionid at the time of transportation, therefore, also may influence the extent of the physiological stress experienced in transfer and acclimation to a laboratory setting. In addition, some bivalves can reduce their metabolism in response to emersion (Wang and Widdows 1991). Pora et al. (1969) showed that oysters held in moist air at 15°C for 23.5 h, with a daily 30 min bath in hypercalcic seawater (Ca content double that of seawater), had lowered metabolic rates which increased survival compared to oysters held in water and starved over 2 wk. Finally, Waller et al. (1995) reported that unionids exposed to the atmosphere for 4 h (and less) had greater survival 4 - 5 mo in the river post-handling than those exposed to the atmosphere for 8 h. Thus, transportation in moist air for short periods (< 4 h) may not adversely affect unionids if glycogen reserves are high. In 1995, we transported unionids out of water 1 - 3 h. They were covered in moist burlap/towels. Ice was placed on top of the wet burlap (not directly on unionids) to keep unionids cool and lower metabolic activity. The ice was presumed to be chlorinated and was kept in plastic bags. Waller et al. (1995) also showed that unionids handled in October when temperatures were cooler had greater survival than those handled in June. Collecting

unionids when metabolic activity is lowest and condition is high, therefore, is recommended to minimize stress. The condition of the unionid depends on reproductive status and metabolic activity of the animal which varies among species. Some long-term brooders may abort glochidia if collected in autumn; however, gravid Villosa iris will hold glochidia in the laboratory for months if temperatures are kept between 12 °C - 16°C (personal observation). Further research to better understand the long-term effects of handling and transfer stress is needed for species being considered for relocation.

The Quarantine Facility. The quarantine facility was assembled in June, 1995 using resources from Virginia Tech, USFWS, USGS, and WVDNR. Groundwater seeping from the Ohio River into a well provided the water source. The facility consisted of fourteen 500 L fiberglass tanks, with insides epoxy-painted or coated (donated by the Aquatic Ecology Lab, Leetown Science Center (LSC), USGS, and the Bowden National Fish Hatchery, USFWS). Because bacterial contamination is often a problem in hatcheries, all donated equipment was washed carefully with a mild, biodegradable detergent and freshwater. An airline was plumbed to each tank, and upwellers were added to circulate the water (Figures 1, 2). Upwellers conveniently fitted into the drain-hole (inside tanks) which gave the upwelling pipe stability inside a tank of water. The upwelling pipe had to be removed from the drain-hole to drain the tank. A 0.5 hp regenerative blower (Sweetwater Model S-31, Aquatic EcoSystems Inc., Apoka, FL) delivered enough air to aerate all 14 tanks. An air cooling line, 1.5 m in length made from 3.75 cm galvanized pipe, was added to dissipate the heat generated from the blower

because air temperature from the blower exceeded 40°C. All tanks were plumbed to a common drain line with a gravity flow discharge; drain lines from each tank to the common line were fitted with ball valves. Only Schedule 40 polyvinylchloride (PVC) piping was used.

Preparation for Quarantine. Because unionids were held in the river prior to transport, they were rinsed with a high pressure hose before being placed in the quarantine facility. After 30 d, each unionid was hand-inspected with a 4X magnifying glass and direct light. In November, 1995, zebra mussels were found after the 30 d quarantine. These unionids repeated an additional 30-90 d in quarantine. This re-quarantine probably reduced their energy reserves, because the facility did not have adequate amounts of algae for feeding unionids in late autumn (Patterson et al. 1997). We recommend the following re-inspection procedures to avoid additional time in quarantine. Unionids should be re-inspected and re-scrubbed for zebra mussels that may have attached to unionids held in the river awaiting transportation. Careful inspection of the umbo area, crenulations of sculptured shells, and the ventral margin where new shell material is produced is advised. This is extremely time-consuming, and condition of mussels will be at risk if they are held out of water for too long, held without food, or held at elevated temperatures. Therefore, rinse unionids and place them in clean tanks in aerated water supplied with food. Then, hand-inspect each unionid, rinse with the high pressure hose again, and place them in their appropriate quarantine tank. Sterilize the tanks that held potentially contaminated unionids with 25 $mg \cdot L^{-1}$ chlorine and a mild detergent prior to reuse.

When the air temperature of the ice was more than 5 °C colder than the quarantine tank water, the unionids were placed in cool well water and gradually warmed to ambient temperature. A temperature change of 5 °C is lethal to most fish (Romaire 1985), and acclimation of 2 °C/h is recommended (G. Libey, Aquaculture Center, Virginia Tech, personal communication). Shumway (1996) reported that the rate of change in water temperature had a greater effect on survival and physiology of oysters than absolute temperature. The temperature of the water from the well at the facility was sometimes 13 °C. The tanks were filled 2 d previous to collecting the unionids so that river temperatures were similar. Acclimation of unionids entering the quarantine facility was rarely necessary.

The ORINWR quarantine facility is on the zebra mussel-infested Ohio River, but it is supplied with clean well-water. Potentially contaminated water, therefore, was drained on site without concern for further spread of the zebra mussel into an uninfested watershed. Brushes, buckets, tanks and miscellaneous equipment were sterilized with a concentrated chlorine solution (25 $mg \cdot L^{-1}$). The insides of the tanks also were scrubbed clean with a chlorine solution, and the equipment was allowed to dry for 4 d (if possible) before reuse (Brown and Gratzek 1980).

Quarantine Water Quality. Little information exists on the water quality requirements of unionids. Imlay (1973) reported that potassium (K) > 4 $mg \cdot L^{-1}$ was lethal to unionids. Alkalinity below 15 $mg \cdot L^{-1}$, hardness less than 47 $mg \cdot L^{-1}$, and pH < 6.1, however, were reported to prohibit unionid growth (Matteson 1955, Clarke and Berg

1959, Harman 1970). Grantham (1969) found no mussels alive where DO occasionally dropped below 3 $mg \cdot L^{-1}$. Adults and juveniles of several unspecified unionid "riffle species" required at least 2.5 $mg \cdot L^{-1}$ dissolved oxygen (DO) for survival at summer temperatures, but required at least 6 $mg \cdot L^{-1}$ DO for normal growth (Imlay 1971). Mouthon (1996) reported that DO less than 7 $mg \cdot L^{-1}$ had a limiting effect on freshwater molluscs in water of pH 8.1- 8.2. Chen (1998) showed that the responses to low DO varied among unionid species. Species adapted to environments which either seasonally or daily experience fluctuations in DO were better able to regulate oxygen consumption under low DO conditions. For example, the riffle-dwelling Villosa iris poorly regulated OC when DO was below 4 $mg \cdot L^{-1}$; whereas, Pyganadon grandis and Elliptio complanata regulated OC at DO >1.5 $mg \cdot L^{-1}$. In addition, the mussels' ability to maintain OC in low DO significantly improved at lower temperatures. Chen (1998) also showed that low DO intolerant species were less stressed (elevated blood glucose levels) when transported in oxygen-saturated water. Thus, threatened and endangered species should be transported in adequately oxygenated water in all salvage efforts.

A water chemistry analysis indicated that the pH (7.0), hardness and alkalinity (90 $mg \cdot L^{-1}$) and potassium levels (1.6 $mg \cdot L^{-1}$) in the well water of our quarantine facility were suitable for holding unionids. We set a minimum threshold of 7.0 $mg \cdot L^{-1}$ DO for maintaining unionids. Dissolved oxygen was measured twice daily (morning and late afternoon), and ranged from 6.0 - 14.0 $mg \cdot L^{-1}$ during the quarantine periods. Freshwater was added to the tanks every 2 d and dissolved oxygen increased with aeration.

Temperature tolerances vary among species of unionids; specifically, 29 °C was lethal for most Anodontoides ferussacianus tested, whereas most P. grandis and Lampsilis radiata luteola survived (Salbenblatt and Edgar 1964). Anodontoides ferussacianus is typically found in lotic environments, whereas the other two species can be found in a variety of habitats from riverine pools, reservoirs, and lakes to fast flowing streams (Bright et al. 1990). Lake-adapted and river-adapted species will likely have different temperature tolerances. Bayne et al. (1973) measured the scope for growth of the marine mussel, Mytilus edulis, at various temperatures and distingished between a "zone of tolerance" (Fry 1947) within which the effects of temperature were minimal, and a zone of stress within which temperature had a deleterious effect on growth. Between 10 °C - 20 °C, scope for growth was constant; between 20 °C - 25 °C, scope for growth was impaired, signaling effects of stress. We set the upper threshold temperature for holding unionids at 28 °C (similar to river temperatures in July).

Temperature was monitored twice daily (morning and afternoon). The summer temperatures in the quarantine facility ranged from 13 °C - 27.5 °C. The temperatures in autumn ranged from 2 °C - 18 °C. The greatest temperature change occurred in early November when the temperature dropped from 14.5 °C to 2.0 °C in 2 d. We saw no evidence of temperature shock from this change. However, we know little about the effect of cold temperatures on unionids. For example, can unionids alter their membrane composition in order to maintain fluidity and membrane integrity under various temperature extremes (Hazel, 1989)? On December 8, 1995, the water temperatures

dropped to freezing, and several hundred unionids died because ice formed at the bottom of some of the tanks.

Unionized ammonia (NH_3) is toxic to most aquatic organisms, but the ammonium ion (NH_4^+) is relatively non-toxic except at extremely high concentrations (Downing and Merkens 1955). Aquatic invertebrates are more sensitive to NH_3 than vertebrates, and the trend in sensitivity to NH_3 seems to be aquatic insects < molluscs < fish (Arthur et al. 1987, Hickey and Vickers 1994). Temperature and pH regulate the proportion of total ammonia that occurs in the unionized form in freshwater; the pH is most important. As temperature and pH increase, there is proportionately more toxic ammonia (Emerson et al. 1975). At pH 7 and 26°C, the percentage of TAN in unionized form is usually 0.60%; whereas at pH 8 and 26°C, more than 5% is unionized. Where temperatures are not lower than 5°C and pH values not higher than 8.0, the European Inland Fisheries Advisory Commission recommends a concentration less than 0.025 $mg \cdot L^{-1}$ NH_3 in salmonid waters (Solbe 1988). Toxic levels of NH_3 for short-term exposure reported for pond fish were between 0.6 - 2.0 $mg \cdot L^{-1}$, and sublethal levels occurred at 0.1 - 0.3 $mg \cdot L^{-1}$ (Boyd 1979). Zischke and Arthur (1987) reported that the lowest NH_3 levels affecting survival, growth, and reproduction in Musculium transversum were 0.09 - 0.16 $mg \cdot L^{-1}$. At pH 7.8 - 8.0, temperature 25°C, and total ammonia nitrogen (TAN) levels of 5 $mg \cdot L^{-1}$, unionized ammonia (NH_3) levels of 0.27 $mg \cdot L^{-1}$ were reported lethal to unionids after 7 d

exposure (Horne and McIntosh 1979). More recently, Hickey and Vickers (1994) reported acute toxicity in Sphaerium novaezelandiae after 96 h exposure to NH_3 levels of 0.33 $mg \cdot L^{-1}$. Goudreau et al. (1993) reported 50% mortality in glochidia of Villosa iris exposed to 0.284 $mg \cdot L^{-1}$ of unionized ammonia. Finally, Scheller (1997) showed that juvenile and adult mussels showed similar sensitivity to unionized ammonia (50% mortality at 0.49 and 0.56 $mg \cdot L^{-1}$ NH_3), while glochidia were the most sensitive to unionized ammonia (0.11 $mg \cdot L^{-1}$ NH_3). Following the European Inland Fisheries Advisory Commission, we set a conservative upper threshold for NH_3 at 0.025 $mg \cdot L^{-1}$. The summer temperatures in the quarantine facility ranged from 13°C - 27.5°C and pH ranged 7.2 - 8.0. If TAN reached 1.0 mg/L in the quarantine tanks, NH_3 would range from 0.002 - 0.066 $mg \cdot L^{-1}$. We measured TAN daily using a freshwater ammonia kit (Mydor, Ft. Lauderdale, FL). We changed the tank water whenever TAN reached 1.0 $mg \cdot L^{-1}$, temperature reached 27.5°C, or every 2 d to avoid potential accumulation of ammonia and other metabolites. TAN reached 1.0 $mg \cdot L^{-1}$ twice in the summer in 2 tanks containing a few dead unionids, and the water was changed immediately. The temperatures in fall ranged from 2°C - 18°C, and pH ranged from 8.1 - 8.5. By adopting a protocol of changing the water every 2 d, TAN was rarely over 0.25 $mg \cdot L^{-1}$, with the exception of the previously mentioned 2 days in summer. Therefore, the NH_3 levels did not exceed 0.025 $mg \cdot L^{-1}$.

If a quarantine facility is supplied by chlorinated city water, dechlorination is necessary. Chlorine (free and combined) is extremely toxic to aquatic species at concentrations as low as 0.1 $mg \cdot L^{-1}$ (Boyd 1982), and the amount of total residual chlorine depends on pH (Romaire 1985). The oyster, C. virginica exhibited 50 % mortality after 48 h exposure to 0.026 mg/L total chlorine residuals (Roberts and Gleason 1978). Glochidia of V. iris showed 50% mortality after 24 h exposure to 0.084 $mg \cdot L^{-1}$ monochloramines (a chlorine residual) (Goudreau et al. 1993). Because our quarantine facility was supplied with well water, dechlorination was not necessary. Depending on the volume, water can be dechlorinated with one to several days aeration (Spotte 1979).

Algae Culture. Lighting inside the quarantine building was not sufficient for indoor algal culture. Thus, in 1995 one epoxy-painted 500 L tank was set outside to take advantage of sunlight. Nitrogen (N), phosphorus (P), and light are usually the most limiting factors to algae growth. An N:P ratio of at least 10:1 will lead to a system with dense algal populations (Golterman 1975). Two tablespoons of plant fertilizer (Stern's Liquid Miracle Gro, Port Washington, NY) provided adequate N and P to stimulate an algal bloom in our outside tank. My objective was to provide the algae with at least 200 $\mu g \cdot L^{-1}$ N and 20 $\mu g \cdot L^{-1}$ P. Commercial fertilizers generally contain high levels of potassium which can be toxic to unionids (Imlay 1973). The algal cell density reached 1 x 10^6 $c \cdot mL^{-1}$ within 5 d. The outdoor tanks provided algae throughout the summer, enough to feed 2700 mussels 3 times weekly. The outside tank did not produce enough

algae for the facility during the cool autumn months. Therefore, algae were collected from local ponds, and grown indoors in glass aquaria to feed unionids in autumn.

In 1997, three 250-L clear, fiberglass tanks (Aquatic Ecosystems Inc., Apoka, FL) and cool-white fluorescent lightning were set up to grow unialgal cultures for feeding quarantined unionids. The green alga, Neochloris oleoabundans, was grown in 20-L clear carboys, and when this culture was between 10^6 and 10^7 c·mL^{-1}, a third of the volume was added to each of three, 250-L algal tanks. Fritz F/2 Media (Aquatic EcoSystems Inc., Apoka, FL) for freshwater algae was used according to specifications by the manufacturer. All algal cultures were aerated. Fresh media and water were added to fill half these tanks. After 24 h, more media and water were added to fill the tanks. These cultures reached 1 x 10^6 c·mL^{-1} within 4 d. High temperatures (>29°C) and insufficient lighting caused my indoor algal cultures to crash. Algae were grown indoors by innoculating with stock cultures in exponential phase, maintaining culture temperatures at 15-22^{0}C, and directing 60-100µE ·m^2·s^{-1} of cool white fluorescent light at each culture tank.

Holding and Maintenance of Unionids. In 1995, unionids were fed 10 L of a dark green culture of algae (cell density above 1 x 10^6 c·mL^{-1}) three times per week. Patterson (1998) provides information on maintaining the condition of unionids in quarantine. Unionids were stocked to maximum capacity in each of the tanks, which ranged 150-250 mussels per tank. Species were not mixed wherever possible, and unionids from different

river locales were never mixed in order to avoid cross contamination in quarantine. In the autumn, each tank of unionids was fed weekly with 2 L of a dense (10^7 c·mL^{-1}) algal suspension.

In 1995, 96.5% of unionids survived the summer 30 d quarantine Q.p. pustulosa exhibited the greatest mortality (43 specimens), which occurred in one tank stocked with 55 P. alatus and 145 Q.p. pustulosa. Dissolved oxygen levels were low (2.0 mg·L^{-1}) when compared to other tanks (6 - 7.2 mg·L^{-1}). A loose belt on the compressor contributed to low DO levels for several hours; however, dissolved oxygen levels came back up to 7.0 mg/L after the belt was repaired. Q.p. pustulosa in the tank with P. alatus continued to die, and DO levels were still low (4 mg·L^{-1}). Once these species were separated, no Q.p. pustulosa died in quarantine. Specimens of P. alatus were very active in their tanks. They may have consumed more oxygen than Q. p. pustulosa to meet their metabolic requirements. We would not recommend mixing other species with salvaged P. alatus in quarantine tanks until the oxygen demand for this species is determined. In addition, we recommend monitoring DO in 4 h intervals for the first day to ensure that oxygen levels in tanks fully stocked with unionids remains above 7 mg·L^{-1}.

Glycogen levels in unionids after 7 d starvation in quarantine were approximately 50% lower than in unionids from the river, and after 30 d, glycogen levels showed a 70-80% reduction (Patterson et al. 1997). Unionids fed twice daily at 1 x 10^5 c·mL^{-1} (4.0 mg dry wt·L^{-1}) of algae for 30 d maintained glycogen levels equivalent to those in the river (Patterson 1998). Although less than 2% of the starved unionids died in quarantine

(Patterson et al. 1997), survival in ponds after 1 yr was 70% compared to 90 % for unionids collected in 1995 and fed 3 times weekly (unpublished data, C. Gatenby). These preliminary results from the LSC ponds indicate that adequate food resources for quarantined animals are important to the survival and reproductive potential of unionids upon relocation to refugia. If resources are not available for rearing algae indoors, fertilizing outdoor ponds to maintain a dense population of algae is an alternative to indoor algae cultures. Because commercial fertilizers are high in potassium, we suggest fertilizing ponds as needed with a 10:1 ratio of N:P (McCombie 1953) using inorganic fertilizers that do not contain potassium. Feeding requirements will vary between species and will depend on metabolic activity at different temperatures. Until these feeding requirements for different species of unionids are known, we recommend unionids be fed twice daily at 4.0 mg dry wt$\cdot L^{-1}$ (Patterson, 1998). A suitable food source should be chosen that is digestible and nutritious to unionids (Gatenby et al. 1997, Patterson 1998).

End of the Quarantine Period. After 30 d all unionids were hand-inspected with a lamp and magnifying glass, for zebra mussels that may have escaped the initial inspection. Once all unionids were examined and no zebra mussels were present, the unionids were relocated to their experimental refugia. Assuming I had removed all attached juveniles and adult zebra mussels during the initial scrubbing and inspection, I presumed a 30 d quarantine period was sufficient time for hidden veligers to grow to a size visible with a magnifying glass. If any zebra mussels were found after 30 d, all unionids in the facility repeated another 30 d quarantine. In October, and again in November, zebra mussels were found. The unionids were quarantined an additional 30 d

each time zebra mussels were found, for a total of 120 d in autumn. Patterson et al. (1997) showed that condition (glycogen reserves) of unionids is at great risk if they are starved over 7 d. All quarantine facilities, therefore, should have resources for feeding unionids throughout the year. A quick freeze in December also caused high mortality of unionids in quarantine, and high indoor temperatures caused algal crashes. Quarantine facilities should be insulated against high heat and extreme cold. In order to shorten the total time in quarantine, we suggest inspecting unionids every 7 d, and to quarantine unionids for 30 d after the last zebra mussel is found.

SUMMARY OF RECOMMENDATIONS

Inadequate information exists on the water quality and feeding requirements of unionids. Users of this report, therefore, should treat our recommendations as guidelines, and note that future research must be conducted to determine the specific tolerances and requirements of unionids proposed for relocation.

1) To minimize stress-related effects of handling, keep unionids cool. Consider salvaging unionids during cooler months (fall) when metabolism is low and energy reserves are high.

2) Keep unionids cool ($< 28^0$C) during handling, , and in a shaded area of the river prior to transport. Monitor temperature and DO in buckets with unionids waiting to be scrubbed, tagged, and transported. Maintain temperatures $< 28^0$C and DO levels above 6 $mg \cdot L^{-1}$, and maintain upper threshold for NH_3 at 0.025 $mg \cdot L^{-1}$.

3) Scrub mussels with plastic bristle brushes or scour-pads on site, but do not remove the protective outer periostracum. Remove all zebra mussels. Do not collect shell-damaged unionids that could sequester zebra mussels and later contaminate the quarantine facility.

4) Transportation of unionids for short periods (< 6 h) in air, wrapped in moist toweling, and cooled does not appear to adversely affect their condition (Chen 1998). Enclose chlorinated-ice in plastic bags and avoid direct contact with the unionids. Transporting unionids in zebra mussel-free water, aerated, and supplied with algal food may further reduce stress. Transportation of endangered species or species adapted to riffle habitats in adequately oxygenated water should be considered, however, due to their potential intolerance of hypoxic and anoxic conditions.

5) Upon arrival at the quarantine facility, re-examine unionids for zebra mussels that may have attached from the river while unionids awaited transportation. Rinse unionids with a high pressure hose and place them in clean tanks supplied with food. Then, hand-inspect each unionid, rinse again, and place in the appropriate quarantine tank.

6) Avoid cross-contamination between tanks of unionids. Sterilize all equipment and waste water with 25 $mg \cdot L^{-1}$ chlorine, and allow equipment to dry for several days prior to reuse.

7) Hold unionids from different collecting sites in separate tanks (if possible) to avoid cross-contamination between individuals.

8) Stock tanks with a single species until dissolved oxygen requirements are known for different species. Monitor DO periodically for the first day to ensure oxygen levels remain above 6 $mg \cdot L^{-1}$

9) Inspect unionids every 7 d, and quarantine for a total of 30 d after the last zebra mussel is found. This may help to reduce total quarantine time if zebra mussels are spotted early in the quarantine period.

10) Care in quarantine:

a) Acclimate unionids to quarantine tank temperatures at $2^{\circ}C \cdot h^{-1}$.

b) Suggested water quality parameters for holding unionids:

i) Upper threshold temperature = 28°C.

ii) DO (summer temperatures ca. 24°C) > 6 $mg \cdot L^{-1}$.

iii) Potassium (K) < 4 $mg \cdot L^{-1}$.

iv) Alkalinity > 15 $mg \cdot L^{-1}$, hardness >50 $mg \cdot L^{-1}$, and pH > 6.5.

v) Upper threshold unionized ammonia (NH_3) < 0.025 $mg \cdot L^{-1}$.

vi) Total chlorine residuals < 0.026 $mg \cdot L^{-1}$. Depending on the volume, water can be dechlorinated using aeration for 24 h to several days.

c) Feed unionids at least twice daily with 1 x 10^5 $cells \cdot mL^{-1}$ algae or 4.0 mg dry $wt \cdot L^{-1}$.

RESEARCH NEEDS

Future research on the protocol for salvage and quarantine of unionids should focus on water quality tolerances of unionids in tanks and ponds (°C, DO, TAN, and pH); nutritional requirements for maintaining condition of unionids in captivity; and stress-related factors associated with transport, handling, and acclimation. In addition, unionid bivalve diseases, such as those associated with viral or bacterial infections, will need to be identified and their epidemiological importance studied. Methods for efficiently removing zebra mussels (primarily how to flush or remove veligers) that would shorten the quarantine period and avoid additional stress are needed. The optimal stocking density per water volume also should be determined for holding and rearing unionids in captive environments.

ACKNOWLEDGEMENTS

I am very grateful to Janet Butler, Janet Clayton, Kari Duncan, Mitch Ellis, Chris Gatens, Christina Kravitz, Debra Neves, Craig Snyder, Bill Tolin, Jack Wallace, Doug Wood, and other volunteers for their assistance in the field work from dawn till dusk. I also am indebted to Jim Dotson for his mechanical expertise, and Jon Cawley for his prowess at computer graphics. Funding for this project was provided by the U. S. Fish and Wildlife Service and U. S. Geological Survey.

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Figure 1. Schematic of Ohio River Islands National Wildlife Refuge quarantine facility: tank design with aeration and drain manifolds. Dimensions are in inches.

Figure 2. Fiberglass tank (500 L) with removable upwelling tube. Dimensions are in inches.

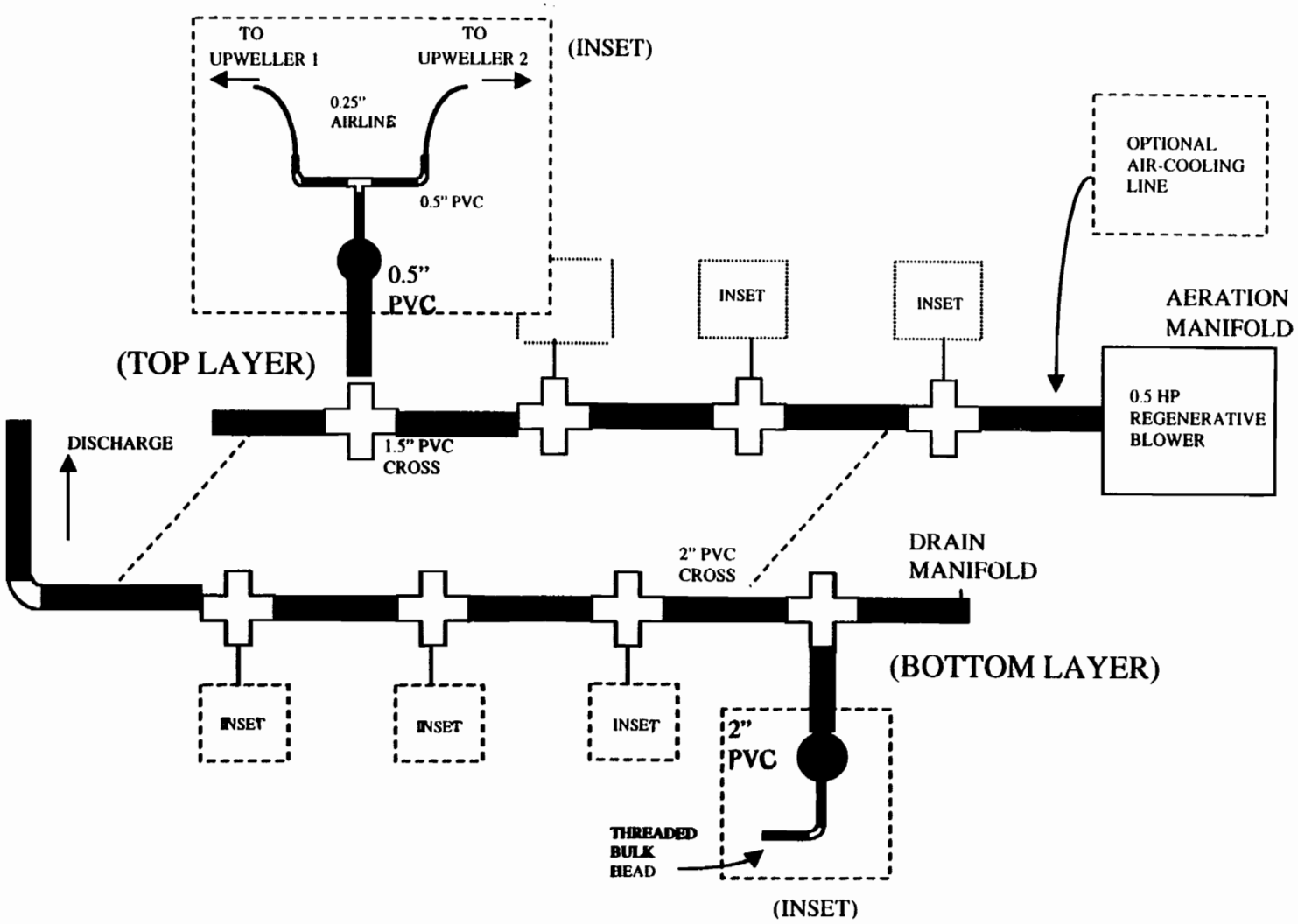

Figure 1. Ohio River Islands National Wildlife Refuge quarantine facility: Tank design with aeration and drain manifolds. Dimensions are in inches.

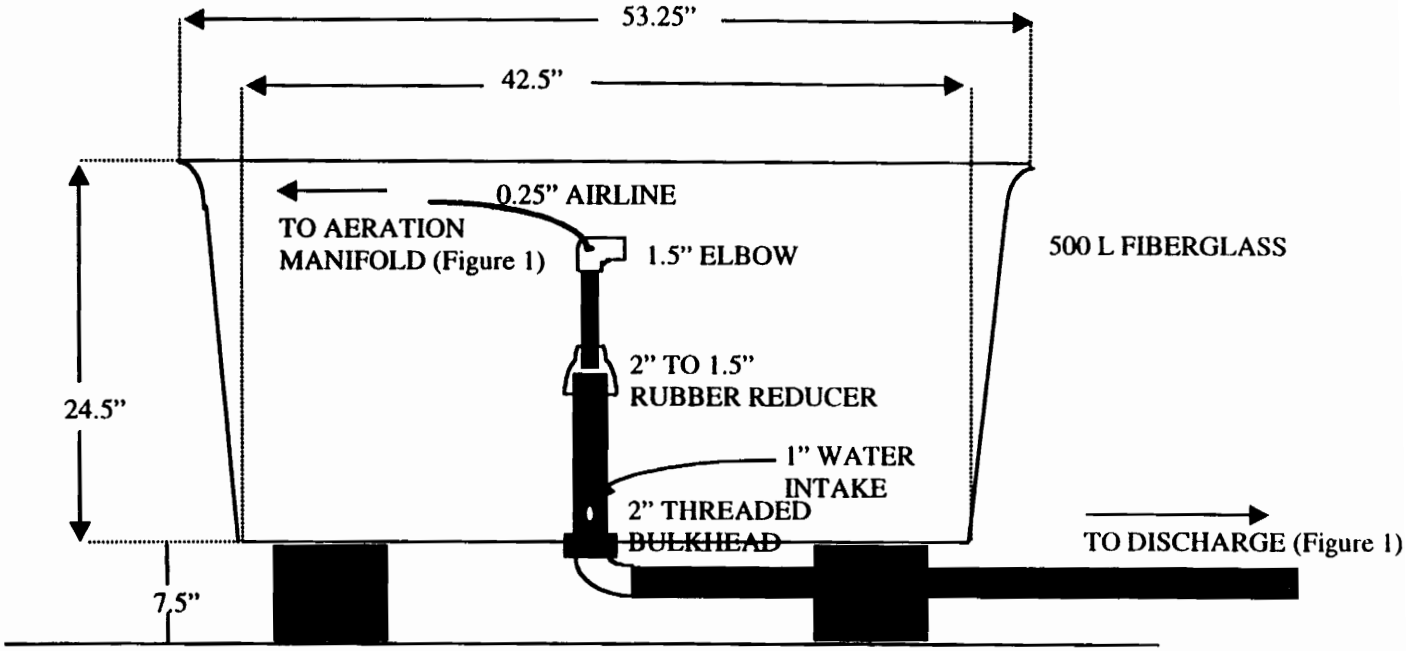

Figure 2. Tank (500 L) with removable upwelling tube. Dimensions are in inches.

CHAPTER 2

A STUDY OF SURVIVAL, REPRODUCTIVE ACTIVITY, AND BODY CONDITION OF FRESHWATER MUSSELS (BIVALVIA: UNIONIDAE) HELD IN LINED PONDS

ABSTRACT

In 1995, 2700 freshwater mussels (Unionidae) of 6 species were collected from the Ohio River and transported to ponds at the Leetown Science Center (LSC), West Virginia. Survival of unionids in lined ponds, glass racks, and suspended pocket nets was studied over 3 y. All unionids were quarantined for a minimum of 30 d, where they were fed live algae daily. A mix of diatoms, green, yellow-green, and golden algae were maintained in the ponds using a strict fertilization protocol throughout the summer. Survival of unionids at the LSC was 73 % after 1 y, 44 % after 2 y, and 5 % after 3y. Most of the mortality occurred when summer temperatures sometimes reached 30°C. Water chemistry analysis indicated pH, dissolved oxygen, alkalinity, and hardness were suitable for unionids. Additionally, unionized ammonia (NH_3), and ammonium (NH_4^+) were not toxic to unionids. Gravid females were rarely found at 1 and 2 y, and none were found at 3 y. Mean glycogen levels of mussels in ponds for 1y were significantly greater than the glycogen levels of mussels in ponds after 2.5 y and 3 y, indicating a chronic decline in condition of mussels held in ponds. Gut content analysis in 1996 indicated that unionids ingested a variety of algal cells from 3 - 80 µm. In contrast, the guts of mussels examined in 1998 were empty, despite the presence of a diverse and dense assemblage of algae and organic detritus in the ponds. The soft bodies of mussels in 1998 were emaciated. Unionids were seemingly stressed by the holding conditions of the LSC ponds, and discontinued feeding.

INTRODUCTION

Due to habitat degradation and the introduction of exotic species, over 100 native freshwater mussel species are at risk of extinction and another dozen commercial species support a declining commercial harvest of shells for the cultured pearl industry in Asia (Williams et al. 1993). For example, the lower Ohio River and its native mussel fauna have become heavily infested with zebra mussels, to the extent that several states fear the eventual loss of many unionid mussel beds and likely entire populations from the river (Chaffee 1997). Unionids can often comprise a significant proportion of the benthic biomass (Negus 1966, Kryger and Riisgard 1988, Strayer et al. 1994), and by feeding at the base of the food chain on microscopic particles and depositing much of what they eat to the benthos as organic by-products, they serve as trophic links in the functional ecology of the system. Recent declines in unionid populations, therefore, may adversely affect aquatic ecosystem integrity. In addition, because of their unusual life history traits, unionids do not recover rapidly once populations have been depleted (McMahon 1991). For these reasons, research aimed at the conservation of freshwater mussels has become a priority in the United States. One suggestion for perpetuating populations of threatened unionids has been to transfer some of these species to temporary refugia and determine whether controlled propagation is a feasible alternative to sustain their long-term survival.

OBJECTIVES

This research is part of an ongoing study on the culture and propagation of freshwater mussels at Virginia Tech. My primary goal was to determine the feasibility of maintaining riverine mussels in 0.25 ha lined ponds. My specific research objectives were to compare survival, condition, and reproductive success among different species of unionids held in different containers and stocked at two different densities in ponds.

METHODS

Acquisition and quarantine of mussels.

Collection and handling of mussels. Beginning in July of 1995, 2657 unionids of six species (Amblema p. plicata, Quadrula p. pustulosa, Pleurobema cordatum, and Elliptio crassidens, Potamilus alatus, and Obliquaria reflexa) were collected from the Ohio River: river miles (ORM) 170, 176, 238, and 292.5. In October 1995, specimens of P. alatus, P. cordatum, and O. reflexa were collected from ORM 238 and 292.5 to achieve the desired quantity of mussels needed for this study (Table 1). These species were targeted because they were 'big river' species found in zebra-infested waters. Unionids were collected using SCUBA. These were tagged, aged, measured, and sexed when possible. Recorded site information included was river mile, temperature, substrate type, and depth.

All unionids were examined and cleaned on site to remove any attached zebra mussels, and then transported to a quarantine facility. Special care was taken to avoid shell-damaged individuals where veligers could hide and potentially infest the quarantine

facility. At the collection site, unionids were kept in large mesh-bags in a shaded area of the river to keep specimens cool until they could be cleaned, tagged, and measured. They were transferred to 20 L buckets during scrubbings and tagging. Water temperature was monitored, and buckets of mussels were frequently iced to keep temperatures below28°C, similar to July river temperatures. The water also was renewed to replenish dissolved oxygen. Plastic tags (Hallprint Pty. Ltd., South Australia, Australia) were glued to the shells in order to follow survival and condition of individuals over time. Total emersion time during the tagging process was approximately 20 min. Zebra mussel-free water was not available at our remote collecting sites. Scrubbed unionids were returned to a shaded area of the river (in mesh bags) until transportation to the quarantine site. Placing unionids in the river was a convenient holding method prior to transport, but could allow reinfestation by zebra mussel veligers present in the river, depending on the time of year.

Transportation to quarantine. The body condition (amount of glycogen) of the unionid at the time of transportation will influence the extent of the physiological stress experienced in transfer and acclimation to a laboratory setting (Dietz 1974, Englund and Pynnonen 1996, Chen 1998). In 1995, I transported unionids in air 1 - 3 h, covered in moist burlap/towels. Ice was placed on top of the wet burlap (not directly on unionids) to keep unionids cool and lower metabolic activity. The ice was presumed to be chlorinated and was kept in plastic bags.

Quarantine. The quarantine facility was built in June, 1995 using resources from this research project. It consisted of fourteen 300 L tanks in which approximately 150-250 unionids were placed for 30 d. Groundwater, seeped from the Ohio River into a

well, provided the water source (Appendix 1). Airline plumbed to each tank provided aeration and circulated the water (Figure 1). Water quality parameters monitored daily were pH, dissolved oxygen, and temperature. Dissolved oxygen ranged 6-9 mg/L in the summer and 9-12 $mg \cdot L^{-1}$ in the fall. The tank water was changed every 3 d to avoid potentially toxic ammonia accumulations, or when the water temperature reached 28°C. Due to insufficient lighting inside the quarantine facility, a tank was set outside and fertilized to encourage a dense algal bloom for feeding quarantined mussels. Mussels in quarantine tanks were fed three times per week with 10 L of a dark green culture of algae; however, they were not fed during the first 2 wk in quarantine while algae grew to densities sufficient for feeding. After 30 d, the unionids were examined again to confirm absence of zebra mussels prior to transfer to the U.S.G.S. Aquatic Ecology Laboratory in Leetown, WV (Leetown Science Center (LSC). Mussels collected in June, 1995 were quarantined only 30 d. Additional time in quarantine was required in October and November, 1995 because juvenile zebra-mussels were found on some unionid mussels after 30 d and 60 d in quarantine.

Leetown Science Center Mussel Refuge

Description of ponds and pond management. Unionids were transferred to three 0.25 ha ponds at the LSC. The ponds were lined with a heavy–duty black rubberized-plastic. The water source was a reservoir on site. The ponds were managed as closed systems; aeration was provided by electric-powered paddlewheels. Only when the environmental conditions of the ponds were outside the prescribed range for maintaining

unionids were the ponds flushed continuously with reservoir water. We prescribed the following temperature, dissolved oxygen (DO), and water chemistry levels for managing the ponds to maintain unionids: i) upper threshold temperature = 28°C (Salbenblatt and Edgar 1964); ii) DO > 6 mg/L (Mouthon 1996); iii) pH > 6.5; and iv) unionized ammonia (NH_3) < 0.025 mg/L (Solbe 1988). When the environmental conditions of the ponds were outside these levels, they were flushed with reservoir water.

Environmental monitoring. Initial water chemical analysis included anions (Cl^-, SO_4^-), various metals (Ca^{+2} , Mg^{+2} Fe^{+2}, Zn^{+2}, and K^+), nitrogen ($N0_3^-$N, NH_4^+-N), phosphorus (ortho-$P0_4^-$), hardness, and pH (Appendix 2). Hardness and alkalinity were (87 and 80 mg/L) at a level believed sufficient for shell growth (i.e., the ponds were not acidic and shell material would not erode) (Matteson 1955, Clarke and Berg 1959, Harman 1970). Flow dynamics also were characterized. Flow rate in four directions (E,W, N, and S) at mid-depth (1m) and near the bottom (2-2.5 m) at 28 different locations within the pond was determined using an electronic flow meter (March-McBirney Inc., Model 2000.) (Figure 2). Water temperature and dissolved oxygen were monitored twice daily, in the morning and afternoon, at the surface and at the bottom of the pond; Secchi disk transparency depth and pH also was measured twice daily, in the morning and the afternoon. Total ammonium nitrogen (TAN) was monitored weekly using a Hach spectrophotometer. Nitrates and phosphates were analyzed at Virginia Tech's Environmental Chemistry Testing Laboratory. Nitrate (NO_3-N) was analyzed using ion

chromatography; orthophosphate (PO_4-P) was analyzed using the ascorbic acid modification of the molybdate spectrophotometric method.

Flow at mid-depth ranged 1.35- 0.03 m/sec (near the paddle-wheel to the opposite end of the pond); flow at the bottom, at the same locations as mid-depth, ranged (-0.18) - 0.33 m/sec. Negative flow measurements indicated that the direction of water movement was opposite of that generated by paddlewheel. Temperature was uniform at all depths, and ranged 22-29.5 °C in the summer, 9.8-24.7 °C in the spring, 3.1-11.5 °C in the fall, and 1.3-3.9 °C in the winter. Dissolved oxygen was uniform at all depths, and ranged 10.0-16.5 $mg \cdot L^{-1}$ in October - March, and 8.0-12.0 $mg \cdot L^{-1}$, in April - September. The pH was lowest in winter and highest in summer, and ranged 7.5 -9.0. Total ammonium (TAN) ranged 0.00 to 0.18 mg/L (November 1995-June 1996) during the months prior to fertilization, and 0.11-0.36 $mg \cdot L^{-1}$ (July 1996-October 1998) during the months in which ponds were regularly fertilized.

Algae management. We identified the algae to genus and quantified cells/mL each month beginning in August 1995 through November 1996, followed by alternate month samples through March 1997. The ponds were fertilized with a nitrogen to phosphorus (N:P) ratio of 10:1 (1.0 mg/L-N, 0.10 mg/L-P) (McCombie 1953) using NH_4NO_3 and $NaHPO_4$ salts. Once I established the dominance of green algae and diatoms, I adopted monthly fertilizations in July through October with an N:P ratio of either 10:1 or 5:1. Fertilization with N:P of 5:1 was adopted when phosphorus was limiting algae growth. Low N:P however, can favor blue-green algae; thus, I monitored the algal species assemblage in the ponds. The appearance of a filamentous, blue-green

algal bloom in June 1996, however, required that we fertilize with a N:P ratio of 20:1. This ratio encouraged growth of diatoms and green algae, and discouraged growth of blue-green algae. Before fertilization, I attempted to physically remove some of the filamentous algae. Because fertilization was discontinued in winter, N:P ratios were low in the following spring. To deter another filamentous blue-green bloom, fertilization with N:P of 20:1 was adopted in May 1997 and 1998. Monthly fertilizations with N:P of either 10:1 or 5:1 followed in July through October, in 1997 and 1998.

Experimental Design. All unionids were transported to the LSC refuge in fish-hauling trucks containing LSC pond water maintained at 20°C and aerated to maintain dissolved oxygen (DO) 7-9 mg/L. At the LSC, unionids were stocked in two different culture containers: commercial dish racks (racks) on the bottom of the pond and nylon pocket nets (nets) held in suspension, with the bottom row of mussels 40 cm from the surface of the pond. Each dish rack (45 cm x 45 cm) contained 25 individual cells measuring 9 cm^2, and the nets contained 30 pockets, 10 cm wide and 18 cm high. Floating booms were constructed from which the dish racks and pocket nets were suspended. Each pond contained 6 booms, on which all 6 species were distributed, in dish racks and nets (Figure 3). Unionids of identical species were stocked at two different densities in each holding container [low stocking density (Lo) = 5 mussels per culture container; high density (Hi) = 20 mussels per holding container] within each pond. I did not test the effect of density on P. alatus and O. reflexa because I did not collect enough specimens.

Amblema plicata was stocked in 9 dish racks and 9 pocket nets in each pond; 4 units of each culture container (racks or nets) were allocated to the high (Hi) density treatment (20 animals per container), and 5 units of each culture container were allocated to the low (Lo) density treatment (5 animals per container). Quadrula quadrula was stocked in 9 dish racks and 9 pocket nets per pond; 5 units of each culture container were allocated to the Hi density treatment, and 4 units of each culture container were allocated to the Lo density treatment. Elliptio crassidens was stocked in 8 dish racks and 8 pocket nets per pond; 4 units of each culture container were allocated to the Hi density treatment, and 4 units of each culture container were allocated to the Lo density treatment. Pleurobema cordatum was stocked in 8 dish racks and 8 pocket nets; 4 units of each culture container were allocated to the Hi density treatment and 4 units of each culture container were allocated to the Lo density treatment. Many unionids collected in October died in quarantine, due to a December freeze. Thus, 64 P. cordatum were stocked in a third pond in 6 racks and 6 nets; the Hi density contained 8 mussels in each container, and 2-4 mussels were stocked in containers allocated to the Lo density treatment. These 64 mussels were not included in the comparison between species because I presumed that these animals were in poor condition and would not survive as well as those mussels entering the ponds in September. Obliquaria reflexa was stocked in 4 dish racks and 4 pocket nets (5 animals per container) in each pond; Potamilus alatus was stocked in 2 dish racks and 2 pocket nets (5 animals per container) in each of 2 ponds, and in 3 dish racks and 3 pocket nets in a third pond (5 animals per container). Similarly, the O. reflexa and P. alatus entering the ponds in March, 1996 were not included in the

statistical comparisons between species and holding methods; however, the final % survival of these mussels was reported (Appendix 3).

Survival. Survival was monitored monthly. Unionids were considered dead if valves were found gaping or their valves did not close when pried slightly open. Mussels were assumed live if valves were closed or difficult to open. I used nonparametric methods to analyze the survival of the mussels that were released into the ponds during September 1995. Kaplan-Meier (KM) estimator was used to compute survival curves, and a Wilcoxon test was used to compare survival curves among treatments (holding container and stocking density). I also used the Cox-proportional hazards model to test for the effect of shell length while controlling for pond (Cochran and Cox 1957, Zar 1974).

Growth. Shell lengths of each individual were measured at the time of collection. At the end of 1 y, at least 100 specimens of each species were re-measured. One-way ANOVA was used to compare mean shell lengths and assess growth over time in the pond refuge.

Reproductive success. Mussels were examined using reversing pliers for charged marsupia, at putative times of gravidity. Examination from June through September. The majority of the species were tachytictic (short-term brooders). Ortmann (1919), however, reported an atypical breeding cycle by P. alatus; females could be gravid from April until December. Holland-Bartels and Krammer (1989) found P. alatus gravid from fall through the winter, releasing glochidia in late spring, which follows a typical bradytictic, long-term brooding cycle (Baker 1928). P. alatus was checked for gravidity

beginning in June through October. Viability of glochidia was confirmed by a saline test (Coker et al. 1921, Zale and Neves 1982). All individuals confirmed as females were noted in order to facilitate reproductive assessment in subsequent years. Because so few gravid females were found, only the total number of gravid unionids was reported.

Unionid physiological condition. I observed great differences in survival among unionid species after 1.5 years in the ponds, and therefore decided to monitor body condition. I measured glycogen because it is the primary energy reserve in adult bivalves and in many aquatic invertebrates (De Zwaan and Zandee 1972, Gabbott 1983, Hummel et al. 1989, Leavitt et al. 1990). Glycogen also has been used as an indicator of fitness in several mussels (Holopainen 1987, Haag 1993, Patterson et al. 1997). My original experimental design did not include sacrificing mussels over time for glycogen analyses; therefore, additional specimens were collected in 1996 and incorporated into the pond study. A. p. plicata and Q. p. pustulosa were collected from ORM 176 where zebra mussel densities were 0.3 zebra mussels/m^2 (P. Morrison, pers. comm.) and where 1995 collections had occurred. 10 A. plicata, 10 Q. p. pustulosa, and 10 P. cordatum also were collected from ORM 176 in May 1998 for comparison of glycogen levels with mussels in ponds. Glycogen levels are known to vary seasonally with reproductive activity and food availability (Gabbott 1983); however, only the glycogen levels in unionids in the Ohio River in May, 1998 was determined for comparison with condition of mussels held in ponds.

Glycogen in mantle tissue was determined and compared between species held in nets. Glycogen from unionids in racks was not determined because we had too few

specimens to compare glycogen between holding containers and among species. Thirty-six A. p. plicata from 3 ponds, and 9 Q. p. pustulosa collected from one pond were sacrificed in July, 1997 to determine condition of unionids after 1 y (1996-1997) in refugia. Eleven A. p. plicata, 10 Q. p. pustulosa, 10 P. cordatum, and 4 E. crassidens were sacrificed in March 1998 from one pond to determine condition after 2.5 y (1995-1998) in the refugia. In July 1998, 10 Q. p. pustulosa, 10 A. p. plicata, and 1 P. cordatum were sacrificed to determine condition after 3 y (1995-1998) in the pond refuge. Only one P. cordatum was used because only one specimen of this species was found alive after 3 y.

The glycogen content was determined using the technique described by Keppler and Decker (1974), with a minor modification (Patterson et al. 1997). A 50-100 mg sample of ethanol-preserved mantle tissue was dissected, blotted dry to remove ethanol and weighed. Tissue samples were homogenized for 2 hr in 3M perchloric acid and neutralized with 2M $KHCO_3$. Glycogen was converted to glucose with amyloglucosidase (Sigma Chemical Co.), combined with a dye solution containing o-dianisidine dihydrochloride, and read in a spectrophotometer at 450 nm. Total glycogen was determined from a standard curve of glycogen from the blue mussel, Mytilus edulis, and expressed in mg glycogen / g ethanol (ETOH)-preserved mantle tissue. Mean glycogen levels were not standardized by total body weight because simple regression revealed no correlation between wet weight and glycogen content ($r^2 < 0.10$; Patterson et al. 1997). The mean glycogen levels of unionids were compared between species and between collection periods using ANOVA. Scheffe's multiple comparison F-test was used to

further evaluate differences between species and collecting period treatment (Zar 1974, Sokal and Rohlf 1981).

The gut contents of ETOH-preserved mussels (15 A. p. plicata and 9 E. crassidens,) from 3 ponds in November, 1996 (1.2 y in ponds) were examined and quantified. In July 1998, the gut contents of A. p. plicata, Q. p. pustulosa, and P. cordatum also were examined. One shell valve was removed, the body washed to be sure no debris was confused with food item/gut contents. Gut contents comprised the digestive gland/stomach complex, and were identified using standard light and phase contrast microscopic technique on an Ausjena/Nomarski microscope.

RESULTS

Algae Assemblage and Cell Density. Algal density in the ponds from August, 1995 – August 1997 ranged 2.0 x 10^3 to 2.9 x 10^5 cells·mL^{-1}, and was dominated by chlorophytes, followed by bacilliarophytes (diatoms), cyanophytes (blue-greens), and miscellaneous chrysophytes and cryptophytes. Algal genera in all ponds were Chlorella, Scenedesmus, and the diatoms, Syndera and Navicula. Other genera of chlorophytes such as Chlamydomonas, Chlorococcum, Pandorina, and Eudorina, were abundant, as were the chrysophytes, Chromulina and Dinobryon, and the cryptophytes, Chroomonas, Cryptomonas, and Tetragonidium (Tables 2a, 2b, and 2c). Finally, the cyanophytes, Merismopedia, Gleocapsa, Schizothrix, Oscillatoria, and Chroococcus, also were present at varying density depending on season and the fertilization protocol.

Survival. The survival rates of mussels differed considerably among species (Table 3, Figure 5). E. crassidens survived poorly throughout the experiment; 19% survival after 2 y. A. p. plicata survived well (70%) until mid-experiment (approximately 600 d) and survived at higher rates than the other species for the entire 3 y study (Figure 5). Q. p. pustulosa and P. cordatum survived at levels intermediate (42% and 41%, respectively at 2 y) to E. crassidens and A. p. plicata. Survival was generally high for 600-700 d for all species, with the exception of E. crassidens, and then a "crash" in survival rates occurred (Figure 5). For the most part, there was no effect due to density, but there was an effect due to holding method. The effect of size (shell length (mm)) on survival varied among species and the length of time in the experiment (Table 4).

There was no effect due to density (Table 5), with the exception that A. p. plicata survived at slightly higher levels when held at higher density ($p = 0.056$; Figure 6). In general, mussels held in nets survived at higher rates. E. crassidens in nets had higher survival than those in racks throughout the experiment ($P < 0.0001$; Figure 7). Holding method had an effect on A. p. plicata ($p = 0.002$), but the effect was insignificant in the first half of the study and then increased at mid-experiment until the study ended (Figure 8). In contrast, the effect of holding method on survival in Q. p. pustulosa was opposite that of A. p. plicata. Q. p. pustulosa in nets had higher rates of survival in the first half of the study ($p = 0.0001$), and then a "crash" in survival occurred after 600 d and the effect of holding method was not significant (Figure 9). Finally, survival of P. cordatum was not affected by holding method ($p = 0.56$; Figure 10).

There was no effect of size (shell length) on the survival rate of E. crassidens (Figure 11). The effect of size on survival of A. p. plicata was apparent only after the "crash" in survival rate, when the smaller mussels had the lowest survival (Figure 12). In contrast, survival was higher for smaller specimens of Q. p. pustulosa and P. cordatum. Smaller Q. p. pustulosa survived at higher rates throughout the experiment (Figure 13). The effect of length on survival of P. cordatum was not apparent until mid-experiment (Figure 14).

Growth. Mean shell lengths from 1995 to 1996 were not significantly different within a species (Table 5); no growth in shell length (SL, mm) occurred in any of the species held 1 y in the ponds.

Reproductive success. Every mussel was checked for gravidity at least once each month beginning in May through October in 1996 and 1997. In 1996, I found only eleven gravid A. p. plicata. I verified gravidity through microscopic examination of a sample of glochidia removed from the marsupia with a needle and syringe. Upon examination following a saline test we found the marsupia charged with viable glochidia. In 1997, I found only 2 Q. p. pustulosa, 1 P. cordatum and no A. p. plicata gravid. We observed a gradual decline in body size (bodies became emaciated over time) throughout the study. In addition, it became easier to pry open the valves when checking for gravidity in 1997 indicating the adductor muscles had weakened over time or the mussels overall condition had weakened.

Gut and Body Condition. In November 1996, the gut contents of A. p. plicata and E. crassidens contained algae similar to those in the ponds. Chlorophytes, primarily

Chlorella, dominated the guts; however, diatoms and Chrysophytes also were common in the guts. Total algal cells were estimated at 5.0 x 10^6 and 6.3 x 10^6 in E. crassidens and A. p. plicata, respectively. Gut content analysis indicated that these unionids ingested a variety of algal cells from 3 - 80 μm. The guts also contained a mix of organic detrital material, likely derived from decaying leaves, algae, and animal material. The ash-free dry weight (AFDW) of gut contents was 19.9 mg and 28.3 mg for E. crassidens and A. p. plicata, respectively. In contrast, the guts of mussels examined in 1998 were empty, despite the presence of a diverse and dense assemblage of algae and organic detritus in the ponds. The soft-bodies of mussels in 1998 also were completely emaciated, indicating that the mussels had discontinued feeding (Figure 15).

Mean glycogen levels (10. 2 ± 4.2 $mg \cdot g^{-1}$) of mussels in the Ohio River in spring 1998 were significantly higher than the mean glycogen levels of all mussels held in ponds after 1, 2.5, and 3 y ($p = 0.0001$) (Table 6). In general, the glycogen levels of mussels in ponds for 1y were significantly higher than the glycogen levels of mussels in ponds after 2.5y and 3y indicating a chronic decline in condition of mussels ($p < 0.05$). In addition, A. p. plicata had greater glycogen levels than the other species at 2.5 y; however, there was no difference in glycogen levels between species at 3 y in the ponds.

DISCUSSION

Survival of unionids held in captivity was high for one year; however, after two years in the ponds, survival was extremely poor, indicating that the pond refuge environment or holding methods were not suitable for maintaining adult riverine mussels.

Many successful stock enhancement programs exist for endangered species (aquatic and terrestrial), and for the recreational and commercial fisheries, such as aquaculture of marine bivalves for the food industry (Ukeles 1971, Pinder and Barkham 1978, Brown and Gratzek 1980, Urban and Langdon 1984, Cohn 1988, Reid 1990, Andrews and Kaufman 1994). Thus, the life histories of these commercially significant species have been studied in great detail. For nearly 85 y, the biology of unionid mussels included only information on distribution, systematics, and some information on fish- host species, breeding season, and habitat preferences (Baker 1928, McMahon 1991).

As early as 1899, the U. S. Bureau of Fisheries was interested in conserving and protecting a national resource in freshwater mussels for the booming pearl button industry (Simpson 1899); thus, the Bureau attempted culture and propagation of freshwater mussels (Lefevre and Curtis 1910a, Surber 1912, Reuling 1919, Coker et al 1921, Howard 1922). These studies reported difficulties in maintaining mussels in captivity which they attributed to water quality and holding conditions (Lefevre and Curtis 1910, Howard 1917, 1922). These early investigators also believed that " finding suitable nutrition . . . " was critical to the success of rearing mussels (Lefevre and Curtis 1910, 1912, Coker et al. 1921, Howard 1922). Inadequate information is available on unionid nutrition other than a few studies to determine suitable diets for rearing juvenile mussels (Coker et al. 1921, Imlay and Paige 1972, Hudson and Isom 1984, Gatenby et al. 1996 and Gatenby et al. 1997). They showed that algae, detritus, and commercial fish food (as dissolved nutrients in the water) were potential food sources for juvenile freshwater mussels. The early investigators did not attempt the long-term maintenance of

broodstock, but when Wilson and Clark (1912) examined the stomach contents of river mussels they reported a small amount of plankton combined with a larger quantity of inorganic and organic detritus. Bisbee (1984) examined the gut contents of unionids in the Wisconsin River and found an abundance of green algae, diatoms, and the blue-green alga, Anabaena.

Enzymatic breakdown of ingested cell walls is an important initial step in digestion. The enzymatic capacity of unionids is unknown; however, many marine bivalves, such as Crassostrea virginica, possess extracellular cellulolytic activity (Brock and Kennedy 1992). Bisbee (1984) detected intact diatoms, green algae, some blue-green algae and some zooplankton in the digestive gland of Ligumia recta which suggests that these algae were suitable for intracellular digestion. It is puzzling how completely undigested algae could be found in the digestive gland, also referred to as the digestive diverticula. In bivalves, as the rate of consumption increases the diverticula becomes full, and excess food is voided as intestinal feces (Thompson and Bayne 1972). It is possible, therefore, to detect undigested food material in the stomach, intestine (mid and hind-gut) and rectum. Upon examination of gut contents (stomach/.diverticula gut complex), Miura and Yamashiro (1990) reported that a unionid, Anodonta calipygos, ingested and digested a variety of green algae.

The nutritional value of various chrysophytes, cryptophytes and bacillariophytes to marine bivalves is well documented (Walne 1970, Ukeles 1971, Enright et al. 1986, Wikfors et al. 1992, Thompson and Harrison 1992, Dunstan et al. 1993). In addition, a variety of green algae have been used to study the feeding requirements of several marine

and freshwater bivalves (Davis and Guillard 1958, Chu and Dupuy 1980, Foe and Knight 1985, Vanderploeg et al. 1995, Gatenby et al. 1996 and 1997). Generally, bivalves regulate their filtration rate within a range of particle concentrations and particle sizes, thereby regulating the amount of material that is ingested (Winter 1973, Higgins 1980, Riisgard and Randlov 1981, Navarro and Winter 1982, Paterson 1986, Kryger and Riisgard 1988, Way 1989, Tankersley and Dimock 1993, Roper and Hickey 1995, Vanderploeg et al. 1995). Therefore, assuming unionids in this study have the enzymatic capacity to digest algae and have similar feeding requirements for particle size and concentration as in previous studies, the algal assemblage in the LSC pond refuge appeared to be suitable food for maintaining unionids.

Howard (1922) suggested that "culture for the adult mussels consisted in providing the best environment for growth", and that growth rate of heavy-shelled species increased in flowing water as opposed to static water. It is generally agreed that flowing water ensures improved water quality by removing waste, increasing dissolved oxygen levels, and providing a constant food supply. The paddlewheels provided uniform dissolved oxygen throughout the ponds, and counts of algae at mid-depth indicated the algae remained in suspension. Despite the advantages to using a paddlewheel, there were areas within the ponds where flow rate was negligible. The flow rate declined further from the paddle-wheel source and was negligible at the bottom. Frequently, it appeared that competing flows lowered the actual rate of flow over mussels in racks. For example, I measured negative (reverse) flows nearest the sides of the ponds. Mussels held in suspension at mid-depth were exposed to greater flows than mussels held

in racks at the bottom. Mussels held in racks at the bottom for one year were covered with decaying leaves and fine silt; flow rate was insufficient to prevent sedimentation onto the mussels. After one year, the racks were raised 18 cm out of the zone of sedimentation; however, decaying leaves still settled onto mussels held in racks which we removed monthly.

Low DO and the production of H_2S by decaying organic matter may have caused mortality in the ponds, especially of mussels in racks on the bottom. In September, 1996, I tested for H_2S using a Hach colorimeter field test kit and tested DO at several locations on the bottom of each pond. I detected no H_2S; however, DO at the bottom microlayer between fine organic matter and water overlay decreased to < 4mg·L^{-1}. Two weeks later (late September, 1996), DO was 7-9 mg·L^{-1} at the bottom which coincided with a decrease in water temperature. In early October, 1996, the mussels racks were raised 18 cm out of the zone of sedimentation; DO remained 7-10 mg/L at this depth throughout the year.

Unionized ammonia (NH_3) is toxic to most aquatic organisms, but the ammonium ion (NH_4^+) is relatively non-toxic except at extremely high concentrations (Downing and Merkens 1955). Aquatic invertebrates are more sensitive to NH_3 than vertebrates, and the trend in sensitivity to NH_3 seems to be aquatic insects < molluscs < fish (Arthur et al. 1987, Hickey and Vickers 1994). Toxic levels of NH_3 for short-term exposure reported for pond fish were between 0.6 - 2.0 mg·L^{-1}, and sublethal levels occurred at 0.1 - 0.3

mg/L (Boyd 1979). Zische and Arthur (1987) reported that the lowest NH_3 levels affecting survival, growth, and reproduction in Musculium transversum were 0.09 - 0.16 mg/L. Where temperatures are not lower than 5°C and pH values not higher than 8.0, the European Inland Fisheries Advisory Commission recommends a concentration less than 0.025 mg/L NH_3 in salmonid waters (Solbe 1988). Therefore, I set a conservative upper threshold for NH_3 at 0.025 mg/L. No unusually high levels of unionized ammonia were detected throughout the study.

The effects of temperature on the survival and metabolic energy expenditure of molluscs have been described in detail for many marine species (Newell 1979, Newell and Branch 1980). The physiological effects of temperature are relatively unknown in freshwater bivalves, among which the unionaceans are least understood (McMahon 1991). We do know that temperature tolerances vary among species of unionids. Specifically, 29 °C was lethal for Anodontoides ferussacianus, whereas Pyganodon (Anodonta) grandis and Lampsilis radiata luteola survived at this temperature (Salbenblatt and Edgar 1964). Anodontoides ferussacianus is typically found in lotic environments. whereas the other two species can be found in a variety of habitats from riverine pools, reservoirs, and lakes to fast flowing streams (Bright et al. 1990). Lake-adapted and river-adapted species will likely have different temperature tolerances. Bayne et al. (1973) measured the scope for growth of the marine mussel, Mytilus edulis, at various temperatures and identified a "zone of tolerance" (Fry 1947) within which the

effects of temperature were minimal, and a zone of stress within which temperature had a deleterious effect on growth. Between 10 °C - 20 °C, scope for growth was constant; between 20 °C - 25 °C, scope for growth was impaired which was attributed to effects of stress. Temperature in the LSC ponds sometimes exceeded 25°C for 10-15 consecutive days, and occasionally exceeded 28°C for 4-5 consecutive days in the summer; mortality also was highest in the summer (Figure 16). Quick (1971) reported 54% mortality after 5-d exposure to 35°C in oysters acclimated to 16°C. The metabolic rate in most temperate animals is greatest in summer and least in winter (Burky 1983, Hornbach 1985). Many ectothermic species display a capacity for metabolic temperature acclimation, which involves adjusting the metabolic rate over a few days to several weeks at the new temperature regime. Thus, the organism would be expected to make compensatory adjustments to energy gain and energy expenditure allowing maintenance of an optimal metabolic rate. In contrast to most ectotherms, however, the unionids, Pyganodon grandis, P. cataracta, Utterbackia imbecilis, and Lampsilis radiata displayed no capacity for metabolic temperature acclimation (Huebner 1982, Pohill and Dimock 1996) and therefore, seasonal or diurnal metabolic fluctuations could cause energetic inefficiency. Because unionids experience seasonal fluctuations in environmental temperature, Pohill and Dimock (1996) suggested that an absence of acclimation may actually conserve energy at low environmental temperatures. Similarly, Marshall and McQuaid (1994) showed that the limpet, Siphonaria oculus, also lacked temperature acclimation, and suggested that this adaptation would conserve energy during times of

food shortages allowing the metabolic rate to decline with a seasonal decrease in temperature. If the unionids in the LSC refuge lack a capacity for metabolic temperature acclimation, then metabolic rates may be supra-optimal during extremely warm temperatures. Thus, energy expenditure was not matched by energy gain. High mortality in the summer could be related to an acute lethal effect of temperature exceeding the metabolic zone of tolerance for the unionids in this study, which is enhanced by the mussels poor physiological condition (low glycogen levels).

Complete analysis of gut contents is impossible because the fragile cells and quickly digested species are lost. The variety of identifiable cells found in the guts in combination with seston analysis of the overlying water did provide useful information on the consumption of particular species of algae that otherwise could only be proven using laborious radio-labeling techniques. The stomachs of mussels were full at one year in the ponds, but empty at 2.5 y and 3 y. Possibly, the mussels became stressed by the conditions in the pond refuge, which resulted in reduced valve gape, and disruption of feeding activity. Bivalves are known to close their valves or reduce valve gape when under stress (Brand and Roberts 1973, Akberali et al. 1981, Trueman 1983). When prolonged valve closure occurs, the pH of the body fluids drops and anaerobic respiration normally occurs (Akberali and Trueman 1985). Mobilization of calcium from the shell is then necessary to buffer the end-products of anaerobic respiration. Under long-term or repeated short-term stress conditions, there will be a greater demand for calcium which leads to a reduction in shell mass (Akberali and Black 1980). If the unionids are under anaerobic conditions, their ability to survive will be directly

proportional to their glycogen content (De Zwaan and Zandee 1972, Hochaachka and Mustafa 1972). For unknown reasons, all species in the ponds in both holding methods discontinued feeding, and slowly starved to death. I believe that the water chemistry and food of the pond refuge was suitable for maintaining unionids. Although it has not been proven whether a burrowing substrate is required by adult unionids, this physical feature of the natural environment was lacking from my pond study.

Whether the lack of a burrowing substrate and high temperatures were or were not the primary stressors that caused the unionids to discontinue feeding, it is obvious that these organisms do not thrive in artificial pond environments. Nor do they appear to thrive in many captive environments (Table 6). Comparisons among studies are difficult because different species, holding containers, and environments were tested. The captive systems that seem most suitable for holding unionids were environments supplied with continuous flowing river water. The provision of a burrowing substrate, however, does not appear to have prevented mortality in several captive-care facilities. High mortality at the federal fish hatchery in Frankfort, TN coincided with high temperatures (32°C) (Dunn and Layzer 1997); however, they also reported high temperatures (34°C) in the embayment of Kentucky Lake where mussels had been held for a year suffering low mortality (<15% mortality).

In assessing the decline in natural oyster reefs due to over-harvest, Kellog (1910) pointed out that "there can be practically no conservation without culture or cultivation". Coker et al. (1921) hypothesized that the mineral requirements of unionids were most likely the limiting factor for growth and not the organic food supply. This would imply

that different species have identical food requirements, and although substantiated with some evidence by Coker et al. (1921), it cannot be accepted as definitive. It is important to determine whether the food requirements of various species are the same, whether there is sufficient food supply for maintaining condition of captive unionids, and whether the mineral content of the water is sufficient to promote shell growth. It also is equally important to determine the parameters for optimum feeding activity, such as preferences for particle size, concentration, and burrowing substrate. Finally, a thorough examination of unionid stress physiology is needed that identifies conditions unsuitable for culture, especially the effect of temperature on feeding activity and metabolic rate.

ACKNOWLEDGEMENTS

I am very grateful to the staff at the Leetown Science Center for their assistance in building and maintaining the mussel refuge. I also thank Christina Kravitz, Tom Bunt, and Geraldine Jones for their incredible dedication to monitoring mussel survival and condition. The U. S. Fish and Wildlife Service and the U. S. Geological Survey provided funding for this project.

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LIST OF APPENDICES

Appendix 1. Water chemistry characterization of the Ohio River Islands National Wildlife Refuge well water supplying St. Mary's Island Quarantine Facility.

Appendix 2. Water chemistry characterization of the LSC pond refuge.

Appendix 3. Percent survival of unionids collected in October 1995, and held in the LSC pond refuge for 3 y. Initial number of unionids stocked in the ponds is given.

Appendix 1. Water chemistry characterization of the Ohio River Islands National Wildlife Refuge well water (n = 1) supplying the St. Mary's Island quarantine facility.

Water Chemistry	Concentration (mg/L)
Calcium	31.29
Magnesium	7.19
Sodium	19.54
Potassium	1.64
Carbonate	65
Sulfate	20
Nitrate-N	< 0.05
Chloride	50
Orthophosphate-P	0.095
Boron	0.038
Aluminum	< 0.025
Zinc	0.080
Copper	< 0.002
Iron	0.724
Manganese	1.592
Total Hardness	100.0
pH	7.0

Appendix 2. Water chemistry characterization of the LSC pond refuge, n=1.

Water Chemistry	Concentration (mg/L)
Calcium	22.43
Magnesium	7.42
Sodium	7.4
Potassium	1.62
Boron	0.007
Aluminum	< 0.025
Zinc	< 0.004
Copper	< 0.002
Iron	< 0.000
Manganese	0.009
Carbonate	80
Sulfate	4.6
Nitrate-N	< 0.05
Ammonia	< 0.02
Chloride	< 40
Orthophosphate-P	< 0
Alkalinity	80.0
Hardness	87
pH	8

Appendix 3. Percent survival of unionids collected in October 1995, and held in the LSC pond refuge for 3 y.

Species	Number of Mussels Stocked 0 y	% Survival 1 y	2 y	3y
Obliquaria reflexa	138	58 %	2 %	0 %
Potamilus alatus	78	23	6	0
Mean Total Survival		41 %	4 %	0 %

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Table 7. Survival (%) of unionids at various captive-care facilities. Holding environment and method is in parentheses under each facility location.

Table 1. The number of unionid mussels salvaged from the Ohio River, and the date transferred to the Leetown Science Center pond refuge in 1995.

Species	Number	Entry Date
Amblema p. plicata	630	9/95
Elliptio crassidens	600	9/95
Obliquaria reflexa	138	12/95
Pleurobema cordatum	481	9/95 (63 in 12/95)
Potamilus alatus	78	12/95
Quadrula p. pustulosa	731	9/95
Total	2657	

Table 2a. Algal genera and density (c/mL x 10^4) in Pond 1 (LSC pond 19).

Date	Chlorella	Diatoms	Scenedesmus	Cosmarium Staurastrum	Chlorococcum Chlamydomonas Pandorina Eudorina	Cryptomonas Tetragonidium Chroomonas	Dinobryon Chromulina	Blue-green algae	Total
Aug-95	16.72	0.0575	0.0581	0.2451	0.1677	0.0039	0.0	0.9237	19.17
Nov-95	0.1203	0.0533	0.0013	0.0041	0.0035	0.0	0.0	0.0147	0.198
Mar-96	1.256	0.1488	0.0059	0.0211	0.0412	0.0	0.0	0.0069	1.675
Jun-96	5.476	1.4324	2.5231	0.0187	0.1414	0.00	0.0	0.2690	10.32
Aug-96	1.6397	2.6290	0.8510	0.7494	0.0493	0.0021	0.0017	1.4183	7.457
Oct-96	2.0204	0.3328	2.2148	0.3877	0.0472	0.0	0.4084	1.4657	6.238
Mar-97	0.0617	0.5854	0.2714	0.1336	0.0145	0.0	1.5983	0.0479	2.355
Jun-97	1.1897	0.6216	0.8756	0.2439	0.0668	0.0	0.0084	2.5567	6.602
Aug-97	7.5446	1.6886	1.3450	2.0519	0.0835	0.0	0.0	0.7706	13.75

Table 2b. Algal genera and density (c/mL x 10^4) in Pond 2 (LSC pond 20).

Date	Chlorella	Diatoms	Scenedesmus	Cosmarium Staurastrum	Chlorococcum Chlamydomonas Pandorina, Eudorina	Cryptomonas Tetragonidium Chroomonas	Dinobryon Chromulina	Blue-green algae	Total
Aug-95	20.7860	0.0991	0.0708	0.0037	0.0652	0.0652	0.0	0.5345	22.14
Nov-95	0.0090	0.0010		0.0001	0.0003	0.0002	0.0005	0.0060	0.210
Mar-96	0	0.1196	0.0016	0.0047	0.2774	0.0044	0.0057	0.0776	0.478
Jun-96	20.509	0.5498	1.5021	0.3927	0.4613	0.2573	0.1275	0.8246	28.56
Aug-96	0.3806	0.2217		0.0317	0.1875	0.8589	0.0113	1.5482	3.52
Oct-96	0.2631	0.1178		0.0166	17.1686		0.3210	6.9802	25.10
Mar-97		0.2859	0.0239		0.0887	1.1286	0.0327	0.2764	1.82
Jun-97	0.0844	0.0668		0.0030	9.4539	3.7404	0.0510	13.7876	27.79
Aug-97		0.0167	0.6225		0.2255	0.6240	0.0334	1.0068	6.454

Table 2c. Algal genera and density (c/mL x 10^4) in Pond 3 (LSC pond 21).

Date	Chlorella	Diatoms	Scenedesmus	Cosmarium Staurastrum	Chlorococcum Chlamydomonas	Cryptomonas Tetragonidium Chroomonas	Dinobryon Chromulina	Blue-green algae	Total
Aug-95	10.185	0.0142	0.0227	0.1541	0.439	0.0	0.0	0.2245	10.72
Nov-95	0.1110	0.0021	0.0001	0.0089	0.0004	0.0	0.0006	0.0140	0.14
Mar-96	0.2008	0.0683	0.0015	0.0079	0.0095	0.0	0.0185	0.0381	0.35
Jun-96	12.35	0.4417	2.415	0.0245	0.0736	0.1767	0.0049	1.0211	17.2
Aug-96	0.8602	0.1712	0.5143	0.0347	0.0351	0.0126	0.0129	0.1583	1.83
Oct-96	2.2137	2.3091	0.9178	0.3175	0.1796	1.3615	0.0013	1.6970	9.17
Mar-97	0.1261	0.0684	0.1991	0.0032	0.0142	0.0	1.0886	0.0086	1.52
Jun-97	5.1757	11.4864	13.8504	0.1217	0.3848	0.0	0.0	0.00	31.20
Aug-97	8.1881	1.8565	1.1163	0.0267	0.0217	0.0	0.0	0.00	11.24

Table 3. Percent survival of unionids held in the LSC pond refuge for 3 y.

Species	Number of Mussels Stocked 0 y	% Survival 1 y	2 y	3y
Amblema p. plicata	630	93	70	19
Elliptio crassidens	600	77	19	0
Pleurobema cordatum	481	82	41	1.4
Quadrula p. pustulosa	731	85	42	0.8
Mean Total Survival (%)		84	43	5.3

Table 4. Wilcoxon test whether survival curves differ among levels of the covariates, length, density, and method of holding mussels in the Leetown Science Center's pond refuge.

Covariate	Amblema p. plicata		Elliptio crassidens		Quadrula p. pustulosa		Pleurobema cordatum	
	Chi-sq	P-value	Chi-sq	P-value	Chi-sq	P-value	Chi-sq	P-value
Length	7.3008	0.0069	0.00164	0.9677	16.6091	<0.0001	6.0784	0.0137
Density	3.6421	0.0563	0.5712	0.4498	0.2378	0.6258	1.5039	0.2201
Method	9.8011	0.0017	17.1143	<0.0001	17.7930	<0.0001	.03395	0.5601

Table 5. A comparison of mean shell lengths at the time of collection and after one year in the LSC pond refuge. P-values are given.

Species	Shell Length (mm) ± SD August, 1995	Shell Length (mm) ± SD August ,1996	P-value
Amblema p. plicata	93.4 ± 33.75, n = 624	93.1 ± 7.2, n = 196	p = 0.932
Elliptio crassidens	104.8 ± 6.62, n = 582	105.2 ± 6.06, n = 154	p = 0.412
Pleurobema cordatum	83.9 ± 8.96, n = 472	84.6 ± 8.54, n = 139	p = 0.423
Quadrula p. pustulosa	54.8 ± 5.2, n = 673	55.1 ± 5.2, n = 147	p = 0.428

Table 6. A comparison of mean glycogen levels (mg glycogen / g dry ETOH-preserved tissue) of unionids in the Ohio River (OR) and in the Leetown Science Center pond refuge after 1 y, 2.5 y, and 3 y. Scheffe's F tested at $\alpha = 0.05$. Means followed by the same upper case superscript at 2.5 y are not significantly different.

	OR 1998	1y	2.5 y	3 y
A. p. plicata	10.2 ± 4.17^{a}	4.3 ± 2.39^{b}	$1.5 \pm 2.23^{c,A}$	1.25 ± 1.18^{c}
E. crassidens			0.37 ± 0.72^{B}	
P. cordatum	7.1 ± 3.56^{a}		$0.07 \pm 0.17^{b,B}$	$0.99 \pm$ na^{b}
Q. p. pustulosa	$13.3 + 3.34^{a}$	$3.0 + 2.15^{b}$	$0.25 + 0.31^{c,B}$	$0.99 + 1.03^{c}$

Table 7. Survival (%) of unionids at various captive-care facilities. Holding environment and method is in parentheses under each facility location.

Facility	Time 1 y	2 y	3 y
Leetown Science Center[1]	84 %	43 %	5 %
(Static Pond/Nets and Racks)			
White Sulfur Springs[2]	89	58	< 10
(Flow-through Pond/Substrate)			
National Fish Hatchery, Genoa, WI[3]	50 - 79	24 - 61	10 - 52
(Flow-Thru Earthen Pond/Suspended or buried in substrate)			
Frankfort Fish Hatchery	< 10		
(Earthern, flow-through pond/ suspended nets)			
Laurel Hill[4]			
(Spring-fed Earthen Pond/Nets)	50 – 90	Survival declined	
Elkhorn Station[4]			
(Raceway,Pond/Nets)	50 - 80	50 - 80[5]	
Minor Clark[4]	> 89		
(Flow-through Raceway/Substrate)			
Kentucky Lake[4]	> 85		
(Flow-through embayment/ Nets)			
Marion, VA State Fish Hatchery[6]			
(Static Pond-Suspended)	25 - 67		
- Bottom	0 - 22		
Reynolds Pond, Blacksburg, VA[6]	14 - 90	< 5	
(Static Pond/suspended and non-suspended baskets with no burrowing substrate)			

1. This study. 2. Kari Duncan, pers. comm. 3. Teresa Naimo, pers. comm. 4. Dunn and Layzer (1997). . Layzer et al. (1999). 5. Burress (1995).

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Figure 16. Freshwater mussel monthly mortality and water temperature in the LSC pond refuge.

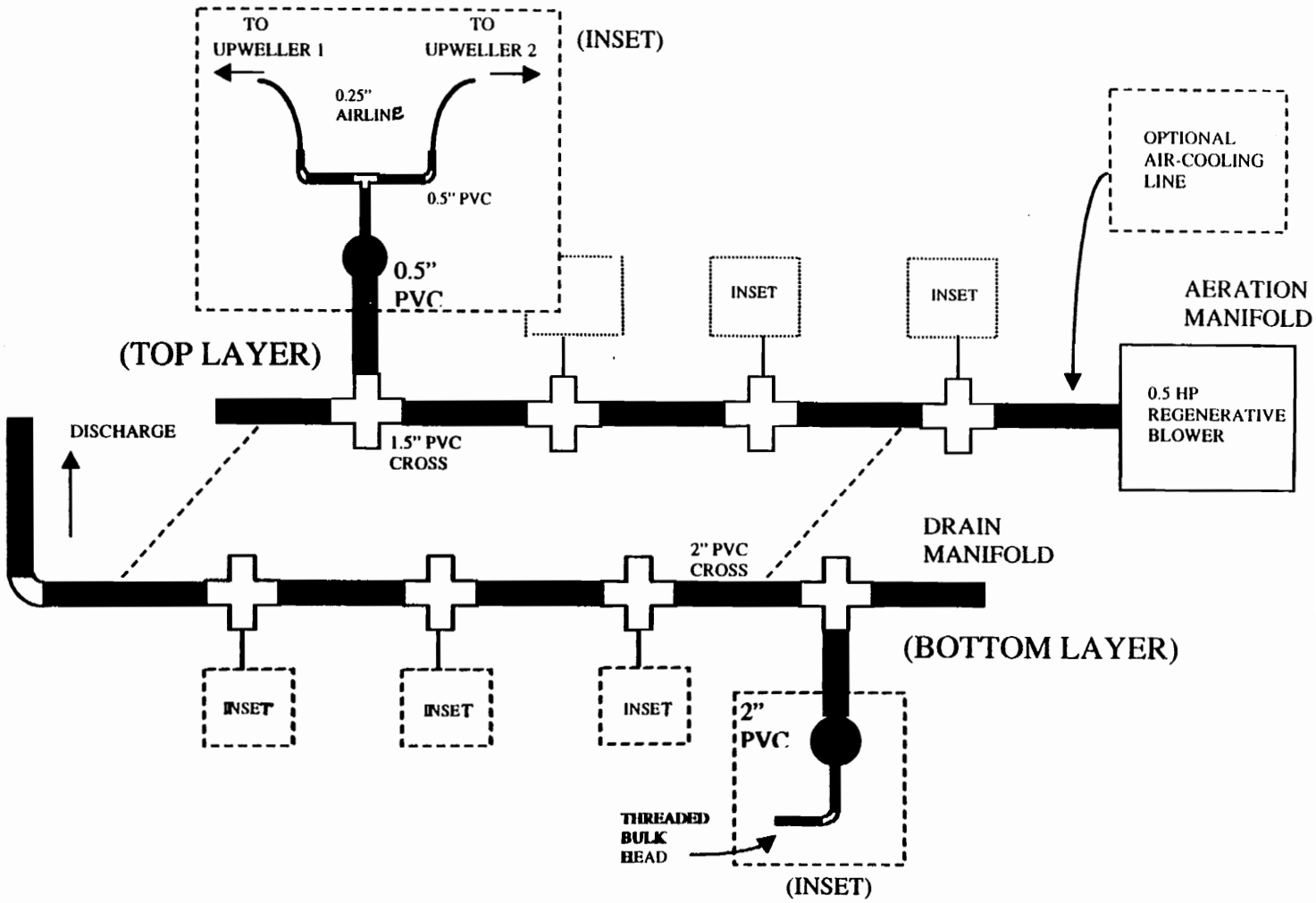


Figure 1. Ohio River Islands National Wildlife Refuge quarantine facility: Tank design with aeration and drain manifolds. Dimensions are in inches.

Figure 2. Distribution of **28** flow rate measurements in the Leetown Science Center pond refuge. Flow rate (cm/s) was measured at mid depth (1 m) and near the bottom (2.5m).

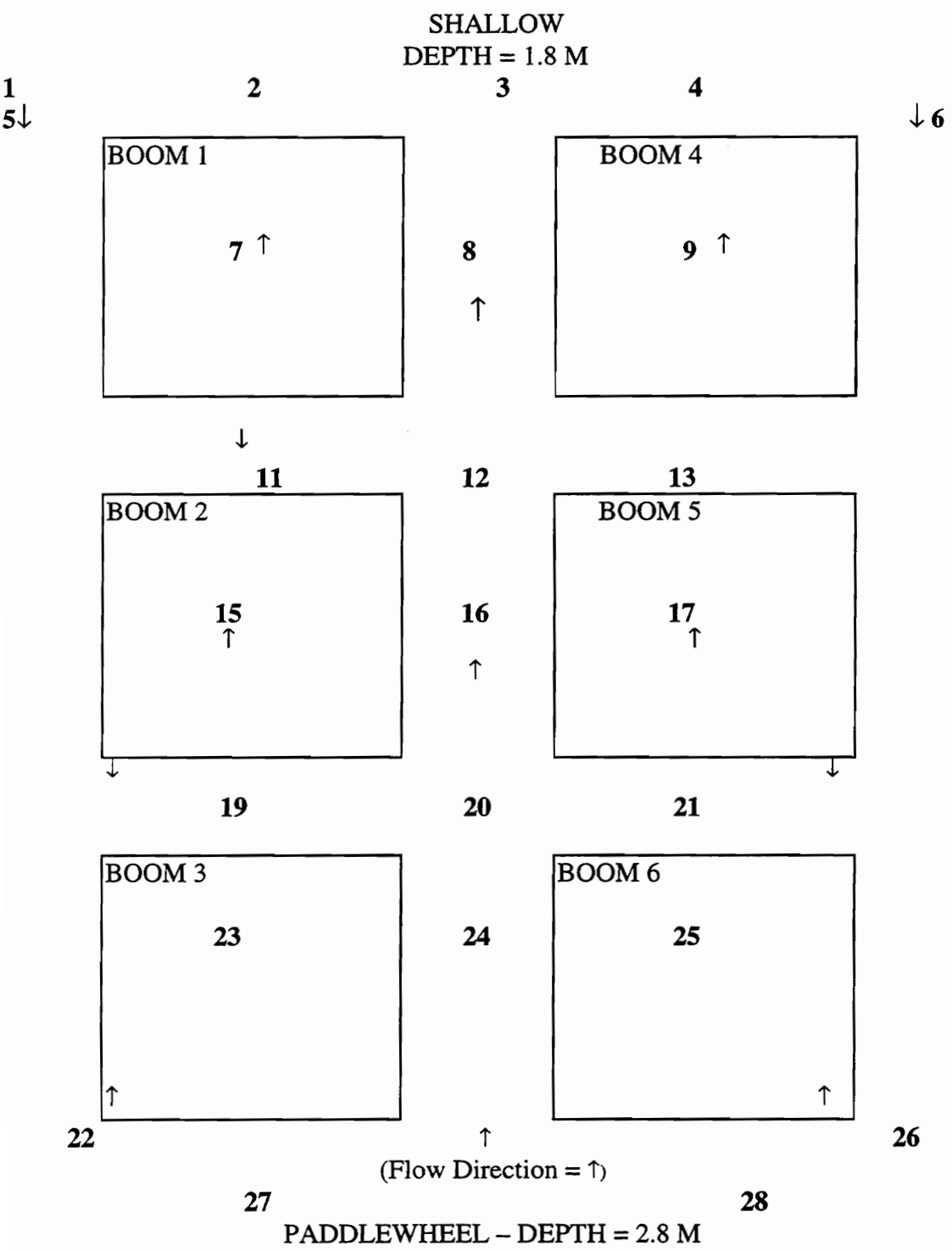
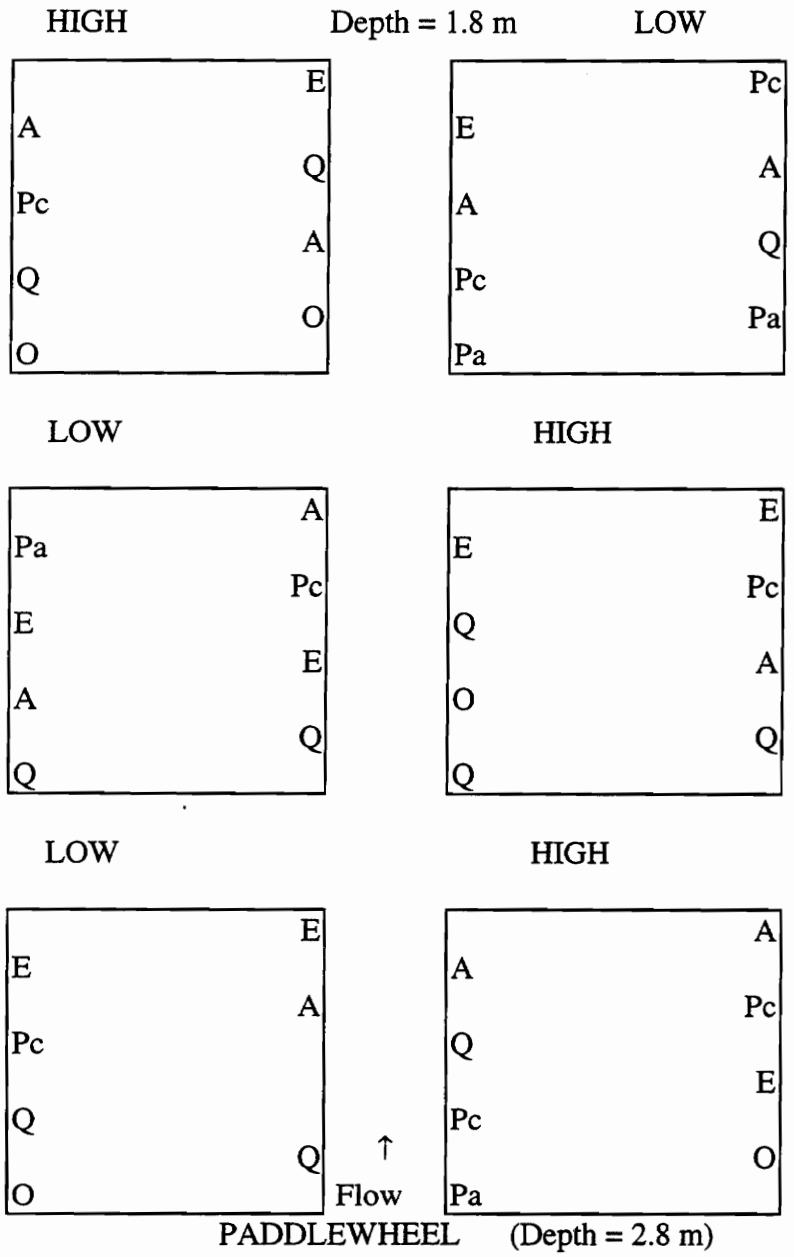
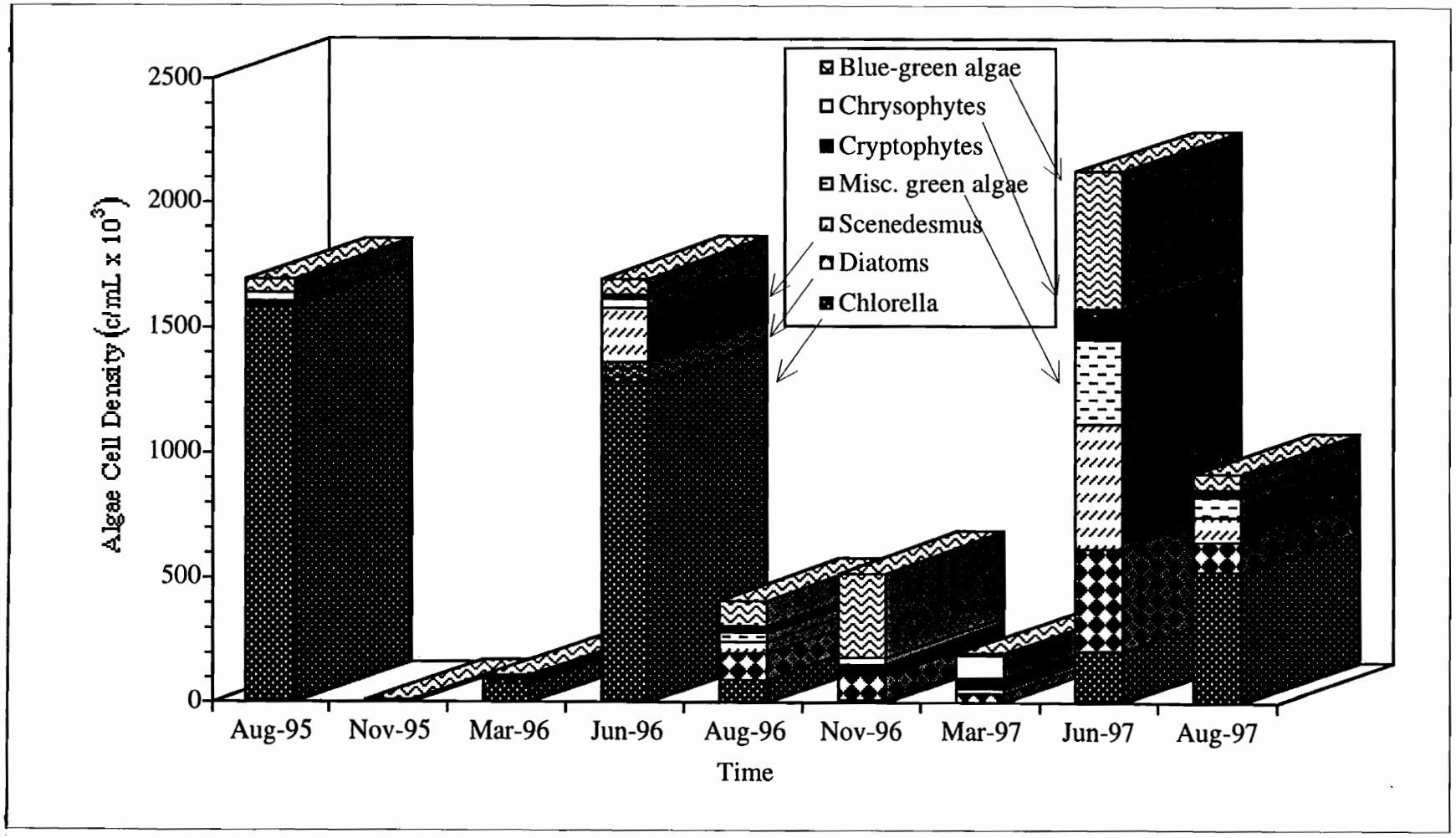


Figure 3. Distribution of freshwater mussels in the LSC pond refuge. A = Amblema plicata, E = Elliptio crassidens, O = Obliquaria reflexa, Pa = Potamilus alatus, Pc = Pleurobema cordatum. Each species is stocked in a pocket net and a dish rack at random locations on the floating booms. LOW = all species were stocked at 5 mussels per holding container; HIGH = all species were stocked at 20 mussels per holding container.





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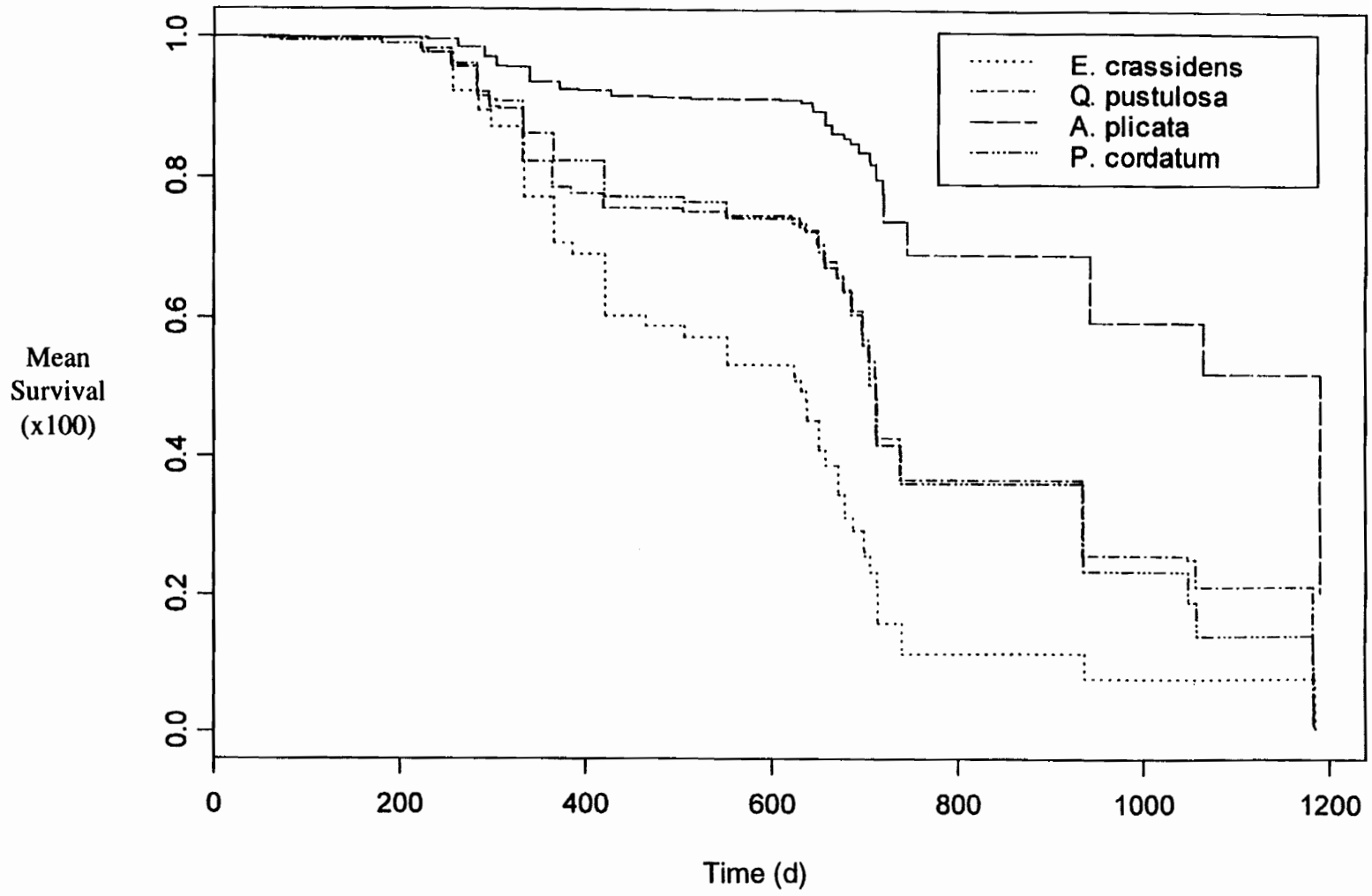


Figure 5. Percent survival of species entering the LSC pond refuge in September,1995.

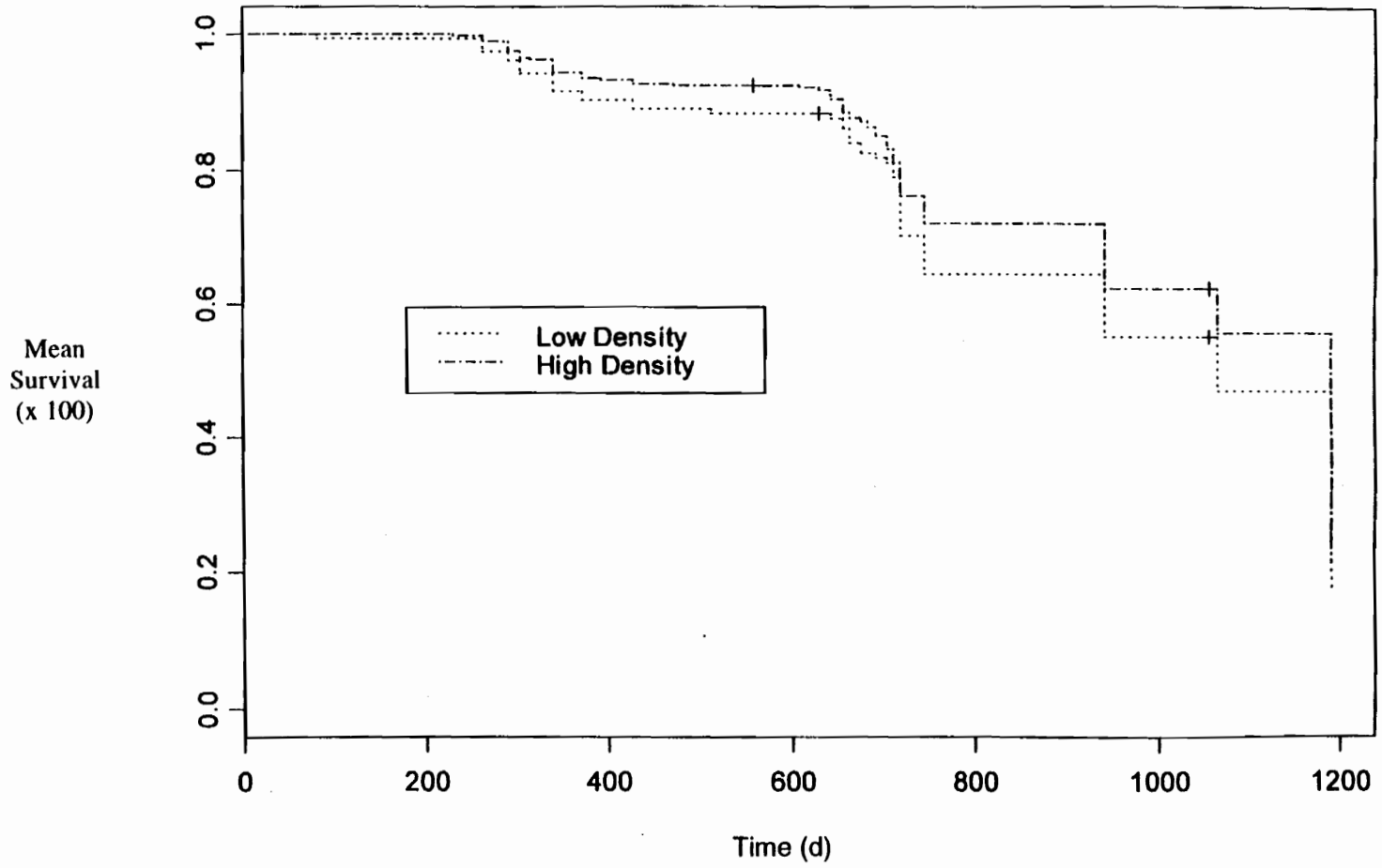


Figure 6. Effect of stocking density on percent survival of Amblema p. plicata in the LSC pond refuge

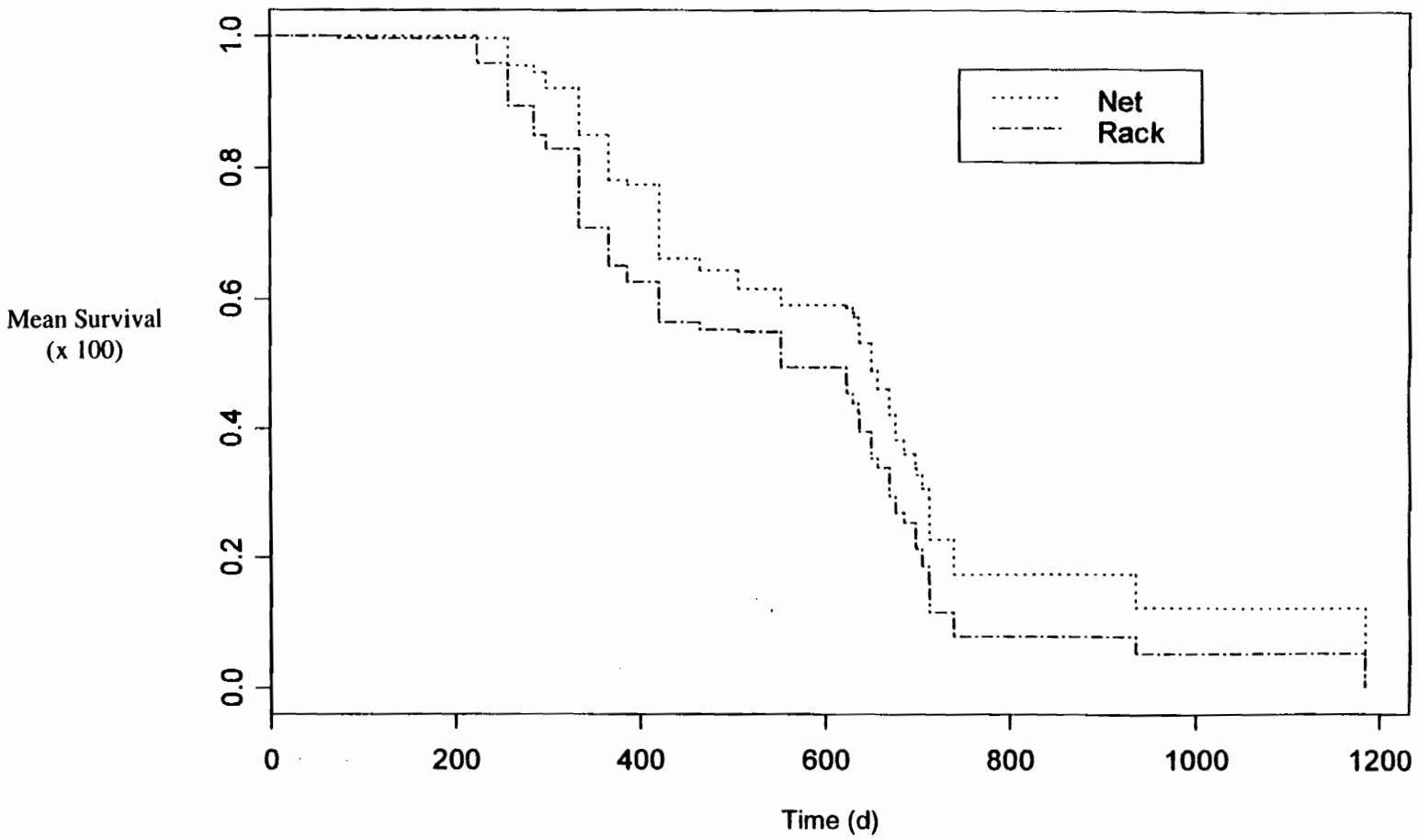


Figure 7. Effect of holding method on percent survival of Elliptio crassidens in the LSC pond refuge.

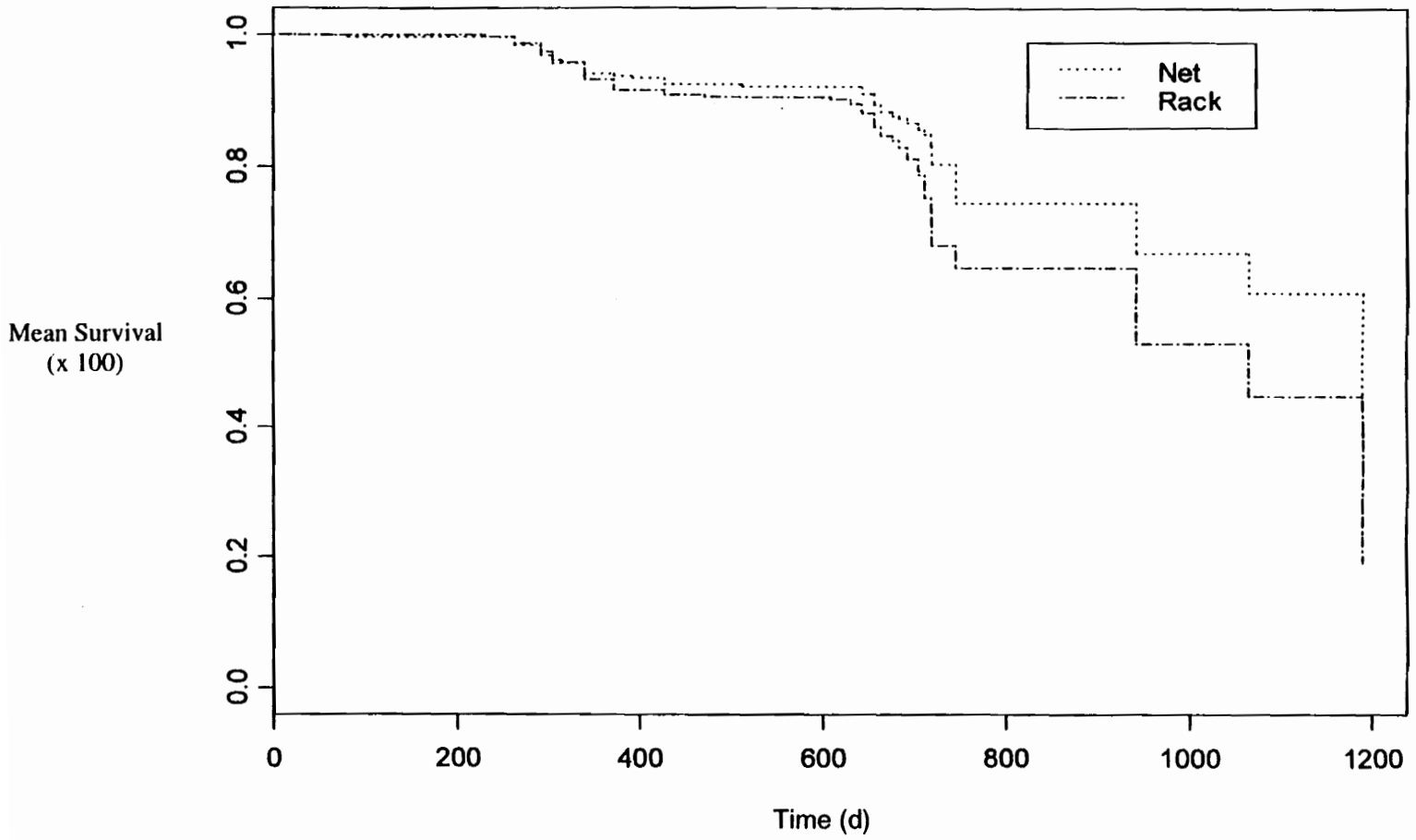


Figure 8. Effect of holding method on percent survival of Amblema p. plicata in the LSC pond refuge..

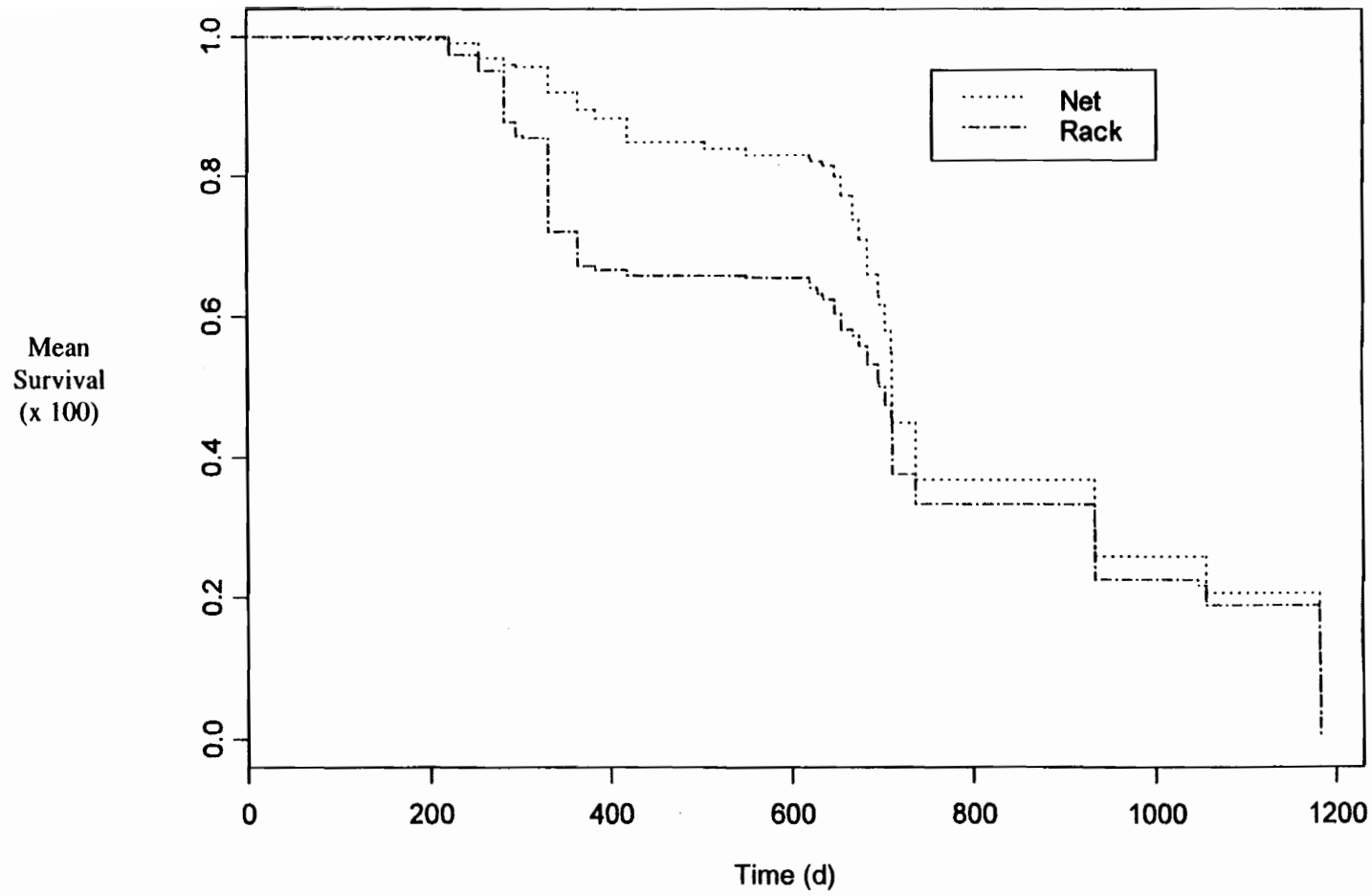


Figure 9. Effect of holding method on percent survival of Quadrula p. pustulosa in the LSC pond refuge.

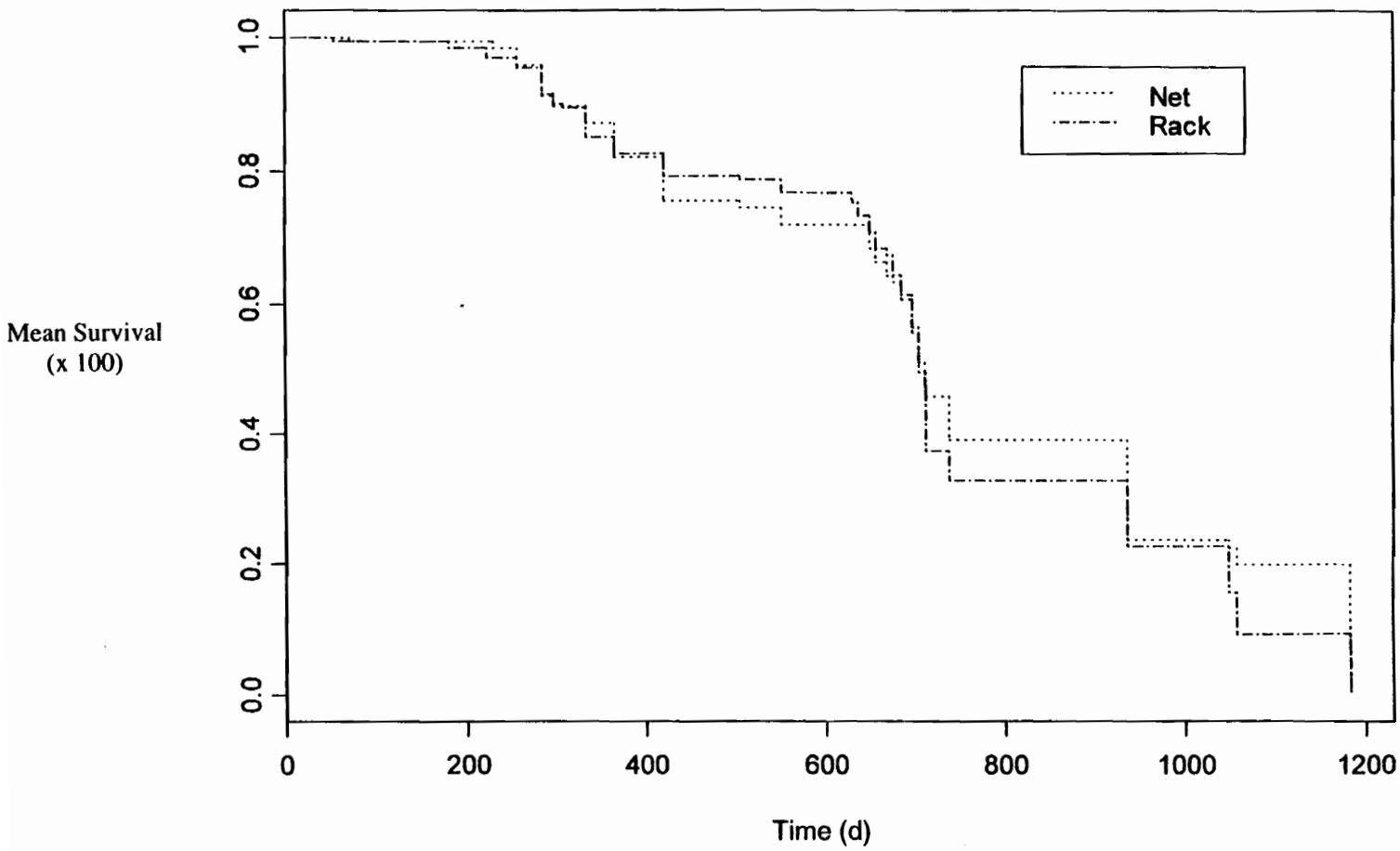


Figure 10. Effect of holding method on percent survival of Pleurobema cordatum in LSC pond refuge.

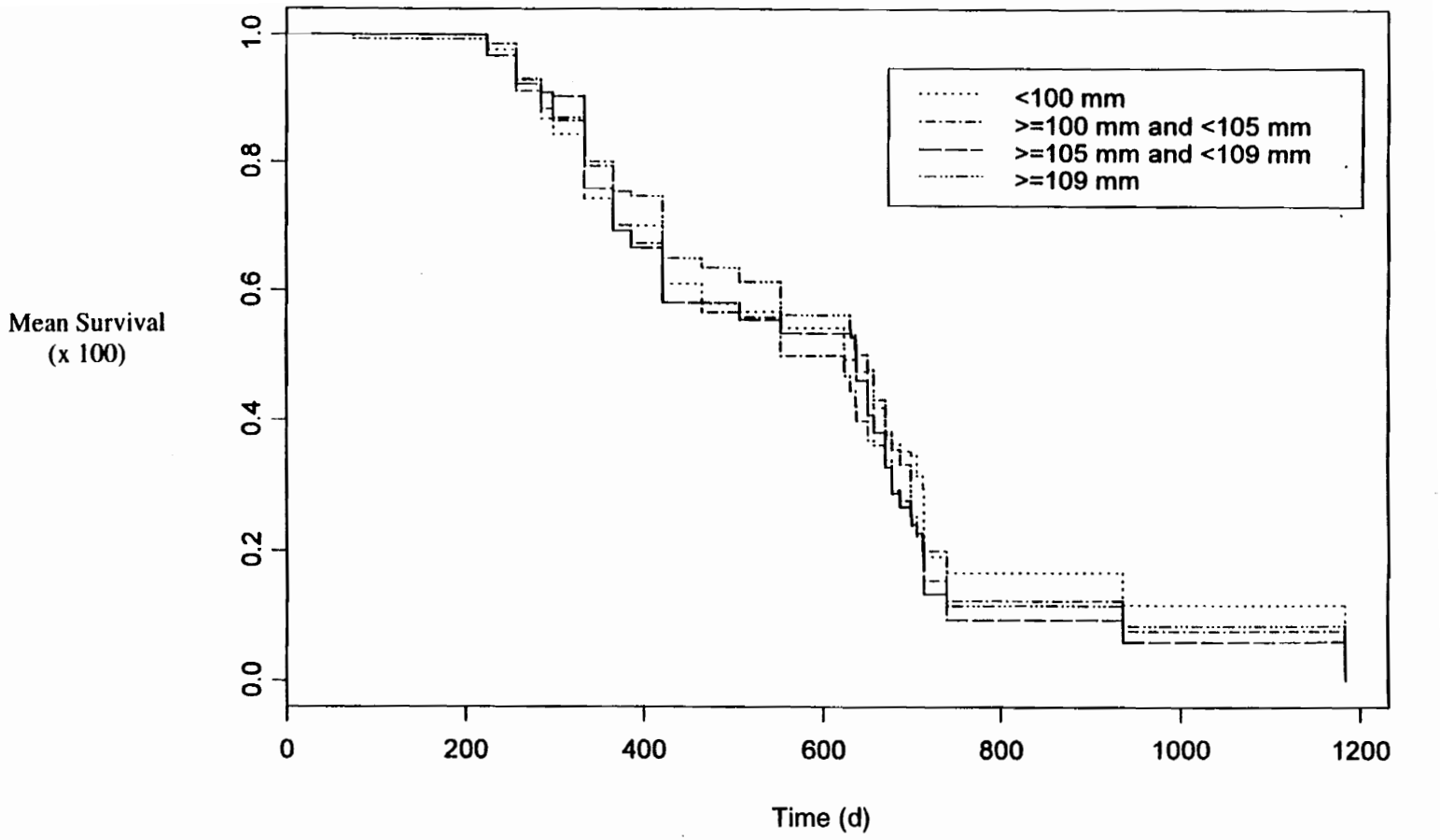
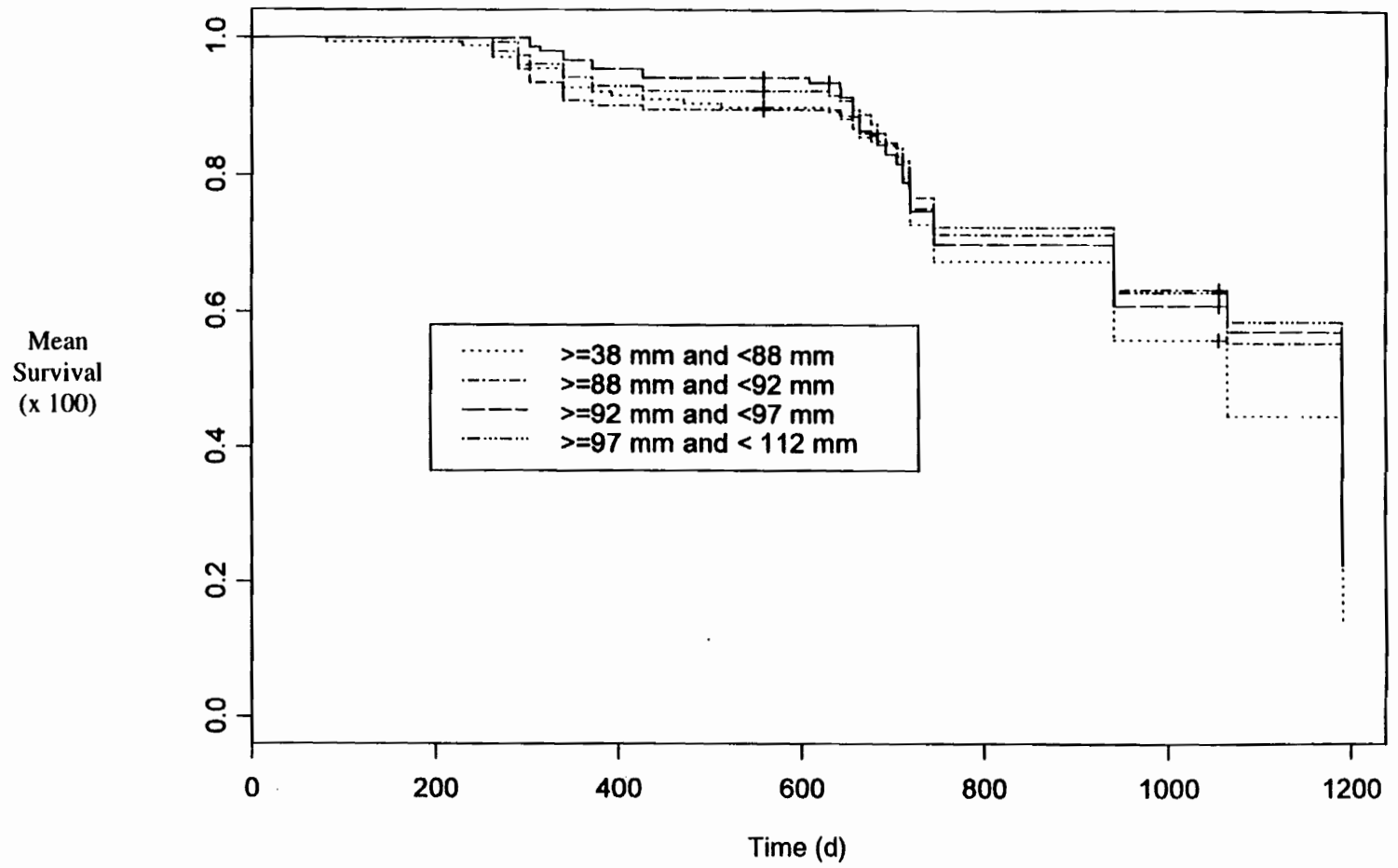


Figure 11. Effect of size (shell length, mm) on percent survival of Elliptio crassidens in the LSC pond refuge.



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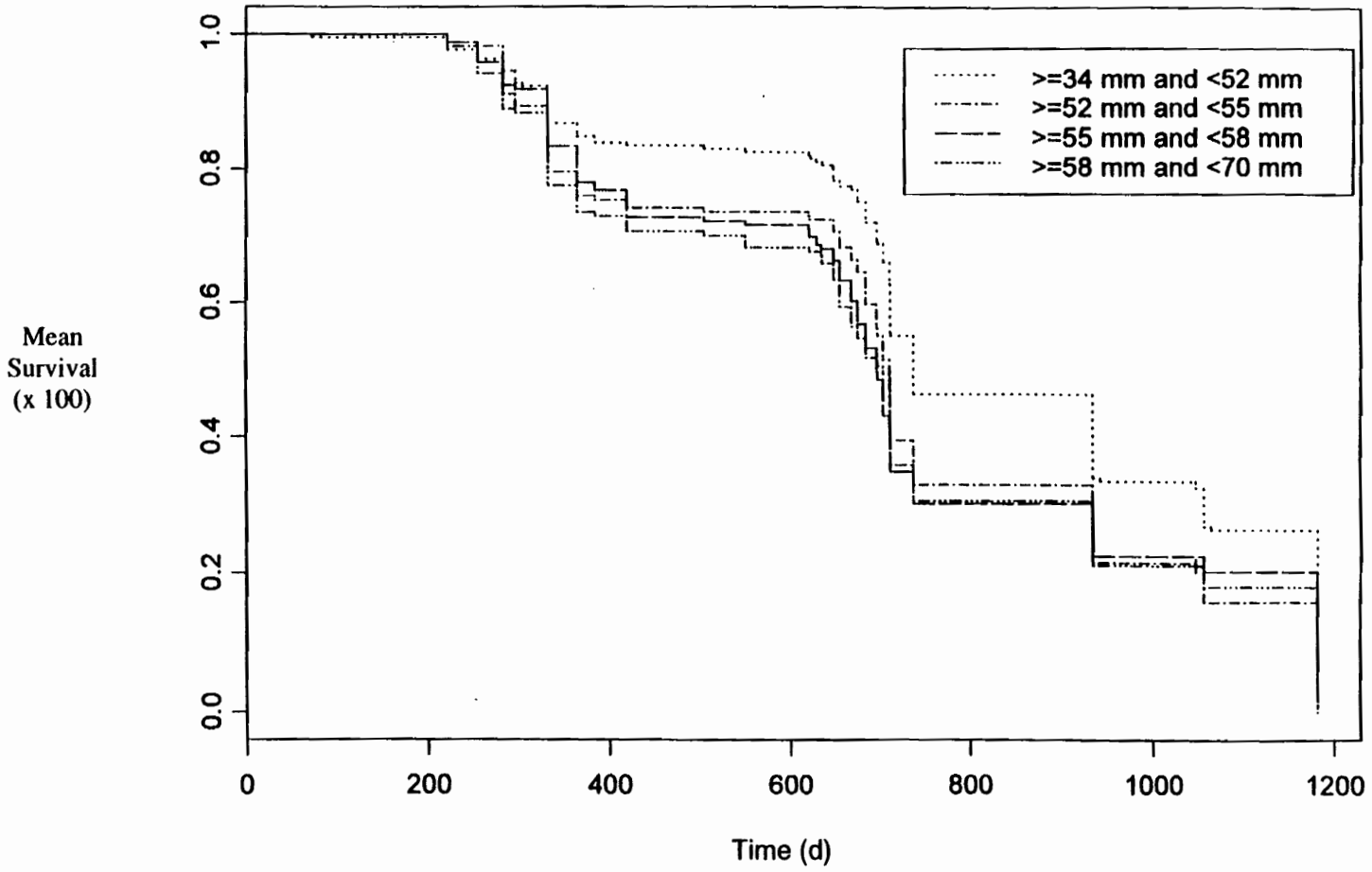


Figure 13. Effect of size (shell length, mm) on percent survival of Quadrula p. pustulosa in LSC pond refuge.

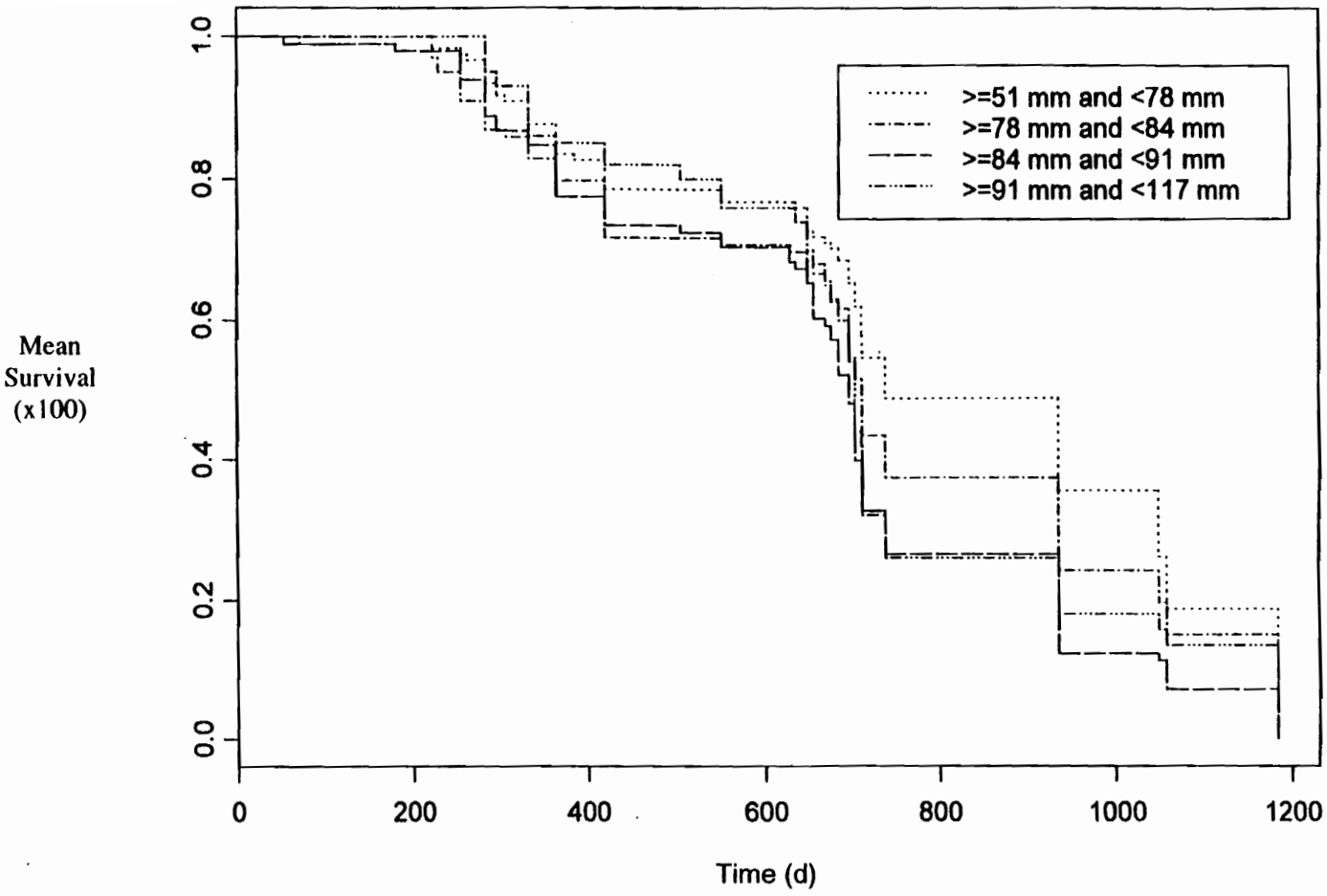
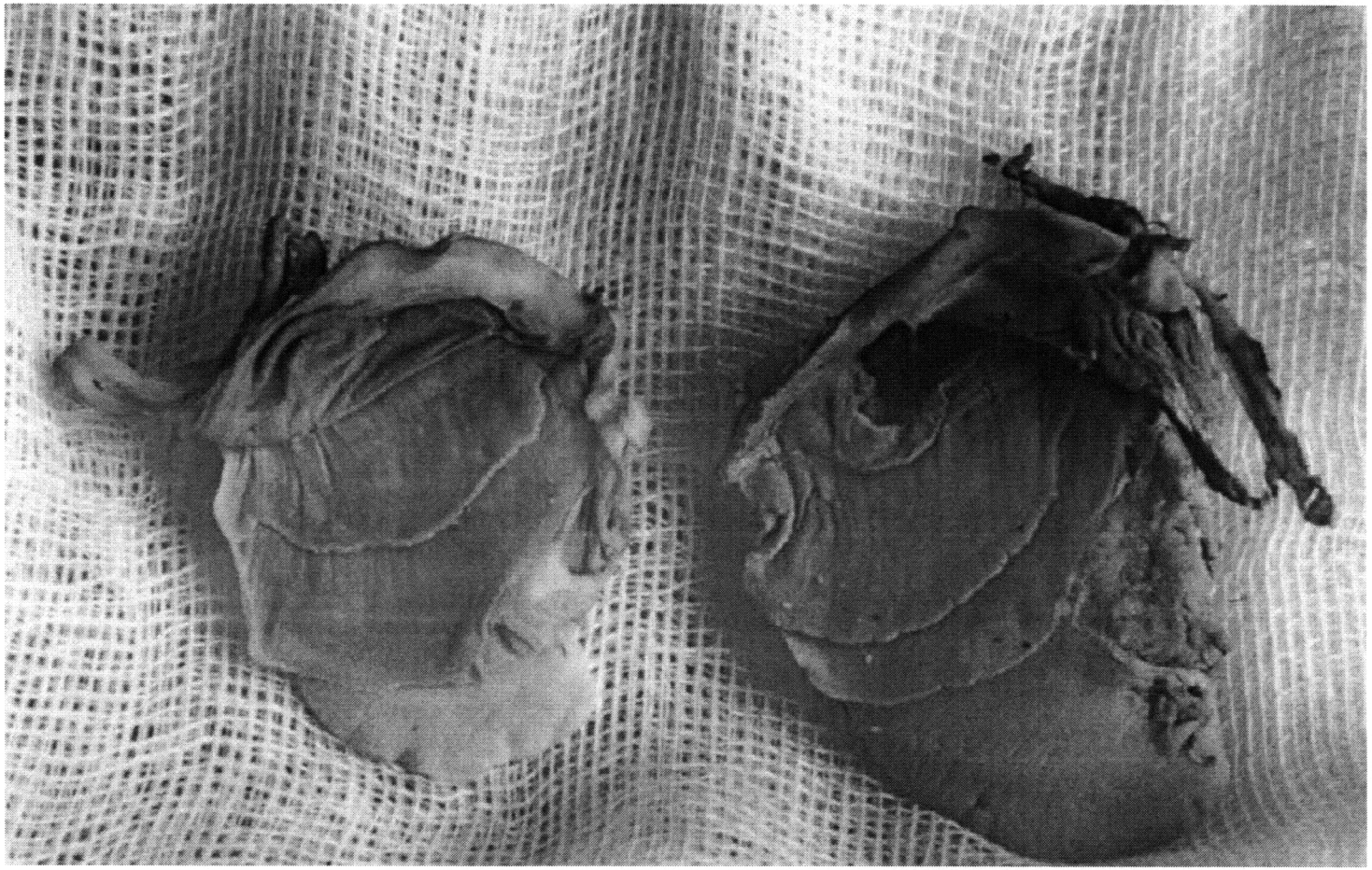


Figure 14. Effect of size (shell length, mm) on percent survival of Pleurobema cordatum in the LSC pond refuge.



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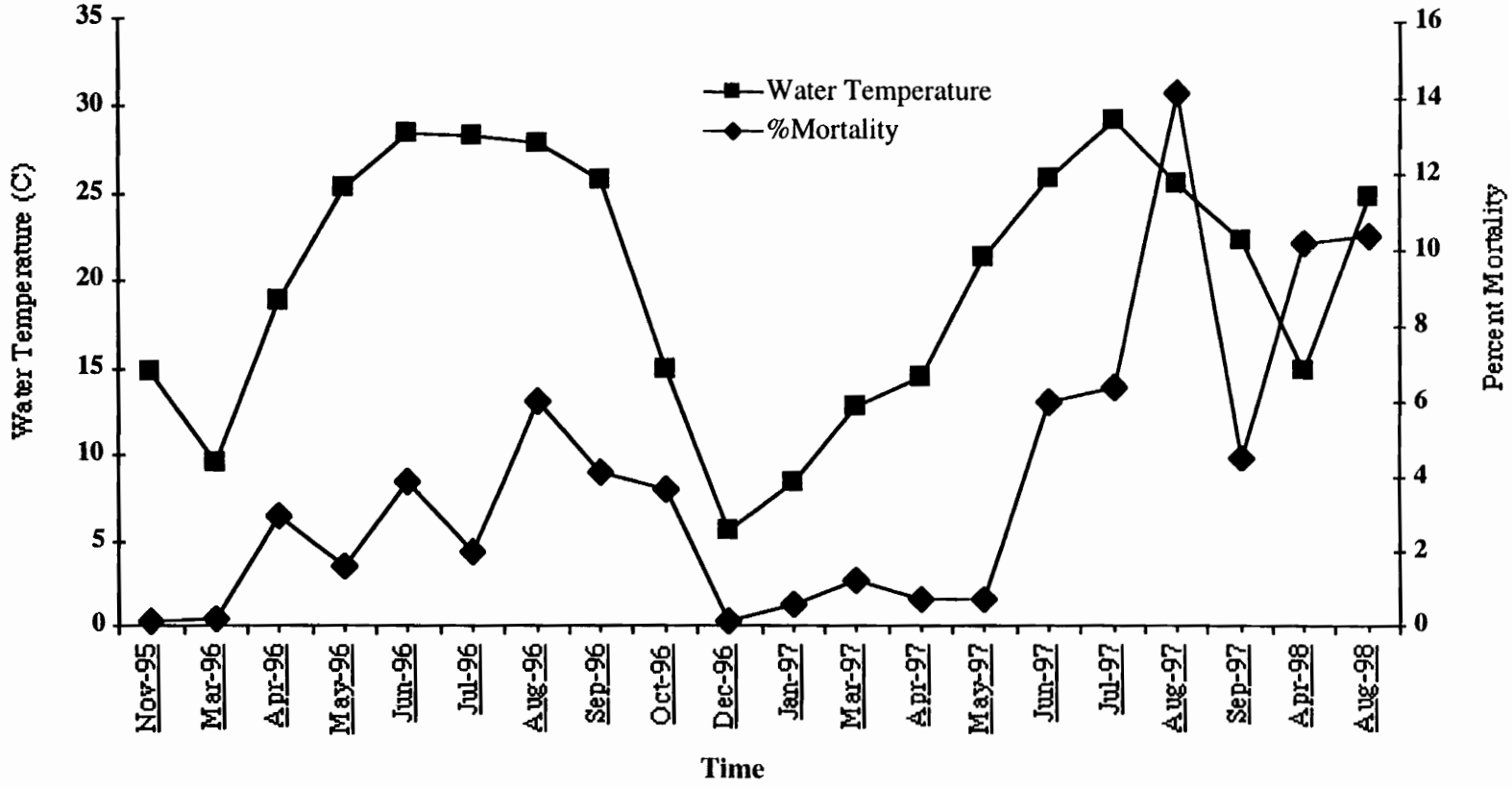

Figure 16. Freshwater mussel monthly mortality and water temperatures in the LSC pond refuge.

CHAPTER 3

FILTRATION RATES OF VILLOSA IRIS (LEA, 1829) (BIVALVIA: UNIONIDAE) FED DIFFERENT RATIONS OF ALGAE

ABSTRACT

This study determined the amount of algae cleared by the freshwater mussel, Villosa iris (Lea, 1829) in 24 h, at several algal cell concentrations, and estimated the algae concentration needed to maintain mussels in captivity. In the first experiment, V. iris was fed a single ration (1.3×10^6 c·mL^{-1}, 8.9 mg dry wt·L^{-1}) of the green alga, Neochloris oleoabundans, and filtration rate (mL·h^{-1}) was monitored for 24 h. Filtration rate declined with time and the algae concentration decreased to approximately 4×10^3 c·mL^{-1}. Filtration rates varied with shell length, ranging 14 - 280 mL·h^{-1} in the first hour and 9 - 34 mL·h^{-1} after 24 h. Mussels initially filtered at a high rate, and probably achieved gut satiation. Then, filtration decreased with time presumably to maintain ingestion rate, and in response to the decline in algae concentration. In the second experiment, mussels were fed one of three rations of N. oleoabundans: ration A was 5.0×10^4 c·mL^{-1} (0.34 mg dry wt·L^{-1}), ration B was 1.5×10^5 c.mL^{-1} (1.0 mg dry wt·L^{-1}), and ration C was 5.0×10^5 c·mL^{-1} (3.4 mg dry wt·L^{-1}). Filtration rate in the first feeding hour was highest in ration B and lowest in ration C. After the first hour, filtration rate declined in ration B but increased in rations C and A. In ration B, I believe that V. iris achieved gut satiation in the first hour using maximum filtration (712.5 mL·h^{-1}·g^{-1}) and then decreased filtration to 259 mL·h^{-1}·g^{-1} thereby regulating ingestion rate during the following 2 h. From the amount of algae cleared in ration B in 3 h, I estimated that V. iris would require 2.24 mg of algae (3.6×10^8 cells of N. oleoabundans) in 24 h or ca. 2.4 % of tissue dry weight.

INTRODUCTION

Freshwater mussels of the Unionacea are among the most widespread of bivalves (Ortmann 1911, Baker 1928, Banarescu 1990). Where mussels are present, they often comprise a significant proportion of the benthic biomass (Negus 1966, Kryger and Riisgard 1988, Strayer et al. 1994). It is ironic, therefore, that little attention has been directed at the role of suspension-feeding mussels in nutrient cycling and in structuring benthic species assemblages. If this role is important, then recent declines in freshwater mussel populations may adversely affect aquatic ecosystem integrity. In addition, conservation methods such as propagation for population restoration would benefit from an understanding of feeding physiology. More specifically, filtration rates and the physical and biological factors affecting filter-feeding behavior must be identified to successfully culture mussels in captivity, and to assess their ecological role in aquatic systems. A dearth of information on the filtration rates of marine bivalves has been published, but few studies on freshwater mussel filtration rates (Paterson 1984, Kryger and Riisgard 1988, Tankersley and Dimock 1993, McCall et al. 1995, Roper and Hickey 1995, Silverman et al. 1995, Vanderploeg et al. 1995). My objectives, therefore, were to determine the amount of algae cleared by the rainbow mussel, Villosa iris (Lea, 1829) in 24 h, investigate the effect of algal cell concentration on filtration rate, and estimate the optimal algal cell concentration for feeding this freshwater mussel in captivity.

I estimated the filtration rate of V. iris from the clearance of suspended material according to Coughlan (1969). Estimation of filtration rates from changes in the suspended particle concentration assumed the following:

a) reductions in particle concentration were from filtration by the mussel,

b) the mussel's pumping rate was constant,

c) particle retention was 100% efficient,

d) the test suspension was at all times homogeneous.

METHODS

Two experiments were conducted to study the filtration rate of V. iris. In the first experiment, specimens of V. iris were fed a single ration of algae, and filtration rate ($mL \cdot h^{-1}$) was monitored for 24 h. In the second experiment, the effect of algal ration (cell concentration) on filtration rate ($mL \cdot h^{-1} \cdot g^{-1}$ dry tissue weight (wt)) was determined while maintaining the initial cell concentration for three 1-h feeding periods.

Mussel Acclimation and Algae Culture.

Twelve male V. iris (shell length 37-52 mm) were collected from Copper Creek, Scott Co., Virginia in June, 1997. An additional 30 specimens (shell length 37-51 mm) were collected in February, 1998. All mussels were transported to the laboratory in aerated river water in a 10 L cooler. In the laboratory, the mussels were acclimated from field temperatures (17°C in summer and 10°C in winter) at $2°C \cdot h^{-1}$ to laboratory temperatures of 20°C. Mussels were transferred to 250 mL aerated chambers with a 50:50 mix of well water and dechlorinated city water (pH 8.0). Prior to the single ration test, mussels were fed 1 X 10^6 $c \cdot mL^{-1}$ (6.9 mg dry $wt \cdot L^{-1}$) of Neochloris oleoabundans (Chantanachat and Bold 1962) and acclimated to the feed for 24 h.

The green alga N. oleoabundans was selected for filtration rate experiments because it was shown to be suitable for rearing unionids (Gatenby et al. 1997, Patterson 1998). Algae were grown in Neochloris media (Gatenby et al. 1997) under continuous white fluorescent light (photon flux: 35 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 20 ± 1°C. Algal cells were counted using a hemacytometer, and cells were harvested for feeding during late-log phase. The cell size of N. oleoabundans ranges 2-20 µm depending on growth phase. Some mussels filter particle sizes of 5-10 µm more efficiently than smaller particles (< 5 µm) (Paterson 1986, Miura and Yamashiro 1990, Tankersley and Dimock 1993). Thus, I harvested N. oloeabundans during log phase growth when the majority of the cells were ca. 6.2 µm in diameter (range 5-10 µm). Ten 100 mL aliquots of algae were dried for 8 h at 90°C (dry wt), and ashed at 450°C for 8 h to obtain ash-free dry wt (AFDW).

Experimental Procedure.

Single ration test. The water in each 250 mL chamber was changed, and mussels were fed 1.3 x 10^6 c·mL^{-1} N. oleoabundans (8.9 mg dry wt·L^{-1}). A high concentration of algae was fed to ensure not all algae were filtered in 24 h. The single ration test began when each mussel exhibited shell gape, and apertures were visible. Water samples were collected after 1, 2, 3, 5, 8, and 24 h, and reduction in particle concentration was determined. Algal cells were not replenished after each sampling interval, simulating an aquaculture practice of feeding mussels only once per day. Three algae-only controls demonstrated that no algae settled out of suspension during the experiment. Particle

concentration at each sampling was determined using a Coulter Counter, Model ZM, aperture 100 μm.

Effect of algal ration test. Thirty V. iris collected in February, 1998 were used to compare filtration rates at different algae concentrations. Mussels were held in 250 mL containers and acclimated for 5 h to the new temperature regime without food. Ten individuals (per treatment) were fed one of three rations (cell concentration) of N. oleoabundans: ration A was $5.0x10^4$ c·mL^{-1} (0.34 mg dry wt·L^{-1}, ca 1.0% of unionid dry tissue wt), ration B was $1.5x10^5$ c.mL^{-1} (1.0 mg dry wt·L^{-1}, ca. 2.8 % of unionid dry wt), and ration C was $5.0x10^5$ c·mL^{-1} (3.4 mg dry wt·L^{-1}, ca.9.7 % unionid dry wt). The experiment began when each mussel exhibited shell gape and apertures were visible. Reductions in particle concentration over time were determined from water samples after three hourly feeding periods. Algal cells were replaced after each sampling interval. Three algae-only controls were tested to verify that no algae settled out of suspension during the experiment. Particle concentration at each sampling period was determined using the Coulter Counter. The presence of feces and pseudofeces was noted for each treatment (cell concentration).

Filtration Rate Calculations.

Single ration test. Filtration rates (FR) in mL · h^{-1} for the single ration test were calculated using the following equation (Coughlan 1969):

$$FR = \{V \cdot (n.t)^{-1}\} \cdot [\ln(conc_o \cdot [conc_f]^{-1})]\}$$

Effect of algal ration test. Hourly weight-specific filtration rates ($mL \cdot h^{-1} \cdot g^{-1}$) for the multiple concentration test were calculated to remove possible effects of size on filtration rates using the following equation (Palmer 1980):

$$FR = \{[V \cdot (n \cdot t)^{-1}] \cdot [\ln(conc_o \cdot [conc_f]^{-1})]\} \cdot W^{-1}$$

where V = volume of feeding chamber, W = dry weight (mg), n = 1 mussel in each feeding chamber, t = duration of sampling interval, and $conc_o$ and $conc_f$ are cell concentration at the beginning and end of each sampling interval, respectively. The mean (SD) dry wt of all V. iris was 289.9 (122.1) mg.

Statistical Analyses

Single ration test. Filtration rates were compared over time. Multivariate repeated measures analysis of variance was used, with shell length (measured from anterior to posterior) included as a covariate. Tests for sphericity indicated that filtration rates were dependent on the time at which samples were collected ($p < 0.01$). Because I suspected that filtration rates were dependent on mussel size (shell length), I also tested for interaction between time and shell length. One-way ANOVA then was used to determine whether mean filtration rates differed between $time_t$ and $time_{t+1}$.

Effect of algal ration test. Mussel filtration rates were compared between feeding hours within each treatment and among treatments. The relationship between shell length and filtration rate, expressed as both $mL \cdot h^{-1}$ and $mL \cdot h^{-1} \cdot g^{-1}$ was examined using simple linear regression to determine whether the effect of shell length on filtration rate was lost when filtration rates were expressed on a per weight basis. Then, the effect of algal

ration (cell concentration) and time on filtration rates (FR = $mL \cdot h^{-1} \cdot g^{-1}$) was analyzed using two-way ANOVA. Filtration rates were considered independent observations (at different times) because the cell concentration was replenished. Due to a significant interaction between time and cell concentration (p=0.0007), one-way ANOVA was used to compare the effect of algal ration within each feeding hour. Duncan's new multiple range test (DNMRT) was used to further evaluate the difference among treatments within a feeding period. ANOVA also was used to examine the effect of time within each treatment (algal ration). Duncan's test (DNMRT) then was used to evaluate the difference in filtration rates between feeding time within a treatment group. Finally, the effect of algal ration on the mean number of cells consumed was analyzed using one-way ANOVA. The mean cells consumed were log-transformed to stabilize the variance. All tests were considered significant at $\alpha = 0.05$.

RESULTS

Single ration test. The multivariate repeated measures tests showed that filtration rates decreased with time ($p = 0.023$), and filtration rate increased with shell length ($p = 0.01$) (Table 1). A significant interaction between time and shell length ($p = 0.0145$) was evident, indicating that the effect of shell length on filtration rate was not consistent over time. With the exception of four larger mussels (shell lengths of 47 to 52 mm), which exhibited an increase in filtration rates at 8 h, filtration rates generally declined with time (Table 1, Figure 1). In the first hour of feeding, filtration rates for all mussels ranged 15 - 280 $mL \cdot h^{-1}$. At 3 h, filtration rates ranged 14 - 100 $mL \cdot h^{-1}$, and at 24 h, the filtration rates

ranged between 6.9 and 34.2 $mL \cdot h^{-1}$ (Table 1). Mean filtration rate at 1 h was greater than that at 2 h ($p = 0.07$). Mean filtration rate at 2 h was significantly greater than at 3 h ($p = 0.02$), and the filtration rate at 8 h was greater than at 24 h ($p = 0.02$) (Table 1). Pseudofeces were produced by all individuals within the first hour of feeding, but were irregularly produced in subsequent hours of the test. Approximately 86.7% of the algal cells (1.5 mg AFDW), was cleared in 24 h (Table 1). One mussel's filtration rate was much greater than the rest, possibly exaggerating the significance of our results. We removed this observation from the data and re-analyzed the tests, with similar results. Filtration rate decreased with time ($p = 0.06$), and there was interaction between shell length and time ($p = 0.04$).

Effect of algal cell concentration test. Linear regressions showed that filtration rates expressed as $mL \cdot h^{-1}$ were dependent on shell length ($p = 0.012$); however, filtration rates expressed as $mL \cdot h^{-1} \cdot g^{-1}$ showed no significant relationship with shell length ($p = 0.448$). Thus, the effect of length on filtration rates, as observed in the single ration test, was removed when filtration rates were expressed on a weight-specific basis. Two-way ANOVA showed that the effect of algal ration changed with time (feeding hour), such that no treatment showed the same effect at each hour. One-way ANOVA, therefore, was used to compare the effect of treatment within each feeding hour. This test showed that filtration rates differed among treatments within each feeding hour and with time among treatments (Table 2, Figure 2).

Duncan's test confirmed that V. iris fed ration A (0.34 $mg \cdot L^{-1}$) had the greatest mean filtration rate (712.5 $mL \cdot h^{-1} \cdot g^{-1}$) at 1 h, followed by ration B (1.0 $mg \cdot L^{-1}$, FR = 365.2

$mL \cdot h^{-1} \cdot g^{-1}$), and then ration C (3.4 $mg \cdot L^{-1}$, FR = 146.2 $mL \cdot h^{-1} \cdot g^{-1}$) (Table 2). Filtration rates did not significantly differ at 2 h between rations A (730.8 $mL \cdot h^{-1} \cdot g^{-1}$) and B (490.4 $mL \cdot h^{-1} \cdot g^{-1}$), but both were significantly greater than ration C (211.4 $mL \cdot h^{-1} \cdot g^{-1}$). Filtration rates in rations A (759.9 $mL \cdot h^{-1} \cdot g^{-1}$) and B (259.0 $mL \cdot h^{-1} \cdot g^{-1}$) at 3 h were significantly different; however, neither A nor B differed from C (423.1 $mL \cdot h^{-1} \cdot g^{-1}$) (Table 2).

One-way ANOVA of within-treatment filtration rates indicated that mean filtration rate of mussels fed ration B (1.0 $mg \cdot L^{-1}$) decreased with time ($p=0.023$). Mean filtration rate of V. iris fed ration A (0.34 $mg \cdot L^{-1}$) increased with time ($p=0.055$), although mean filtration rates from feeding hours 2 and 3 were not significantly different. Mean filtration rate of V. iris fed ration C (3.4 $mg \cdot L^{-1}$) increased with time ($p=0.028$).

The total number of cells cleared by V. iris during all three feeding periods was significantly different among treatments (Table 3). Mussels fed ration C cleared the greatest number of cells (9.38×10^7 cells, 0.64 mg dry wt), followed by mussels fed ration B (4.6×10^7 cells, 0.32 mg dry wt), and then A (1.76×10^7 cells, 0.12 mg dry wt). A greater percentage of the available algae, however, was cleared from ration A (the lowest cell concentration) than from rations B and C (Table 3). Pseudofeces were produced by all mussels in ration C. Pseudofeces were not observed in rations A and B. At these cell concentrations, the average filtration rate was 618.6 $mL \cdot h^{-1} \cdot g^{-1}$ and 487.3 $mL \cdot h^{-1} \cdot g^{-1}$, respectively for rations A and B; however, the average filtration rate in ration C was only 260.2 $mL \cdot h^{-1} \cdot g^{-1}$.

DISCUSSION

Bivalve filtration rates are a function of physiological and environmental factors including gill type, body size, body condition, temperature, particle size and type, particle concentration, and current speed (Walne 1972, Foster-Smith 1975a, Winter 1978, Leff et al. 1990, Vanderploeg et al. 1995). Generally, bivalves regulate their filtration rate within a range of particle concentrations, thereby regulating the amount of material that is ingested (Winter 1973, Higgins 1980, Riisgard and Randlov 1981, Navarro and Winter 1982, Way 1989, Roper and Hickey 1995). At low concentration, filtration rate is increased to maximize ingestion. At slightly higher particle concentration excess material is rejected as pseudofeces (Iglesias et al. 1992). Filtration rate declines with further increases in concentration, presumably to decrease the amount of food entering the gut and to allow the digestive gland to efficiently digest and assimilate material entering the stomach (Riisgard 1991). Otherwise, excess material may bypass the digestive gland and pass through the gut undigested as intestinal feces (Foster-Smith 1975a, Winter 1978, Widdows et al. 1979). Bivalves more adapted to turbid water, such as Corbicula sp., Dreissena polymorpha, Crassostrea virginica, and Scrobicularia plana, tend to maintain high filtration rate even at high particle concentrations (Hughes 1969, Morton 1971, Haven and Morales-Alamo 1978, Lauritsen 1986, Reeders et al. 1993, Fanslow et al. 1995) and consequently must produce copious amounts of pseudofeces to regulate their ingestion rate (Bricelj and Malouf 1984). Conversely, species such Hyridella menziesi, Mercenaria mercenaria, Arctica islandica, and Cerastoderma edule that are less successful at exploiting turbid environments tend to control ingestion

primarily by reducing filtration rates as particle concentration is increased (Winter 1970, Foster-Smith 1975a, Bricelj and Malouf 1984, Roper and Hickey 1995).

Single ration test. The filtration rate of V. iris declined by over 56 % within 2 h, concomitant with a decrease in algal cell concentration (Figure 1, 3). I fed V. iris a dense suspension of algae (1.3 x 10^6 c·mL^{-1}, 8.9 mg·L^{-1}), which elicited pseudofeces production within the first hour. Pseudofeces can foul the pallial apparatus and lower subsequent filtration rates (Foster-Smith 1975a, Winter 1978, Sprung and Rose 1988). Alternatively, greater filtration during the first feeding hour may have reflected an empty gut, with all or most food from the previous acclimation period having been assimilated. Higgins (1980) found that oysters (C. virginica) fed under batch regimes totally cleared the algae from suspension after 24 h, and final concentrations were usually lower than 10,000 c·mL^{-1}. The introduction of food to unfed oysters induced valve gape and increased filtration rates (Higgins 1980). The provision of food to starved M. edulis also stimulated an increase in ventilation, filtration, and O_2 consumption followed by reduced filtration rate upon satiation of the digestive system (Thompson and Bayne 1974, Bayne et al. 1976, Riisgard 1991). V. iris initially filtered at a high rate presumably to achieve gut satiation, and then filtered at a lower rate either to maintain a constant ingestion rate (Figure 1) or in response to the decline in particle concentration. After 24 h in the single ration test, the amount of algae in the feeding chambers ranged 0.3-7.0 mg·L^{-1} (0.212-4.78 x 10^5c·mL^{-1}). Production of pseudofeces decreased with time and with the decline in algae concentration in the feeding chambers. In Figure 1, all cells filtered were treated as if they were ingested.

Another plausible explanation for a decline in filtration rate is stress. Jorgensen et al. (1988) found that marine mussels decreased valve gape, and thus reduced their filtration rate in response to suboptimal conditions. High algal concentrations may constitute suboptimal conditions (Mohlenberg and Riisgard 1979). Environmental conditions that cause stress to freshwater mussels need further investigation to better understand the observed changes in filtration rates, which corresponded to changes in particle concentration in our feeding trials.

Diurnal feeding rhythms and filtration rhythms associated with digestive processes and motor activity also have been documented for several bivalves, including the freshwater mussels Anodonta cygnea and Unio pictorum (Morton 1970, Salanki et al. 1974, Winter 1978, Benedens and Hinz 1980). The observed increase in filtration rate after 8 h by four larger mussels in this study may indicate that V. iris possesses filtration activity which is environmental, or digestive in nature.

Effect of algal concentration. Filtration rates in the first hour increased with algal concentration to 1.0 $mg \cdot L^{-1}$ (1.5 x $10^5 c \cdot mL^{-1}$), but decreased with further increase in the particle concentration (3.4 $mg \cdot L^{-1}$, 5 x $10^5 c \cdot mL^{-1}$). After 1 h, however, filtration rates declined in ration B (1.0 $mg \cdot L^{-1}$), but increased in rations C and A. In ration B, I suspect that V. iris maximally filtered (712.5 $mL \cdot h^{-1} \cdot g^{-1}$) in the first hour, achieved gut satiation, and decreased its filtration rate thereby regulating ingestion rate. Riisgard (1991) reported a decrease in filtration rate by M. edulis after an initial period of maximal filtration rate. This behavior seemed uninfluenced by the algae concentration, indicating that M. edulis was not adjusting to ambient algae

concentrations. Rather, after gut capacity was exceeded, this marine mussel apparently reduced valve gape, which the authors inferred would reduce filtration rate and ingestion rate. This relationship between filtration rate and ingestion rate following gut satiation is common among bivalves (Mitroposkij 1966, Foster-Smith 1975a, Hornbach et al. 1984, Way 1989, Riisgard 1991).

Very low algae concentrations have been shown to support maximum growth of marine mussels in laboratory experiments (Kiorboe et al. 1980). However, low concentrations may lead to shell closure, reduced filtration rate, and reduced metabolism (Riisgard and Randlov 1981, Jorgensen et al. 1986). At 0.34 $mg \cdot L^{-1}$ of N. oleoabundans (ration C), pseudofecal production was not observed, and V. iris increased its filtration rate between 1 h and 2 h. I believe that gut satiation was not achieved in the first hour; thus, V. iris increased its filtration rate to achieve gut fullness. Between feeding periods 2 and 3, V. iris appeared to regulate its filtration rate at ca. 700 $mL \cdot h^{-1} \cdot g^{-1}$, while producing no pseudofeces. Perhaps V. iris achieved gut fullness during the second feeding period, thereby regulating ingestion rate by maintaining a high filtration rate. Alternatively, if gut satiation was not achieved, V. iris filtered at a maximum rate to obtain adequate food for metabolic functions. Similar filtration rates (ca. 700 $mL \cdot h^{-1} \cdot g^{-1}$) have been reported for bivalves fed a low concentration of similar-sized live algae (Table 4). Continued maximal filtration at low food concentrations could be energetically costly, and calorically inadequate for growth and reproduction. In Willows (1992) model for optimal digestive investment, he cautions that at low concentrations of food, energetic

costs of filtration become a significant component of the overall costs for digestion and will determine the upper limit for filtration rate.

Filtration rate of V. iris fed at the highest concentration (3.4 $mg \cdot L^{-1}$) increased over time. Pseudofeces were also produced over time, but it was not quantified. High filtration rates at higher concentrations can overload the pallial feeding apparatus so that a large proportion of material is rejected as pseudofeces (Foster-Smith 1975a, 1975b, Riisgard 1991). The ingestion rate and growth rate of M. edulis increased in response to the addition of silt to the algae; however, high mortality also was reported when mussels were fed at a high ration (3.21 $mg \cdot L^{-1}$ algae + 12.5 $mg \cdot L^{-1}$ silt) (Thompson and Bayne 1972). Thompson and Bayne (1972) suggested that M. edulis may suffer nutritive stress when feeding for long periods in high concentrations of suspended particles. If the pallial apparatus was obstructed, V. iris may have increased pseudofecal production to clear gill structures, and subsequently increased filtration rates to ingest adequate amounts of food.

Bricelj and Malouf (1984) suggested that in mixed algal-sediment suspensions, a reduction in clearance rate may be less advantageous than pseudofecal production as a strategy for maximizing absorption of organic material. Bivalve species that are better adapted to turbid environments would control ingestion by producing copious amounts of pseudofeces. Stenton-Dozey and Brown (1992) concluded that long-term cycles of food availability, characteristic of northern cline environments, also may be a major factor in determining the feeding strategy of bivalves. In this study, mussels were collected from a small stream in southwestern Virginia that experiences seasonal increases in turbidity following rain and snow melt. V. iris is likely not adapted to highly turbid environments

and, according to Bricelj and Malouf (1984), would regulate its ingestion rate by reducing filtration rate in high particle concentrations. Yet when fed at the highest algal concentration, V. iris produced pseudofeces and increased filtration rate over 3 h. Navarro et al. (1992) showed that some bivalves would regulate ingestion rate in response to rising particle concentrations by both reducing their clearance rate or increasing the amount of filtered material as pseudofeces. The mechanism employed varied with food quality. Admittedly, more information on freshwater mussel feeding requirements is needed to understand the filtering behavior of V. iris recorded under laboratory conditions.

As previously mentioned, all suspension-feeding bivalves appear to regulate their filtration rates within a range of suspended particle concentrations. Filtration rate differs among bivalves, and is affected by pseudofeces production, temperature, particle size and particle type (Winter 1969, Foster-Smith 1975b, Sprung and Rose 1988, Kryger and Riisgard 1988). Physiological adaptations to turbid environments may influence feeding behaviors and feeding tolerances. This relationship between food concentration and filter-feeding behavior has to be considered when prescribing a feeding regime for the captive care of unionids. As suggested by Winter (1978), the pseudofeces-free cell density is likely the optimum food concentration at which filtration activity is reduced to a low-energy consuming level and all algal cells filtered are ingested. Mussels (37- 52 mm) fed between 0.34 $mg \cdot L^{-1}$ and 1.0 $mg \cdot L^{-1}$ of N. oleoabundans produced no pseudofeces, indicating that all filtered material was ingested. After 3 h, more energy was acquired by mussels fed 1.0 $mg \cdot L^{-1}$ than mussels fed 0.34 $mg \cdot L^{-1}$. I concluded,

therefore, that V. iris required at least 2.24 mg of algae (3.6×10^8 cells of N. oleoabundans) in 24 h or ca. 2.4 % of tissue dry weight..

Little is known of feeding rhythms in freshwater mussels; thus, it must be determined whether V. iris should be reared on a continuous feeding regime or by batch feedings. Mohlenberg and Riisgard (1979) showed that filtration rates in marine bivalves were often underestimated due to experimental conditions that did not simulate the habitat of infaunal bivalves. Palmer (1980) also cautioned against using unialgal tests to extrapolate filtration rates in nature, where suspended material is a mix of algae and detritus. Clearly there is a need for more freshwater mussel feeding experiments, using knowledge of actual seston concentrations and particle sizes from native environments. Ultimately, a comprehensive study of factors affecting mussel filtration rates (e.g., temperature, particle size, particle concentration, food quality) is needed to assess their capacity for removing suspended particles and their trophic importance in the benthos.

ACKNOWLEDGEMENTS

I am grateful to Dr. Daniel A. Kreeger, Philadelphia Academy of Natural Sciences for his advice and generosity in helping to design this study and interpret the results. I also am very grateful to Dr. Bruce C. Parker for allowing me access to his Coulter Counter and proofreading this manuscript. This research was sponsored by the Biological Resources Division of the United States Geological Survey.

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Table 4. Filtration rates (FR) of several freshwater bivalves fed similar rations (cell density) of algae.

Table 1. Filtration rates ($mL \cdot h^{-1}$) and total algae filtered (mg dry weight) by individual Villosa iris, each fed a single ration of algae (1.3×10^6 $c \cdot mL^{-1}$), during 24 h. P-values are reported from the 1-way ANOVA contrast tests of mean filtration rate (mL.h-1) between consecutive feeding periods. SL = shell length.

	Feeding Period (h)						Algae Filtered
SL(mm)	0-1	1-2	2-3	3-5	5-8	8-24	x 10^8 alga cells (mg)
52	87.29	52.1	37.0	29.2	29.4	21.5	2.514 (1.7)
51	280.22	120.9	100.1	70.7	53.8	25.7	2.596 (1.8)
50	98.58	43.5	34.1	20.7	32.9	22.6	2.090 (1.4)
48	115.57	61.5	41.0	40.9	23.9	25.2	2.398 (1.6)
48	74.21	38.4	32.9	28.2	38.3	34.2	2.529 (1.7)
47	31.10	27.2	21.9	15.5	19.0	21.3	2.306 (3.4)
45	86.65	47.6	35.1	24.3	20.4	20.7	2.272 (1.6)
43	33.13	26.0	21.6	13.5	12.5	11.6	1.968 (1.4)
43	13.80	18.8	15.2	14.1	10.4	12.2	1.846 (1.3)
41	42.35	30.5	20.0	14.5	10.5	12.5	2.038 (1.4)
39	36.46	16.4	15.4	16.0	11.9	6.5	2.170 (1.5)
37	16.49	9.8	10.4	7.2	7.0	8.9	1.824 (1.3)
Mean	94.49	41.0	32.1	24.6	22.5	19.4	2.213 (1.5)
p-values	0.07	0.02	0.125	0.902	0.024		

Table 2. Hourly filtration rates ($mL \cdot h^{-1} \cdot g^{-1}$) of Villosa iris for 3 feeding periods and at 3 algae rations: Ration A = $5x10^4$ $c \cdot mL^{-1}$, Ration B = $1.5x10^5$ $c \cdot mL^{-1}$, and Ration C = $5.0x10^5$ $c \cdot mL^{-1}$. Values in rows followed by the same lower case letter are not significantly different; values in columns followed by the same upper case superscript are not significantly different by Duncan's Multiple Range Test (DMRT), $\alpha = 0.05$.

Time Interval (h)	Ration A	Ration B	Ration C
1	365.2 b,A	712.5 a,A	146.2 b,A
2	730.8 a,B	490.4 a,A,B	211.4 b,A
3	759.9 a,B	259.0 b,B	423.1 a,b,B

Table 3. Total algal cells filtered and the percent of organic material filtered (ash-free dry weight (AFDW)) by V. iris at 3 different algae rations during 3 feeding periods. Algal rations: Ration A = $5x10^4$ c·mL^{-1}, Ration B = $1.5x10^5$ c·mL^{-1}, and Ration C = $5.0x10^5$ c·mL^{-1}. Values in columns followed by the same uppercase superscript are not significantly different by DMRT ($\alpha = 0.05$).

Feeding Ration (AFDW)	Algal Cells Filtered (AFDW)	Percent of Organic Material Filtered
A (0.30 mg)	1.76×10^7 cells (0.11 mg) A	36 %
B (0.9 mg)	4.6×10^7 cells (0.28 mg) B	31 %
C (3.0 mg)	9.38×10^7 cells (0.57 mg) C	19 %

Table 4. Filtration rates (FR) of several freshwater bivalves fed similar rations (cell density) of algae.

Species	Algae	Algae Ration (10^4 c·mL^{-1})	FR (mL·h^{-1}·g^{-1})
Anodonta anatina[1]	Chlorella vulgaris	1.2	827-923
Unio pictorum[1]	Chlorella vulgaris	1.2	1060-1525
Unio tumidus[1]	Chlorella vulgaris	1.2	875
Unio crassus[1]	Chlorella vulgaris	1.2	1233-1532
Villosa iris[2]	Neochloris oleoabundans	5 x 10^4 c·mL^{-1}	730
Dreissena polymorpha[1]	Chlorella vulgaris	1.2 x 10^4 c·mL^{-1}	7692

1. Kryger and Riisgard (1988).
2. This study.

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Figure 1. Mean filtration rate and ingestion rate in specimens of Villosa iris batch fed 1.3 x 10^6 algal cells·mL^{-1} for 24 h.

Figure 2. Effect of algal cell concentration on filtration rates in specimens of Villosa iris. Cell concentrations: A = 50,000 c·mL^{-1}, B = 150,000 c·mL^{-1}, C = 500,000 c·mL^{-1}.

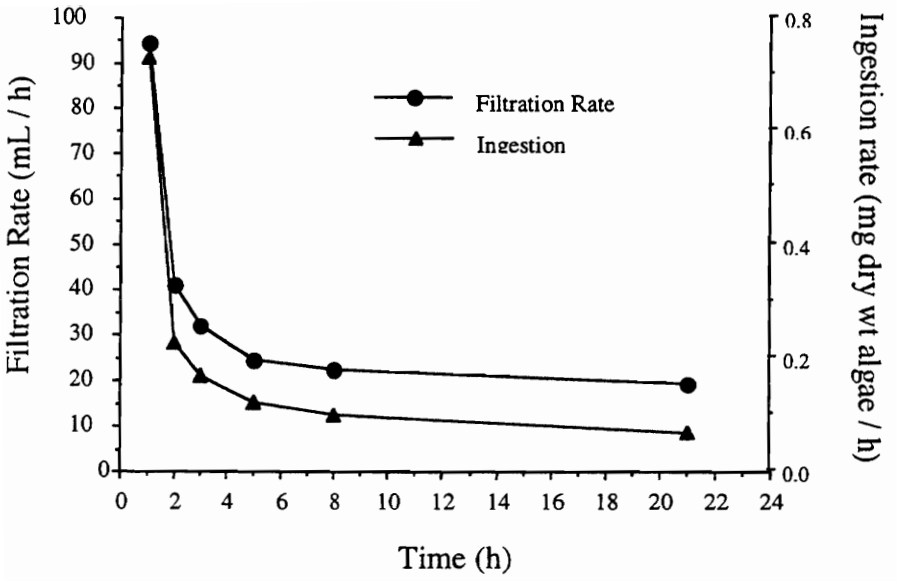

Figure 1. Mean filtration rate and ingestion rate in specimens of Villosa iris batch fed 8.9 mg dry wt algae / L for 24 h.

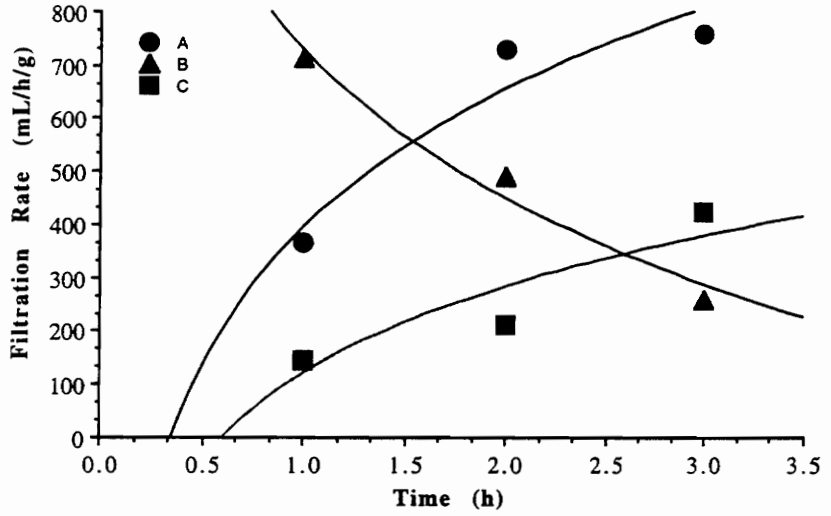

Figure 2. Effect of algal cell concentration on filtration rates in specimens of Villosa iris. Cell concentrations : A = 50,000 c/mL, B = 150,000 c/mL, and C = 500,000 c/mL.

CHAPTER 4

INGESTION AND ASSIMILATION OF NEOCHLORIS OLEOABUNDANS BY THE FRESHWATER MUSSEL, VILLOSA IRIS (LEA 1829), AT THREE ALGAE CONCENTRATIONS

ABSTRACT

Assimilation efficiencies (AE) and carbon budgets were determined for the rainbow mussel, Villosa iris (Lea 1829), using radio-labeled cultures of Neochloris oleoabundans (Chantanachat and Bold 1962) at three cell concentrations. Approximately 70% of the ingested carbon was assimilated (assimilation efficiency) by V. iris fed 5 x 10^5 cells · mL^{-1} (3.4 mg dry weight · L^{-1}); 22% of the ingested carbon was defecated, 8% excreted as waste, 24% respired, and 46% incorporated into tissues. At 5 x 10^4 cells · mL^{-1} (0.34 mg dry weight · L^{-1}), 39% of the ingested carbon was defecated, 13% excreted as waste, 20% respired, and 26% incorporated into tissues. Assimilation efficiency at this cell concentration was 47.5 %. At 5 x 10^3 cells · mL^{-1} (0.034 mg dry weight · L^{-1}), 41% of the ingested carbon was defecated, 6% excreted as waste, 4% respired, and 49% incorporated into tissues. Assimilation efficiency at this cell concentration was 40%. Assimilation efficiencies for V. iris were dependent of both cell concentration and total ingestion. In addition, gut purge time was greater (21 h) for mussels fed the highest concentration of algae than mussels fed the two lower concentrations (9 h). Thus, total assimilation was maximized at 5 x 10^5 cells · mL^{-1}. This indicates that in this study, V. iris had the greatest amount of energy available for growth, reproduction, and body condition when fed 3.4 mg dry weight · L^{-1}

INTRODUCTION

The Unionidae is the largest family of freshwater pearly mussels, world-wide in distribution, with the greatest diversity (nearly 300 species) of freshwater mussels in the continental United States (Ortmann 1911, Baker, 1928, Banarescu 1990). Within the last fifty years, significant declines in freshwater mussel populations have occurred due to river channelization and dredging, impoundment, and water pollution (Bogan 1993). More recently, the non-indigenous zebra mussel (Dreissena polymorpha) has invaded the Mississippi and Laurentian drainage systems, threatening many vulnerable species with possible extinction (Herbert et al. 1991, Ricciardi et al. 1998).

Freshwater mussels provide significant ecological and economic benefits to the nation. They are ecologically important as a food source to many organisms and they play a role in nutrient cycling and structuring benthic species assemblages (Negus 1966, Strayer et al. 1994). In addition, the harvest and exportation of shell material for the cultured pearl industry has been valued annually at $40-$50 million. Thus, the gradual decline of freshwater mussel populations throughout North America will affect the ecosystem dynamics in some river systems and the economic welfare of many commercial shellers.

To preserve the ecological and economic values of freshwater mussels to society, propagation of mussels for stock enhancement and preservation of endangered species has become a priority in the United States. Salvaged mussels, however, are experiencing poor survival in pond refugia, and show signs of stress in other captive settings (unpublished data, C. Gatenby). The success of these conservation programs is currently

limited largely by our inadequate understanding of the feeding physiology and nutritional needs of freshwater mussels. Some studies have been conducted on particle selection and filtration rates of freshwater mussels (Walz 1978, Paterson 1986, Kryger and Riisgard 1988, Miura and Yamashiro 1990, Tankersley and Dimock 1993, McCall et al. 1995, Roper and Hickey 1995, Silverman et al. 1995, Vanderploeg et al. 1995); but less information is available on gut contents (Coker et al. 1921, Gale and Lowe 1971, Bisbee 1984, and Parker et al. 1999). No published information is available on digestive processes such as gut retention time, assimilation efficiency, and nutrient utilization by freshwater mussels. Bivalve energy stores decline without proper feeding, and decreased energy reserves in adult bivalves negatively affect growth rates and developing offspring (Bayne and Thompson 1970, Gabbot and Walker 1971, Bayne 1972, Helm et al. 1973, Bayne et al. 1975, Patterson et al. 1997). Ultimately, food resources for maintaining the health of freshwater mussels in captivity should be digested and assimilated with high efficiency, while providing the necessary nutrients for growth and survival. Assimilation and gut retention time must be measured and integrated with ingestion before food utilization can be determined and suitable diets developed for rearing mussels in captivity.

Detritus is a major food source for mussels in natural systems (Coker et al. 1921, Patterson 1998). In hatchery settings, however, the consistent production of algae that has been nutritionally characterized would be more practical than producing a detritus food source for the large-scale feeding of captive freshwater mussels. Radio-labeled algae cultures have been widely used to study carbon assimilation in bivalves including

Corbicula fluminea, (Lauritsen 1986), Crassostrea virginica, (Newell and Langdon 1986, Crosby et al. 1990), Mytilus edulis (Kreeger et al. 1996, Wang and Fisher 1996), Mytilus trossulus (Kreeger and Langdon 1994), Ostrea edulis (Allen 1962), Argopecten irradians concentricus (Peirson 1983), and Geukensia demissa (Kreeger et al. 1988, Kreeger et al. 1990, Kreeger and Newell 1996). Many of these studies indicate that the efficiency of carbon assimilation is highly dependent on the concentration of food. My objectives were to feed the freshwater mussel, Villosa iris (Lea 1829), a unialgal culture of radio-labeled Neochloris oleoabundans at three cell concentrations and determine: 1) gut passage and gut retention time; 2) the amount of carbon ingested at each cell concentration; 3) the proportion of the ingested carbon assimilated at each cell concentration; and 4) which cell concentration maximized total assimilation.

METHODS

Algae

The green alga N. oleoabundans was selected for assimilation and ingestion experiments because it was known to be a suitable food for rearing freshwater mussels (Gatenby et al. 1997, Patterson 1998). Algae were grown in Neochloris media (Gatenby et al. 1997) under continuous white fluorescent light (photon flux: 35 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 20 ± 1°C. Alga cells were counted using a hemacytometer, and cells were harvested for feeding during late-log phase. Some freshwater mussels filter particle sizes of 5-10 μm more efficiently than smaller particles (< 5 μm) (Paterson 1986, Miura and Yamashiro

1990, Tankersley and Dimock 1993). I harvested the algae during log phase growth when the majority of the cells were ca. 6.2 μm in diameter.

When cell concentrations reached 1 x 10^5 cells · mL^{-1}, algae were inoculated with 100μCi · L^{-1}, [^{14}C]-sodium bicarbonate (57 μCi · mmol $^{-1}$) (ICN Pharmaceuticals, Inc., Irvine, CA), stoppered without aeration, and allowed to grow under the conditions stated above until cell concentrations reached 2 x 10^6 cells · mL^{-1} (> 4 doublings). Cultures were then centrifuged at 7000 - 10,000 rpm for 25 min, the medium decanted, and the algae rinsed with a $(NH_4)_2HPO_4$ solution (pH 7.9-8.5) for a total of 3 washings. Algae were resuspended in autoclaved, distilled water and 2 mL aliquots were radio-assayed to verify that counts were over 100,000 cpm. Final specific activity of the stock algae culture was 0.025 dpm · cell $^{-1}$.

Mussel collection and acclimation

In June 1999, 54 male V. iris (shell lengths, 43-52 mm; dry tissue weights [DW], 0.20-0.46 g) were collected from Copper Creek in Scott County, Virginia. Mussels were scrubbed to remove epiphytes and placed in 250 mL experimental feeding chambers at 18^{0}C. Mussels were fed non-labeled N. oleoabundans at 1 x 10^5 cells · mL twice daily, and allowed to acclimate for 2 wk in the feeding chambers prior to use in the gut passage and assimilation experiments. Feeding chambers were vigorously aerated to maintain algae cells in suspension; water was changed each morning prior to the first feeding.

Delivery of labeled algae

Mussels were placed in new 250 mL feeding chambers, and after the mussels exhibited shell gape and aperture opening, they were fed ^{14}C-labeled algae using a pulse-chase approach (Kreeger and Newell 1996). The pulse chambers were sealed, but a small hole in the lid allowed a 1-ml pipette to be inserted for the delivery of air to maintain algae in suspension. Labeled algae were fed to mussels at three different concentrations: A= 5 x 10^3 cells $\cdot$ mL^{-1} (0.034 mg $\cdot$ L^{-1}), B= 5 x 10^4 cells $\cdot$ mL^{-1} (0.34 mg $\cdot$ L^{-1}), and C= 5 x 10^5 cells $\cdot$ mL^{-1} (3.4 mg $\cdot$ L^{-1}). The chase chambers will be described further, but these were not aerated to prevent respired CO_2 from escaping into the atmosphere.

Gut-passage time

Four mussels per algal concentration were fed radio-labeled algae for 1 h. The mussels were then returned to their unlabeled diet, and feces were collected by filtering the contents of the feeding chambers onto Whatman GF/C filter at 0.5 h intervals for 3 h and then every 3 h up to 48 h. Filters were homogenized and placed in scintillation vials with 10 mL of Beckman Ready Safe (BRS) scintillation cocktail prior to analysis. I estimated gut passage time from the time of the first "spike" in radioactivity to the time when radioactivity had decreased to background levels. I removed the pseudofeces material before filtering and radio-assaying contents of gut passage chase chambers.

Ingestion and Assimilation

Pulse. Eight mussels were allowed to feed on radio-labeled algae for 2 h at three algal concentrations (A, B, C). Upon removal from the pulse chambers, shells were scrubbed to remove any attached ^{14}C-labeled algae. Four additional mussels per treatment were fed radio-labeled algae for 2 h and sacrificed immediately after the pulse to directly calculate ingestion. Gut passage time experiments indicated that a 2 h pulse allowed for sufficient labeled algae to be ingested, but not defecated or excreted.

Chase. At the end of the pulse, mussels were transferred to sealed 250 mL feeding chambers containing a mix of well and city water (pH 8.2), and fed unlabeled, autoclaved N. oleoabundans for 24 h. During the 24 h chase, I changed the water and replenished the algae every 3 h at the same concentrations as in the pulse to prevent changes in feeding physiology. According to gut passage experiments, the duration of the chase (24 h) was sufficient to allow mussels to purge their guts of unassimilated ^{14}C. For example, Geukensia demissa (Kreeger et al. 1988) and Mytilus trossulus (Kreeger 1993) defecated > 95% of the total fecal ^{14}C within 18 h.

Processing Post-Chase. After the chase, the mussels were removed and the chase chambers were sealed until analysis. Mussels were scrubbed, and any attached feces were thoroughly rinsed back into the chase chamber. Bodies were removed from the shell and frozen (-10°C) until analysis. A buffer solution (pH=9) was added to the chase water to trap dissolved $^{14}CO_2$. Feces were collected from the buffered chase water by filtering onto pre-ashed (450°C, 24 h), Whatman GF/C filters. The amount of respired $^{14}CO_2$ and excreted ^{14}C was determined using the "Difference Method" of Kreeger and

Langdon (1994). Two 5 mL aliquots of the buffered chase water were collected after filtering fecal samples. One 5 mL aliquot was acidified (pH<4) and was placed overnight in a dessicator containing 10 mL of concentrated HCl to evolve dissolved (respired) $^{14}CO_2$.

Radio-Assay Procedure

All components were assayed in quintuplicate (5 replicate counts of specific activity) for each mussel per treatment, with the exception that defecation was assayed by summing all filters used to collect the buffered chase water for each mussel.

Respiration and Excretion. I added 15 mL of BRS cocktail to the 5 mL Difference Method aliquots. Specific activities, determined with the liquid scintillation counter (LSC) (Beckman LS 6000SC; Beckman Instruments, Inc., Fullerton, CA), were then corrected for the volumes in the purge beakers (ca. 1200 ml due to 8 water changes) to calculate total ^{14}C respired or excreted by each mussel.

Defecation. Filters were placed in scintillation vials and homogenized in 3 mL of deionized water. The homogenate was digested with 1 mL of tissue solubilizer (Scintigest) in a 55 ^{0}C water bath overnight. I then added 10 mL of BRS cocktail and 130 µL of glacial acetic acid to reduce chemiluminescence of the tissue solubilizer prior to analysis with the LSC.

Ingestion and Incorporation. Mussel tissues were macerated with scissors and homogenized in 20 mL distilled water. Eight, 200 µL aliquots of the tissue homogenate were then removed. Five aliquots were added to a dried, pre-weighed vial, dried (100°C,

24 h), and weighed. I digested the other five aliquots with 2.5 mL of tissue solubilizer (Scintigest) in a 55 ^{0}C waterbath overnight (Lauritsen 1986). After digestion, I added 10 mL of BRS cocktail and 130 µL of glacial acetic acid for radio-assay. These ^{14}C activities were then corrected for the total dry tissue weight of mussels. In a preliminary control test of homogenization, I found no significant difference ($p = 0.749$) between the dry weights (6.4 mg $\pm$ 0.65) of 5, 200 uL aliquots of homogenized tissue. In addition, I found no significant difference ($p = 0.726$) between the mean dry weights (36.0 mg $\pm$ 13) of 116, 1 mL homogenized aliquots used in the assimilation experiment.

The contents of the pulse chambers also were filtered through Whatman G/FC filters to calculate the amount of radio-label used (i.e., ingestion). This value was compared to other ingestion values obtained by the summation of energy components and the direct sacrifice of mussels. Single filters were homogenized with 200 mL distilled water in scintillation vials, and 10 mL of BRS was added prior to analysis with the LSC.

Energy Budget Calculation

A complete ^{14}C-budget was calculated for each mussel as follows:

$$I = T + D + R + E,$$

where ingestion (I), tissue incorporation (T), defecation (D), respiration (R), and excretion (E) were expressed in units of disintegrations per minute per gram tissue dry weight (Kreeger and Langdon 1994). Assimilation efficiencies (AE) were estimated as the proportion of ingested ^{14}C that had been incorporated and respired, as follows (Kreeger and Langdon 1994):

$AE = [(T + R)/I] \times 100\%$.

Ingestion was computed by adding the mean activities of the ^{14}C-energy budget components. Kreeger and Newell (1996) found measurements of ingestion using the removal of labeled particles from suspension to be more variable than summation of budget components, and they concluded that the latter approach was more reliable. In this study, total ingestion (774, 872.3 dpm) obtained from the summation of energy budget components for all mussels was not significantly different ($p=0.17$) to that obtained from the loss in radioactivity from suspension (460,780.09 dpm). Mean ingestion values obtained from the sacrifice of mussels (0.15 ± 0.189 µg C · g DW mussel tissue^{-1}) immediately following the pulse and from summation of energy components (0.218 ± 0.277 µg C · g DW $^{-1}$) also were similar ($p=0.195$). Thus, I concluded that the components of the energy budget accounted for all of the ^{14}C activity ingested by mussels, and we used this when comparing assimilation and ingestion among treatments.

Pearson correlation coefficients were calculated for ingestion vs. assimilation efficiency, ingestion vs. total assimilation, ingestion vs. tissue dry weight, and assimilation efficiency vs. tissue dry weight at each cell concentration using Statview 512+ (Brainpower, Inc.). Total ingestion, total assimilation, and assimilation efficiency at each cell concentration were compared with ANOVA. If significant differences were detected, the Scheffe F-test was used to determine the statistical significance of individual treatments, $\alpha = 0.05$.

^{14}C-Retention Efficiency

Prior to running the gut passage and assimilation experiments with mussels, I simulated an experiment without mussels following established methodology for determining defecation, excretion, and respiration, including tests with $^{14}CO_2$ traps. The pH of the chase water was 8.2; thus, $^{14}CO_2$ should remain in solution. I compared the retention efficiency (proportion of total ^{14}C-activity (dpm) remaining in the experiment) between sealed chase chambers and aerated chambers. I included a "respiration-trap" containing 5 mL of a 2:1 mixture of ethylene glycol monomethyl ether and ethanolamine in the sealed chambers to determine whether any $^{14}CO_2$ evolved out of solution (Pierson 1983). I also tested the hypothesis that live algae may cause color quenching (phyto-pigments distort accurate radioactivity counts) and that live algae take up $NaH^{14}CO_3$, thus potentially lowering accurate measurement of respiration radioactivity. Algae rations were tested in quadruplicate, and were the same rations tested with mussels.

I quantified the ^{14}C-activity remaining in the sealed chambers after 3 h, and in the aerated chambers after 24 h, and tested this in quadruplicate. Four of the sealed chase chambers received an aliquot of autoclaved algae at the concentration tested in the assimilation experiment, and four received no algae. Four aerated chambers received autoclaved algae, and four aerated chambers contained live algae. I delivered 30 μ Ci $NaH^{14}CO_3$ to each chase chamber; 10 mL of BRS cocktail and 1 mL of methanol were added to enhance mixing of the cocktail with the ethanolamine trap mixture (Peirson 1983). Filters were homogenized and 10 mL BRS was added prior to reading radioactivity. Preliminary control trials indicated that a single filter caused no quenching.

Total ^{14}C-activity (dpm) used (initial $_{dpm}$ – final $_{dpm}$) was compared within treatments using ANOVA. Retention efficiency values were arcsine-transformed and compared among chambers with no algae, autoclaved algae, and live algae ignoring chamber type (sealed vs. aerated). I then compared arcsine transformed retention efficiency between aerated chambers and sealed chambers containing autoclaved algae. All aerated samples also were compared using internal standards to test the hypothesis that the observed loss in radioactivity was attributed to aeration.

I recovered 96 ± 12 % of ^{14}C-activity in the sealed chase chambers without algae; the traps accounted for 0.09 ± 0.03% of the ^{14}C-activity. There was no difference between initial and final radioactivity levels ($p = 0.0054$). I recovered 100.0 ± 5 % of ^{14}C-activity in the sealed chambers containing autoclaved algae; the traps accounted for 0.17 ± 0.17 %. In addition, there was no difference in retention efficiency (Scheffe's $F = 0.379$) between the sealed chambers containing autoclaved algae and those containing no algae. This indicated there was no color quenching by any concentration of algae in the sealed chambers, nor did I lose radioactivity to the method employed. Retention efficiency for the aerated chambers was significantly lower (37.2 ± 26 % and 25.6 ± 5 % for chambers with autoclaved algae and live algae, respectively) than the retention efficiency of the sealed chase chambers ($p<0.05$, Scheffe's $F \geq 37.09$ for all four comparisons. A final ANOVA comparing total ^{14}C-activity recovered (dpm) plus an added internal standard (dpm) for the aerated chase chambers containing live algae and autoclaved algae to the expected values of ^{14}C-activity (dpm) indicated that the observed decrease in retention efficiency was not due to color quenching ($p = 0.114$).

I concluded that aeration caused an undesirable loss in radioactivity; therefore, no aeration was used in the chase chambers in further experiments. I also decided against using traps in the assimilation and gut passage experiments because little to no ^{14}C was detected in the traps. In previous trials, I noted that the trap chemicals, ethylene glycolmonomethyl ether and ethanolamine, were lethal to mussels when they were accidentally spilled into the feeding chambers.

RESULTS

Gut Passage

Minimum gut passage time for mussels fed ration C was 1.5 h. Two other mussels in treatment C began defecating at 2 and 2.5 h. The four mussels in treatment C purged their guts within 21 h; radioactivity in ration C was equivalent to background levels at 24 h, 36 h, and 48 h. Minimum gut passage time for mussels fed ration B appeared to be 2.5 h (2 mussels); however, ^{14}C-specific activity was significantly higher than background levels for one mussel in ration B at 1.0 h. I suspected that pseudofeces or labeled algae adhering to the shell caused this high radioactivity. Three of the four mussels in ration B defecated at 2.5, 3.0, and 6.0 h. Mussels in ration B appeared to completely purge their guts within 9 h. Because the specific activity of ration A was low due to the low concentration of algae, I did not observe a significant difference in radioactivity between 1.0 and 6.0 h. Minimum gut passage time for two mussels fed

ration A was 1.0 h; specific activities dropped to background levels for one of these mussels at 2 h through 23 h. Total gut purge time was 9 h for 3 mussels fed ration A.

Ingestion and Assimilation

Total carbon ingested (666.2 ± 213.9 μg C · g dry tissue weight $^{-1}$) was significantly higher for mussels fed the highest concentration of algae ration C (Table 1), than mussels fed either rations B or A (95.0 ± 30.3 and 23.7 ± 5.4 μg C · g dry tissue weight^{-1}, respectively) ($p = 0.0001$). Total carbon ingested also was significantly higher for mussels fed ration B than mussels fed ration A ($p=0.0$). Total assimilation (T+R) of carbon (dpm · g mussel^{-1}) was linearly related to total ingestion (dpm · g mussel^{-1}) for all treatments (Figure 1; $R=0.978$, $p=0.0001$). Assimilation, therefore, was significantly higher for mussels fed ration C (461.3 ± 213.0 μg C · g dry tissue weight $^{-1}$) than mussels fed either ration B or ration A (46.0 ± 18.2 and 9.4 ± 2.1μg C · g dry tissue weight $^{-1}$, respectively) (Table 1, $p = 0.0001$). Total assimilation also was higher for mussels fed ration B than ration A ($p = 0.0001$). In general, mussels fed ration B incorporated less of the ingested material than mussels fed the other rations (Table 1).

Assimilation efficiency was significantly higher for mussels fed ration C (70.2 ± 12.3%) than for mussels fed rations B (47.5 ± 9.3%) and A (40.1± 6.3%) ($p = 0.0001$) (Table 1). Despite differences in total ingestion, and assimilation, assimilation efficiencies at the lower cell concentrations (40 % and 47.5%) were not significantly different ($F=0.131$, $p>0.5$). In general, assimilation efficiency increased with increasing ingestion for mussels pooled from each treatment (Figure 1; $R = 0.85$, $p = 0.0001$).

Assimilation efficiency of mussels within a treatment, however, was not dependent on total ingestion for mussels fed rations A and B (R=0.4, p=0.315; R=0.44, p=0.281, respectively) but was dependent on ingestion for mussels fed ration C (R=0.74, p=0.04).

Individual budget components as a percentage of ^{14}C ingested were not similar among treatments (p = 0.0001). However, the amount of ingested energy that was voided (D + E = 55.9% and 52.5% for A and B, respectively) and the amount of energy assimilated (T+R = 43% and 46% for A and B, respectively) was similar between treatments A and B, as seen in the carbon budget equations (F = 0.34 and 0.13).

Mussels fed ration A ingested ≈100 % [7.5 ± 0.6 µg dry weight C, or 23.7 ± 5.4 µg dry weight C (g dry tissue weight)$^{-1}$)] of the available carbon (Tables 2 and 3). Of the carbon ingested by mussels, 13.4% was defecated [3.2 ± 1.4 µg dry weight C (g dry tissue weight)$^{-1}$)], 42.8% excreted as waste [10.2 ± 2.6 µg dry weight C (g dry tissue weight)$^{-1}$)], 13.6% respired [3.3 ± 1.3 µg dry weight C (g dry tissue weight)$^{-1}$)], and 30.5% incorporated into tissues [7.1 ± 1.3 µg dry weight C (g dry tissue weight)$^{-1}$)]. The energy budget calculated for V. iris at this cell concentration was:

100% I = 30% T + 13% D + 14% R + 43% E.

Mussels fed ration B ingested ≈23 % [31.7 ± 5.7, or 95.0 ± 30.3 µg dry weight C (g dry tissue weight)$^{-1}$)] of the available carbon (Tables 2 and 4). Of the carbon ingested by mussels, 39.1% was defecated [37.9 ± 10.0 µg dry weight C (g dry tissue weight)$^{-1}$)], 13.4% excreted as waste [12.5 ± 3.6 µg dry weight C (g dry tissue weight)$^{-1}$)], 20.4% respired [20.4 ± 6.9 µg dry weight C (g dry tissue weight)$^{-1}$)], and 26.1% incorporated

into tissues [25.6 ± 10.0 µg dry weight C (g dry tissue weight)$^{-1}$)]. The energy budget calculated for V. iris at this cell concentration was:

100% I = 26% T + 39% D + 20% R + 13% E.

Mussels fed ration C (3.4 mg dry weight L^{-1}) ingested ≈47 % [240.5 ± 75 µg dry wt C, or 666.2 ± 213.9 µg dry wt C (g dry tissue weight^{-1})] of the available carbon (Table 2 and 5). Of the carbon ingested by mussels, 21.9% was defecated [145.8 ± 40.0 µg dry weight C (g dry tissue weight)$^{-1}$)], 8.1% excreted as waste [53.6 ± 10.0 µg dry weight C (g dry tissue weight)$^{-1}$)], 23.8% respired [158.7 ± 70.0 µg dry weight C (g dry tissue weight)$^{-1}$)], and 46.2% incorporated into tissues [307.9 ± 248.6 µg dry weight C (g dry tissue weight)$^{-1}$)]. The energy budget calculated for V. iris at this cell concentration was:

100% I = 46% T + 22% D + 24% R + 8% E.

DISCUSSION

Assimilation efficiency for Villosa iris ranged 40 to 70 % and was similar to those obtained for several marine and freshwater bivalves. For example, Corbicula fluminea assimilated 47 to 57% of various green alga diets that were ingested and efficiency varied depending on the alga species (Lauritsen 1986). Walz (1978) reported 40.1% assimilation efficiency for Dreissena polymorpha fed 2 mg L^{-1} Nitzschia actinastroides. Typical assimilation efficiencies for the blue mussel, Mytilus edulis have been reported between 52 and 89% (Thompson and Bayne 1972, Thompson and Bayne 1974, Kiorboe et al. 1980, Riisgard and Randlov 1981). The ribbed mussel, Geukensia demissa,

assimilated 77% of the chrysophyte, Isochrysis galbana (Kreeger and Newell 1996). Finally, the scallop, Argopecten irradians, assimilated 77.5-99.0% of several different algae (Peirson 1983). Assimilation efficiency in filter-feeders, however, is known to be affected by many factors including the physiological condition of the animals and the quality and quantity of food (Bayne and Newell 1983, Peirson 1983, Kreeger 1993, Kreeger et al. 1995, Wang and Fisher 1996).

The quantity of food had a dramatic effect on the assimilation efficiency of the Mytilus edulis, fed unialgal cultures of Tetraselmis suecica. Assimilation efficiencies were 85% at 1 x 10^3 cells $\cdot mL^{-1}$ (0.66 mg $\cdot L^{-1}$) and 0% at 2.5 x 10^3 cells $\cdot mL^{-1}$ (1.65 mg $\cdot L^{-1}$) (Thompson and Bayne 1972). Similarly, when fed Dunaliella primolecta at cell suspensions between 5 x 10^2 (0.06 mg $\cdot L^{-1}$) and 8 x 10^3 cells $\cdot mL^{-1}$ (0.90 mg $\cdot L^{-1}$), assimilation efficiencies of the black mussel, Choromytilus meridionalis, reached 80% and then dropped to 0% between 3 x 10^4 (3.36 mg $\cdot L^{-1}$) and 4 x 10^4 cells $\cdot mL^{-1}$ (4.48 mg $\cdot L^{-1}$) (Griffiths 1980). Assimilation efficiency declined with increasing cell concentration, from 73.4 % to 49.8% for Mytilus chilensis fed 1.5 x 10^4 cells $\cdot mL^{-1}$ (0.8 mg$\cdot L^{-1}$) to 4.0 x 10^4 cells $\cdot mL^{-1}$ (2.14 mg$\cdot L^{-1}$). In contrast, at much higher algal concentrations of Phaedactylum tricornutum (1 x 10^5 cells $\cdot mL^{-1}$; 4.4 mg $\cdot L^{-1}$), the assimilation efficiency of Crassostrea virginica remained around 70% (Langefoss and Maurer 1975). Because of this discrepancy, some authors have concluded that assimilation efficiency is dependent on the amount of algae ingested rather than concentration of the suspension directly (Foster-Smith 1975a, Navarro and Winter 1982). Interestingly, assimilation efficiency for Villosa iris in this experiment was dependent on both cell concentration and total

ingestion. Assimilation efficiency increased from 40% to 67% with increasing cell concentration ($5x10^3$ cells·mL^{-1} to $5x10^5$ cells·mL^{-1}). This was most likely related to the increased gut passage time observed in the previous test. Increased gut passage and gut retention time allows further digestion of food components and increased assimilation (Wang et al. 1995).

It is generally accepted that molluscs, deposit-feeders and filter-feeders, exhibit one of two opposing strategies when feeding on indigestible food: (1) increase gut residence time to give the digestive processes longer to act on the food, and thus maximize nutrient gain; or (2) reduce gut passage time of the food item. Obviously, there would be little benefit in holding indigestible food in the gut longer for more efficient digestion. One explanation for increased gut residence at higher concentrations of N. oleoabundans is that it is readily digestible (Gatenby et al. 1997). Total assimilation as a percent of ingestion, therefore, was greater for mussels fed the higher ration of algae because of increased gut residence time and the greater amount of material available for assimilation. There was no difference in total gut purge time and assimilation efficiency between the two lower concentrations of algae, indicating a relationship between gut residence time and assimilation efficiency.

Wang and Fisher (1996) found that the chlorophytes Chlorella autotrophica and Nannochloris atomus were not readily assimilated (8-21%) by Mytilus edulis. Inefficient assimilation of chlorophytes has been reported in clams, oysters and bay scallops (Floyd 1953, Peirson 1983, Bass et al. 1990). The presence of a resistant substance resembling sporopollenin in the outer cell wall may make some chlorophytes resistant to bivalve

digestion (Atkinson et al. 1972, Bricelj et al. 1984, Wang and Fisher 1996). For example, Peirson (1983) showed that bay scallops, Argopecten irradians concentricus, ingested nearly equal portions of the available Chlorella autotrophica and Chroomonas salina (77% and 81%, respectively). However, the scallops assimilated 84% of the ingested C. salina and only 17% of the ingested C. autotrophica. The chlorophyte Neochloris oleoabundans was assimilated by Villosa iris with relatively high efficiency (~70%). The presence of a thin cell wall (Prescott 1978) and naked zoospores during asexual reproduction (Bourrelly 1966) likely enhanced the digestibility of N. oleoabundans. Gut content analyses of juvenile V. iris reared on N. oleoabundans showed large masses of chlorophyll and few intact cells indicating that juveniles had ingested and digested this alga (Gatenby et al. 1997). This algal species, therefore, can serve as a good food resource for freshwater mussels held in captivity.

Filtration rate, ingestion rate, gut residence time, and assimilation rate are all interrelated. The concentration at which filtration and ingestion is regulated to optimize energy costs and benefits is also the concentration at which assimilation efficiency is highest (Navarro and Winter 1982, Iglesias et al. 1992). This optimum concentration, however, differs with species of bivalves (Winter 1973, Foster-Smith 1975b, Higgins 1980, Riisgard and Randlov 1981, Sprung and Rose 1988, Kryger and Riisgard 1988, Way 1989, Roper and Hickey 1995). Even if energetic costs of pumping water and processing particles in the gills and labial palps are assumed to be low (Jorgensen et al. 1986, Widdows and Hawkins 1989), the benefits derived from ingesting greater amounts of algae of high energetic value would counterbalance the cost of increased filtration.

Further demands of sorting material for ingestion and for rejection of pseudofeces with increasing particle concentration will eventually impose a limitation on growth potential (Bayne et al. 1989). At the pseudofeces-free cell density of 1.5 x 10^5 c·mL^{-1}, V. iris appeared to regulate filtration (unpublished data, C. Gatenby) suggesting this was the optimum food concentration at which filtration activity was reduced to a low-energy consuming level (Winter 1978). An algal density of 5 x 10^5 cells · mL^{-1}, however, led to the highest total assimilation of carbon. By maximizing total carbon assimilation, V. iris had the largest amount of energy available for growth, reproduction, and maintenance food. I recommend that mussels held in captivity should be fed no less than 1.5 x 10^5 c·mL^{-1} (1.0 mg dry wt · L^{-1}, ca. 0.3 % of unionid dry wt) and as high as 5 x 10^5 cells · mL^{-1} (3.4 mg dry weight · L^{-1}, 0.97% of unionid dry wt). The feeding physiology and metabolic rates (basal, reproductive, and growth) have been poorly studied in freshwater bivalves. Studies on gut residence time in freshwater mussels, as well as additional ^{14}C tracer studies with algae that differ in their nutritional value (biochemical composition) and relative lability or refractivity, are needed to confirm the effects of food concentration and food quality on assimilation efficiency.

ACKNOWLEDGMENTS

This project was funded by the Biological Resources Division of the United States Geological Survey. I would like to thank R. I. E. Newell and D. A. Kreeger for access to

their labs and introduction to the methods for ^{14}C feeding studies. I also thank Matt Patterson for helping to develop the methods, and Tracy Booth for her tireless assistance.

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LIST OF TABLES

Table 1. Ingestion and assimilation (μg dry weight C · g dry tissue weight^{-1}), and assimilation efficiency of Villosa iris fed three different rations of algae: Ration A = 0.034 mg dry wt·L^{-1}, Ration B = 0.34 mg dry wt·L^{-1} and Ration C = 3.4 mg dry wt . L-1.

Table 2. The proportion of available carbon (μg) ingested by mussels fed 3 algal rations.

Table 3. ^{14}C budget components of Villosa iris fed ^{14}C-labelled Neochloris oleoabundans at 5 x 10^{5} cells · ml^{-1} (3.4 mg · L^{-1}).

Table 4. ^{14}C budget components of Villosa iris fed ^{14}C-labelled Neochloris oleoabundans at 5 x 10^{4} cells · ml^{-1} (0.34 mg · L^{-1}).

Table 5. ^{14}C budget components ofVillosa iris fed ^{14}C-labelled Neochloris oleoabundans at 5 x 10^{3} cells · ml^{-1} (0.034 mg · L^{-1}).

Table 1. Ingestion and assimilation (μg dry weight C · g dry tissue weight^{-1}), and assimilation efficiency of Villosa iris fed three different rations of algae: Ration A = 0.034 mg dry wt·L^{-1}, Ration B = 0.34 mg dry wt·L^{-1} and Ration C = 3.4 mg dry wt . L-1. Means within a row followed by the same lower case letter were not significantly different by Scheffe's F-test ($\alpha = 0.05$). N = 8 mussels per ration.

	Ration A	Ration B	Ration C
Ingestion	23.7 ± 5.4 a	95.0 ± 30.3 a	666.2 ± 213.9 b
Assimilation	9.4 ± 2.1 a	46.0 ± 18.2 a	461.3 ± 213.0 b
Assimilation Efficiency	44.1 ± 6.3% a	47.5 ± 9.4% a	70.0 ± 12.2% b

Table 2. The proportion of available carbon (μg) ingested by mussels fed 3 algal rations. The mean (± SD) ^{14}C-activity [dpm] is given in brackets and the total dry weights of carbon ingested (μg dry weight C) is given in parentheses. N = 8 mussels per ration.

Treatment	Total Carbon Available (μg)	^{14}C-ingested (%) [dis · min^{-1} x 10^4] (μg dry weight C)
Ration C: 5×10^5 cells · ml^{-1}) (3.4 mg · L^{-1})	510	47.2 [87.46 ± 27.3] (240.5 ± 75)
Ration B: 5×10^4 cells · ml^{-1} (0.34 mg · L^{-1})	51	62.2 [11.54± 2.1] (31.7 ± 5.7)
Ration A: 5×10^3 cells · ml^{-1} (0.034 mg · L^{-1})	7.5	100 [2.7 ± 0.2] (7.5 ± 0.6)

Table 3. ^{14}C budget components of Villosa iris fed ^{14}C-labelled Neochloris oleoabundans at 5 x 10^5 cells · ml^{-1} (3.4 mg · L^{-1}). The mean (± SD) ^{14}C-activity of each component is expressed as a percentage of ^{14}C ingested, with actual ^{14}C-activity [dpm · g dry mussel tissue weight $^{-1}$] given in brackets and total dry weights of carbon (µg dry weight C · g dry tissue weight^{-1}) given in parentheses.

Budget component	^{14}C-component as percentage of ingestion [dis · min^{-1} x 10^4 · (g dry tissue weight)$^{-1}$] (µg dry weight C · (g dry tissue weight)$^{-1}$)
Ingestion (I)	100 [242.2 ± 77.78] (666.2 ± 213.9)
Defecation (D)	21.9 ± 10.4 [53.1 ± 14.6] (145.8 ± 40.0)
Excretion (E)	8.1 ± 2.5 [19.5 ± 5.2] (53.6 ± 10.0)
Respiration (R)	23.8 ± 11.5 [57.7± 26.2] (158.7 ± 70.0)
Incorporation (T)	46.2 ± 20.2 [112.0 ± 90.4] (307.9 ± 248.6)
Total Assimilation (A)	70.0± 12.3 [167.7 ± 77.4] (461.3 ± 213.0)

Table 4. ^{14}C budget components of Villosa iris fed ^{14}C-labelled Neochloris oleoabundans at 5 x 10^4 cells · ml^{-1} (0.34 mg · L^{-1}). The mean (± SD) ^{14}C-activity of each component is expressed as a percentage of ^{14}C ingested, with actual ^{14}C-activity [dpm · g dry mussel tissue weight $^{-1}$] given in brackets and total dry weights of carbon (µg dry weight C · g dry tissue weight^{-1}) given in parentheses.

Budget component	^{14}C-component as percentage of ingestion [dis · min^{-1} x 10^4 · g dry tissue weight^{-1}] (µg dry weight of C · g dry tissue weight^{-1})
Ingestion (I)	100 [34.6 ± 11.0] (95.0 ± 30.3)
Defecation (D)	39.1 ± 8.0 [13.2 ± 4.3] (37.9 ± 10.0)
Excretion (E)	13.4 ± 1.9 [4.5 ± 1.4] (12.5 ± 3.6)
Respiration (R)	21.4 ± 3.0 [7.4 ± 2.5] (20.4 ± 6.9)
Incorporation (T)	26.1 ± 8.8 [9.3 ± 4.7] (25.6 ± 10.0)
Total Assimilation (A)	47.5 ± 9.4 [16.7 ± 6.6] (46.0 ± 18.2)

Table 5. ^{14}C budget components of Villosa iris fed ^{14}C-labelled Neochloris oleoabundans at 5 x 10^3 cells · ml^{-1} (0.034 mg · L^{-1}). The mean (± SD) ^{14}C-activity of each component is expressed as a percentage of ^{14}C ingested, with actual ^{14}C-activity [dpm · g dry mussel tissue weight $^{-1}$] given in brackets and total dry weights of carbon (µg dry weight C · g dry tissue weight^{-1}) given in parentheses.

Budget component	^{14}C-component as percentage of ingestion [dis · min^{-1} x 10^4 (g dry tissue weight)$^{-1}$] (µg dry weight of C (g dry tissue weight)$^{-1}$)
Ingestion (I)	100 [8.6 ± 2.0] (23.7 ± 5.4)
Defecation (D)	13.4 ± 3.7 [1.2 ± 0.4] (3.2 ± 1.4)
Excretion (E)	42.8 ± 3.0 [3.7 ± 0.0.9] (10.2 ± 2.6)
Respiration (R)	13.6 ± 2.5 [1.2 ± 0.4] (3.3 ± 1.3)
Incorporation (T)	30.5 ± 5.2 [2.6 ± 0.5] (7.1 ± 1.3)
Total Assimilation (A)	44.1 ± 6.3 [3.4 ± 0.7] (9.4 ± 2.1)

LIST OF FIGURES

Figure 1. Linear relationship between total assimilation and total ingestion (R=0.98) for Villosa iris fed the green alga, Neochloris oleoabundans.

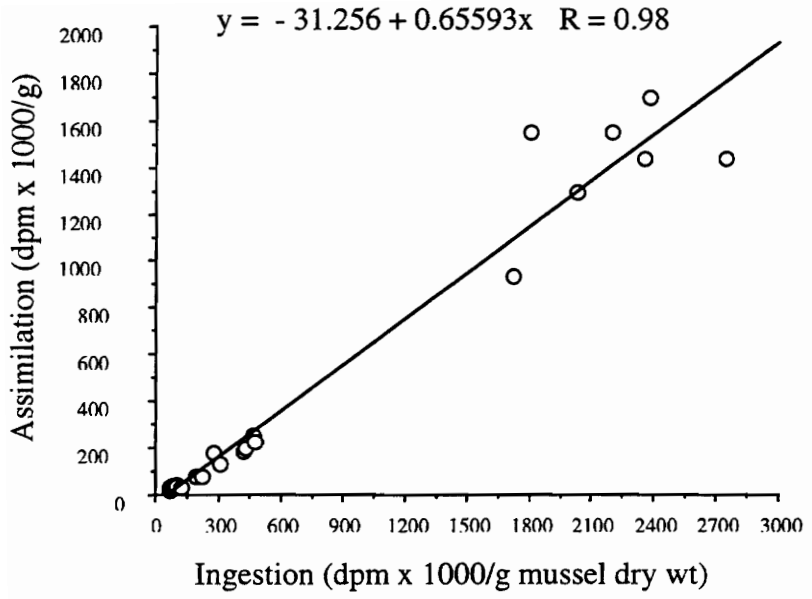

Figure 1. Linear relationship between total assimilation and total ingestion (R = 0.98)

CHAPTER 5

BIOCHEMICAL COMPOSITION OF FOUR ALGAE PROPOSED AS FOOD FOR CAPTIVE FRESHWATER MUSSELS

ABSTRACT

The gross composition (protein, carbohydrate (CHO), and lipid) as well as the fatty acid and sterol composition of four algae (the chlorophytes, ,Bracteacoccus grandis, Neochloris oleoabundans, and Scenedesmus quadricauda, and the bacillariophyte, Phaeodactylum tricornutum) was examined at four different phases of growth. The CHO content of N. oleoabundans and P. tricornutum increased with growth phase, and was significantly greater at late stationary phase (33.1% and 39.4%, respectively) than that of the other algae. At early log phase, B. grandis contained significantly more CHO (24.5%) than the other algae. S. quadricauda had highest CHO at log and stationary phase (38.05% and 26.5%, respectively). The total lipid content increased with growth phase for N. oleoabundans and P. tricornutum. Lipid content of B. grandis decreased with age, and S. quadricauda showed no difference in lipid content among growth phases. Lipid content (31.9%) of N. oleoabundans was greater than the other species; however, mean value was not significantly different from those of S. quadricauda and P. tricornutum (24.4 % and 21.8 %, respectively). The mean fatty acid content of the four algae ranged 15.5% to 45% of the total lipid dry wt. The unsaturated fatty acid content generally increased with growth phase for all algae. The three most abundant fatty acids in the three green algae were 18:1, 18:2, and 18:3. The most abundant fatty acids in P. tricornutum were 16:0, 16:1, and 20:5 (EPA). Generally, there was no effect of phase on sterol content, with the exception of S. quadricauda, which showed a significant increase in sterol content with growth phase. The sterol content of S. quadricauda and B. grandis (19.5 ± 17.7 μg mg lipid^{-1} and 19.3 ± 12.6 μg mg lipid^{-1} respectively), was similar and

significantly greater than the sterol content of P. tricornutum (7.4 ±3.8 μg· mg lipid^{-1}) and N. oleoabundans (5.9 ±0.01 μg · mg lipid^{-1}) ($p = 0.0002$). The sterol composition of B. grandis was dominated by Δ 5,7 stigmastenol and Δ 5 sitosterol, with lesser amounts of Δ 5-ergostenol and Δ 7 ergostenol. The sterol composition of S. quadricauda and N. oleoabundans was the same (Δ 5,7,22 ergostatrienol, Δ 5,7-ergostadienol, and Δ 7 ergostenol). The sterol Δ 5, 22 ergostadienol was predominant in P. tricornutum, with Δ 7, 22-ergostadienol present in minor concentrations. Maximum sterol content (% lipid wt) was found in S. quadricauda at stationary phase and in B. grandis at late stationary phase (5 % and 4.4%, respectively). Mean sterol content of the four algae ranged 1.0 % to 1.8 % of lipid wt. There was no effect of growth phase or species on the protein content (% of algal dry wt) ($p = 0.0.875$). Over all growth phases, there was no difference in the mean protein content among species ($p = 0.408$). The protein content ranged 60.6 ± 17.1 to 70.3 ± 9.5 % of algal dry wt.

INTRODUCTION

Microalgae play an important role in mariculture as food for molluscs, some crustaceans, and fish species. The successful growth and development of cultured animals reared on microalgae is dependent on the proportion and availability of the biochemical constituents in the algae. Various golden-brown flagellates (Pavlova lutheri and Isochrysis sp.), diatoms Thalassiosira pseudonana, Skeletonema costatum, Chaetoceros calcitrans, and C. gracilis, the green (prasinophyte) alga Tetraselmis suecica and the yellow-green (eustigmatophyte) Nannochloropsis oculata have been utilized successfully as food sources for bivalve molluscs (Walne 1970, Enright et al. 1986).

It is well known that nutritional requirements of marine bivalves vary seasonally with changing reproductive condition and seasonal variation in the quality and quantity of available food (Burt 1955, Soniat et al. 1984, Hawkins and Bayne 1985, Berg and Newell 1986, Asmus and Asmus 1991, Kreeger 1993, Kreeger et al. 1995). The lipid composition of the bivalve diet has been suggested as more critical than protein and carbohydrate composition for promoting growth and development in larvae (Holland 1978, Webb and Chu 1983). Kreeger (1993) showed, however, that adult Mytilus trossulus had a greater requirement for protein during the latter stages of gametogenesis, while carbohydrates became important in early fall as storage energy. It is generally agreed that carbohydrates serve as a primary energy reserve in most adult bivalves, whereas the larvae (Holland 1978, Napolitano et al. 1988) accumulate lipids in the form of triacylglycerols.

Fatty acids are the fundamental, structural components of all lipids. Saturated and monoenoic fatty acids can be synthesized by aquatic invertebrates; however, it has not been shown conclusively whether aquatic invertebrates are capable of de novo synthesis of polyunsaturated fatty acids (PUFAs) which are essential for growth, development, and cellular function (Morris and Sargent 1973, Waldock and Holland 1984, Chu and Greaves 1991). The variability in both the number of carbon atoms and the number of double bonds determines to a large extent the physical properties of biological membranes (Hazel 1990). In addition, PUFAs are believed to be precursors to prostaglandins (Castell 1970, Cohen et al. 1988). It has been shown that many marine invertebrates, including bivalves, show a very low capacity to elongate and desaturate fatty acids to PUFAs; thus, they must satisfy some of their essential fatty acid requirements through dietary sources (Chu and Greaves 1991, Zhukova et al. 1998). Sterols also are lipid components of eukaryotic organisms associated with a number of functions such as structural components of cellular membranes, hormone synthesis, and hormone regulation (Nes 1974, Freedland and Briggs, 1977). Sterol metabolism in molluscs differs between classes. Gastropods synthesize cholesterol de novo and can also dealkylate phytosterols to cholesterol. Bivalves, however, are capable of limited synthesis of cholesterol, and some C28 and C29 sterols, but sterol metabolism appears to proceed at a slow rate (Trider and Castell 1980, Webb and Chu 1983, Napolitano et al. 1993).

OBJECTIVES

In this chapter, I examine the biochemical composition of 4 freshwater algal species from the classes Chlorophyceae (3 species) and Bacillariophyceae (1 species). The gross composition (protein, carbohydrate, and lipid) as well as the fatty acid and sterol composition of these algae were examined at four different phases of growth. The nutritional value of algae has been shown to be a function of the digestibility of the cells and the growth phase of the algal culture (Wilson 1979, Chu and Dupuy 1981, Wikfors et al. 1992). These algae have been used in the culture of juvenile freshwater mussels (Gatenby et al. 1997), and one of the chlorophytes was digestible by adult mussels (Chapter 4). These algae grow well under a variety of environmental conditions (Tornabene et al. 1983, Yongmanitchai and Ward, 1991, Arredondo-Vega et al. 1995, Carten et al. 1996), making them well suited to mass culture.

METHODS

Microalgal cultures. The chlorophytes, Bracteacoccus grandis (UTEX #1246) and Neochloris oleoabundans (UTEX #1185) were obtained from the University of Texas. The bacillariophyte, Phaeodactylum tricornutum (UTEX 640) also was obtained from the University of Texas. The chlorophyte, Scenedesmus quadricauda was obtained from the Virginia Tech Aquaculture Center. This alga dominates the culture tanks used to feed freshwater mussels at Virginia Tech's Aquaculture Center. The algae were cultured in media ideal for growth of each species (Appendix 1). P. tricornutum was cultured in Bold's Basal Medium with soil extract added (Nichols 1973); N.

oleoabundans was cultured in a vitamin-enriched media, and Bracteacoccus grandis and S. quadricauda was cultured in our "Chlorella" media (OCM) (Martek, Inc., Columbia, MD). All media were prepared fresh from respective dry chemicals. Starter cultures (in mid-log phase) were inoculated into 30 mL of media in 100 mL test-tubes. Cultures were placed in a water bath maintained at 17 ± 1°C under continuous cool white fluorescent light of 60 $\mu m \cdot m^{-2} \cdot s^{-1}$ photon flux. The cultures were aerated with 1-2% CO_2 added.

Cell growth rates were followed by direct microscopic cell counts, and dry cell weight was determined for each batch of algae harvested. Algae were harvested at early log, late log, stationary, and late stationary growth phase. For example, N. oleoabundans was at late log phase after 10 d in culture (Figure 1). Similar growth curves were constructed for individual species, and growth phases identified.

Carbohydrate analysis. The carbohydrate content of 15 mg of freeze dried algae was determined spectrophotometrically using a modification of the procedure by Pakulski and Benner (1992) (unpublished data, S-C. Huang), and standardized with potato starch (soluble grade, Baker, 4006-4). Samples were hydrolized with 1.0 ml 36N H_2SO_4 for 2 h at room temperature, diluted to 9 mL, and heated for 3 h at 100 °C. Then, 0.1 mL aliquots were diluted with 10 mL deionized water, neutralized with 0.5 mL 1.0N NaOH, treated with 0.2 mL freshly prepared 10% KBH_4 (kept cold because reaction was very exothermic), and incubated in the dark for 4 h at 18°C. After incubation, 2 mL 2N HCL was added to each test tube. Three new test tubes then received 1 ml of the above hydrolysate as samples, and 2 tubes as blanks. Samples and blanks received 0.1 mL periodic acid and were incubated in the dark for 10 min, followed by 0.1 mL of sodium

arsenite and incubated again for 10 min in the dark. Each tube then received 0.2 mL of 2N HCl, 0.2 mL of 3-methyl-2-benzothiazolinone hydrazone hydrolchloride (MBTH). These tubes were sealed and heated for 3 min at 100°C. After heating, 0.2 mL ferric chloride solution was added, and samples were incubated for 30 min in the dark. After color development, 1.0 mL of acetone was added to stop the colorizing reaction. A 200 µL sample of the supernatant was transferred to three replicate wells in a 96-well microplate, and absorbance was read immediately at 665 nm with a microplate reader.

Lipid analysis. The lipid content of 500-1000 mg wet wt of algae was determined following Orcutt and Patterson (1975). Lipids were extracted with a mixture of chloroform-methanol-water (1:2:0.8, v/v/v; 4 x 3mL $CHCl_3$:MeOH:H_20 and 1 x 1 mL NaCl, centrifuged between extractions). Lipid extracts were stored in hexane under N_2 at 10°C overnight. The lipids were then hydrolyzed using 16% KOH in 80% MeOH at 70°C for 2 h, evaporated to dryness under N_2. The lipid fractions corresponding to total fatty acids and sterols were separated using silica gel G thin layer chromatography (Orcutt et al. 1984). The solvent system used to separate the lipid classes was hexane:diethyl ether: acetic acid (80:20:1, v/v/v). Each lipid class was identified by co-chromatography using a mixture containing authentic lipid standards. The total fatty acids were methylated in BCl_3-MeOH for 15 min at 45°C and fatty acids partitioned in hexane. Total fatty acids were analyzed by gas liquid chromatography (GLC) using a 0.75 mm (i.d.) x 30 m glass column with Supelcowax 10 (polyethylene glycol) stationary phase. Operating conditions for GLC of fatty acids were: column, isothermal at 195°C; inlet, 300°C; detector, 470°C. Trimethysilyl (TMS) ether derivatives of sterols were formed by heating

at 55°C in bis-trimethyl acetamide (BSA) for 45 min and were analyzed by GLC using a 0.75 mm i.d. x 60 fused silica glass column packed SPB-1. GLC analyses were cnducted isothermally at an oven temperature of 270°, an inlet temperature of 280°C, and detector temperature of 310°C. Helium was used as a carrier gas. Identification and quantification of fatty acids and sterols was based on GLC retention times compared to known standards and published literature (Patterson 1971). Quantification of sterols and fatty acids were based on internal standards of coprostanol and heptadecanoic acid (C:17) respectively. Lipid standards used for TLC and GLC were purchased from Supelco, Bellefonte, PA.

Electron ionization mass spectra were obtained for the fatty acids and sterols extracted from samples of each species. A Hewlett-Packard 5790 gas chromatograph fitted with a splitless injector and a 30 m by 0.32 mm (i.d.) HP-5 capillary column coated with a 5% diphenyldimethyl siloxane phase was used for the analysis of sterols. A 30 m by 0.32 mm (i.d.) Omegawax capillary column (Supelco) was used to analyze the fatty acids. Both columns were held at 95°C for 1 min., then temperature programmed at a rate of $15°C \cdot min^{-1}$ to 300°C. Helium was used as the carrier gas at a pressure of 15 PSI and 18 PSI for sterols and fatty acids, respectively. Electron ionization spectra were obtained at 70eV with a source block temperature of 200°C using a VG-700 mass spectrometer (Manchester, U.K.).

Protein analysis. The protein content of 4 mg freeze-dried algae was determined spectrophotometrically using a Pierce test kit (BCA 23225) based on the procedure of Lowry et al. (1951) and Kreeger et al. (1997), and standardized with bovine serum

albumin. Freeze-dried algae were prepared for analysis by first suspending the algae in 4mL 1.0N NaOH in a 15 mL polypropylene test tube. The algae were homogenized for 60 s and sonicated for 10 s to transform the algae into a slurry and help break down the algal cell walls. Samples were diluted to 8 mL with 0.1N NaOH, heated to 60°C for 45 min, mixed for 10 s with a Vortex Genie, and then centrifuged (800 g, 5 min). Samples were held on ice whenever possible throughout the procedure. Samples (10 µL) of each supernatant were transferred to each of triplicate wells in a 96-well microplate. BCA reagent (200 µl) was added to each well. After a 30 min incubation period at 37°C, the microplate was analyzed at 562 nm with a microplate reader (Molecular Devices, Thermomax).

Statistical analysis. The biochemical composition percentage of total algal dry weight was calculated from the absolute concentrations for each biochemical component. Percentage data were arcsine square root-transformed prior to all statistical analyses (Sokal and Rohlf 1981). Absolute concentrations (µg) of fatty acids and sterols also were calculated as a percent of total lipid wt, and arcsin square root-transformed prior to statistical analyses. Concentrations (µg/mg lipid) of saturated and unsaturated fatty acids were compared between species among growth phases and within species between growth phases. Concentration values and percent composition values were analyzed by Two-Way Analysis of Variance (ANOVA) with phase and species as the dependent variables. When no phase effect was detected by the Two-Way ANOVA, One-Way ANOVA followed by Fisher's Protected Least Significant Difference (FPLSD) compared

differences among species (Lentner 1993). I used One-Way ANOVA and Fisher's PLSD to compare differences between phases within a species.

RESULTS

Carbohydrate. There was a significant effect of growth phase and species on the carbohydrate (CHO) content (% of algal dry wt) of all algae examined according to 2-way ANOVA ($p = 0.00$). In general, the CHO content increased with growth phase (age of the algal culture) with the exception of S. quadricauda. The CHO content of S. quadricauda showed an increase in CHO from early log to late log, followed by a decrease in CHO at late stationary phase. CHO content also differed among species within a growth phase. At early log phase, B. grandis contained more CHO (24.5%) than the other species of algae (Table 1). S. quadricauda was most abundant in CHO at late log phase (38.05%), and CHO was significantly greater than that of the other species at late log phase. The CHO content of B. grandis and P. tricornutum was most abundant at late stationary phase (43.4 % and 39.4 %, respectively), and was significantly greater than the CHO content of other algae (Table 1).

Lipids. The 2-Way ANOVA showed that there was a significant effect of growth phase and species on the lipid content (% of algal dry wt) of the algae ($p = 0.0128$, and 0.0001). One Way ANOVA showed conclusively that the total lipid content increased with growth phase for N. oleoabundans (Table 2). N. oleoabundans was most abundant in lipids at stationary phase (58.3 ± 8.3 %), and this was significantly greater than the lipid content of the other species. Lipid content of B. grandis decreased with age; P.

tricornutum and S. quadricauda showed no difference in lipid content between growth phase (Table 2). The difference in total lipid content among species was apparent at late log ($p = 0.08$), and was especially significant at stationary phase and late stationary phase ($p = 0.0003$, and $p = 0.049$) (Table 2). Ignoring phase, One-Way ANOVA showed there was a significant difference in the total mean lipid content among species (Table 2). Namely, B. grandis contained the least amount of lipid (10.65 ± 4.8_%) compared to all other algae. Lipid content of N. oleoabundans was greater than all other species (31.85 ± 9.4 %), however, this was not significantly different from S. quadricauda and P. tricornutum (24.4 ± 16.8 % and 21.8 ± 4.3 %).

Two-Way ANOVA detected an effect of phase on the TFA content of total lipid; however, this effect differed among species ($p = 0.0035$). The fatty acid concentration generally increased with growth phase in all species, although this was not significant for B. grandis and S. quadricauda (Table 3). The differences in TFA among species became apparent at stationary and late stationary phase. TFA concentration was greatest in P. tricornutm (632.2 ± 120.3 μg · mg lipid^{-1}) at stationary phase ($p = 0.0176$), and this was significantly greater than the TFA concentration of the other species at stationary phase (Table 3). Interestingly, the TFA concentration was lowest in N. oleoabundans at stationary phase but was highest at late stationary phase ($541.3 + 152.2$ μg · mg lipid^{-1}) ($p = 0.0189$) (Table 3). Ignoring phase, One-Way ANOVA showed that there was a significant difference in the mean fatty acid concentration among the algae; namely S. quadricauda contained significantly less TFA (174.5 ± 48.8 μg · mg lipid^{-1}) than all other algae (Table 3). Mean TFA content (% lipid wt) of the algae ranged from $15.5\ \% \pm 7.3$ to

40.0 ± 3.0 % (Table 3). Mean TFA % of total algal dry wt ranged from 3.3% ± 1.9 to 10.6% ± 7.8; N. oleoabundans and P. tricornutum had the largest TFA content (% algal dry wt) (10.6 % and 8.2 %) (Table 3).

A Two-Way ANOVA also showed that there was an effect of phase and species on the total saturated fatty acid (SAFA) concentration (μg · mg lipid^{-1}) and the unsaturated fatty acid content (UFA) (μg/mg lipid) of the algae. The SAFA concentration generally increased with growth phase (Table 4). SAFA concentration was highest at stationary phase in P. tricornutum and was highest at late stationary phase in N. oleoabundans. There was no significant difference in the SAFA concentration of B. grandis between growth phases, however, SAFA was highest at late stationary phase. Nor was there a difference in the SAFA concentration of S. quadricauda between growth phases (Table 4).

The unsaturated fatty acid concentration increased with growth phase for all algae, but this increase was significant only for P. tricornutum and N. oleoabundans (Table 5). There was a significant difference in the UFA concentration amng algae at the 3 later stages of growth. The UFA concentration of S. quadricauda was lower than the UFA concentration of the other algae at all phases of growth. The UFA concentration of N. oleoabundans and B. grandis was significantly greater than that of the other algae at late log phase. UFA concentration of B. grandis and P. tricornutum was significantly greater than that of N. oleoabundans and S. quadricauda at stationary phase. N. oleoabundans contained the greatest UFA concentration among algae and between

growth phases at late stationary phase. In addition, the total mean UFA concentration of N. oleoabundans was significantly greater than that of the other algae (Table 5). One-Way ANOVA followed by FPLSD also showed that the UFA content was higher than the SAFA content in N. oleoabundans, B. grandis, and P. tricornutum at each growth phase ($p < 0.05$) with the exception that the SAFA content was higher than the UFA content at late stationary phase in P. tricornutum (Figure 2). The SAFA content of S. quadricauda was greater than the UFA content at all growth phases.

The three most abundant fatty acids in the three green algae were 18:1, 18:2, and 18:3 (Table 6). In addition, 16:0 was a major component of the fatty acid composition of B. grandis and S. quadricauda, whereas 16:0 was common in N. oleoabundans, but not as abundant as the fatty acid 16:1. The fatty acids 16:2 and 16:3 also were common but not abundant in N. oleoabundans. B. grandis and S. quadricauda contained minor amounts of 16:1, and 16:2 S. quadricauda contained minor amounts of the long-chain saturated fatty acid, 20:0. Unlike the other green algae, N. oleoabundans contained a long-chain saturated fatty acid, 24:0, and two short-chain fatty acids 14:0 and 15:0. N. oleoabundans also contained the unsaturated fatty acid 20:1. The most abundant fatty acids in P. tricornutum were 16:0, 16:1, and 20:5. The polyunsaturated fatty acids 18:2, 18:3, 18:4, and 20:4 also were common in P. tricornutum, although not as abundant as 16:0 and 20:5. Interestingly, P. tricornutum also contained minor amounts of the short-chain saturated fatty acid 14:0, and the long-chain saturated acid, 18:0 (Table 6).

Two-Way ANOVA detected an effect of species on the sterol concentration of total lipid; generally, there was no effect of phase on the sterol content, with the

exception of S. quadricauda which showed a significant increase in sterol content with growth phase (Figure 3). One-Way ANOVA showed that the sterol content of S. quadricauda and B. grandis (19.5 ± 17.7 μg·mg lipid^{-1} and 19.3 ± 12.6 μg·mg lipid^{-1}, respectively) was similar and significantly greater than the sterol concentration of P. tricornutum (7.4 ± 3.8 μg·mg lipid^{-1}) and N. oleoabundans (5.9 ± 0.01 μg·mg lipid^{-1}) ($p = 0.0002$). The mean sterol content (% lipid wt) of the four algae ranged from 1.0 ± 0.4% to 1.8 ± 1.8 % (Table 7).

Ten sterols were identified in the four algae, and composition varied between only 3 sterols present in two species (N. oleoabundans and S. quadricauda) to 6 sterols in B. grandis and three sterols in P. tricornutum (Table 7). The sterol composition of B. grandis was dominated by Δ 5, 22 stigmastenol (poriferasterol) and Δ 5 stigmastenol (clionasterol) with lesser amounts of Δ 5-ergostenol and Δ 7 ergostenol. The sterol composition of S. quadricauda and N. oleoabundans was the same (Δ 5, 7, 22 ergostatrienol (ergosterol), Δ 5,7-ergostadienol, and Δ 7 ergostenol), although S. quadricauda contained greater amounts of each sterol. The predominant sterol in P. tricornutum was Δ 5, 22 ergostadienol (brassicasterol); Δ 7, 22-ergostadienol was present in minor concentrations in P. tricornutum (Table 7). Minor amounts of Δ 5 cholestenol (cholesterol) also were detected but because it co-eluted with the standard, the actual cholesterol content in P. tricornutum could not be quantified.

Protein. There was no effect of growth phase or species on the protein content (% of algal dry wt) of the algae examined according to Two-way ANOVA ($p = 0.875$). One-way ANOVA verified that there was no difference in the protein content between growth phases for B. grandis, N. oleoabundans, and P. tricornutum (Table 8). The protein content at early log phase in S. quadricauda, however, was significantly less than the protein contents at later stages of growth. Ignoring phase, One-Way ANOVA also showed that there was no difference in the mean protein content among species ($p = 0.408$). The protein content ranged from 60.6 ± 17.0 to 70.3 ± 9.5 % of algal dry wt. (Table 8).

DISCUSSION

The algae tested in the current study were chosen on the basis of their potential suitability as foods for cultured freshwater mussels, taking into consideration their size, growing properties, and freshwater nature. The gross composition of algae refers to the relative proportions of carbohydrate, lipid, protein, and minerals (the latter were not evaluated). The importance of gross composition in determining the nutritional quality of algae is poorly understood, with researchers claiming either total carbohydrate or protein to be more important depending on animal species and life stage (Langton et al. 1977, Flaak and Epifanio 1978, Webb and Chu 1983, Wikfors et al. 1984). At the same time, others report that lipids are more important for developing larvae as an energy source for developing organelles and metamorphosis, followed by protein and then carbohydrates (Chu and Dupuy 1981, Wikfors et al. 1984, Enright et al. 1986, Napolitano et al. 1990).

Wikfors et al. (1992) also showed that post-set clams fed a diet high in protein and lipid had the fastest growth rate. Adult bivalves, on the other hand, direct protein toward tissue growth and maintenance, and utilize carbohydrate as an energy source either for immediate respiration or long-term storage (Gallager and Mann 1982). Lipids also are important to adults for maintaining cellular function, synthesis of hormones, transportation of cholesterol, activation of several enzymes, and the regulation of processes by prostaglandins (Castell 1970, Pike 1971). Finally, lipids are very important during gametogenesis, especially in females to provide an energy source for subsequent embryo development (Pollero et al. 1983).

Carbohydrate. In this study, I observed carbohydrate levels ranging from 5.0 – 39.6 % of the algal cell dry wt depending on growth phase and algal species. Carbohydrate content increased significantly at late stationary phase in N. oleoabundans and P. tricornutum. Brown and Jeffrey (1992) reported carbohydrate levels ranging from 5.9% to 16.7 % in 10 species of algae from the green algal classes Chlorophyceae and Prasinophyceae. Generally, the CHO content was similar among species, and was similar to that reported in an earlier study of diatoms and prasinophytes cultured under similar conditions (Brown 1991, Brown and Jeffrey 1992). Similarly, Wikfors et al. (1992) reported CHO levels ranging from 6.5% to 31% of algal dry wt for 19 uni-algal diets from the classes Prasinophyceae and Bacillariophyceae. Differences in CHO were attributed to differences in growth media, with the nitrogen-deficient media producing algae with the highest CHO content. Wikfors et al. (1992), however, did not find a strong correlation between CHO content of diet and the juvenile hard clam (Mercenaria

mercenaria) growth rate, but rather protein and lipid content had a stronger effect on growth rate. The apparent unimportance of dietary carbohydrate within the range of the diets tested was surprising because carbohydrate glycogen is very important in the metabolic processes of bivalves (De Zwaan 1983).

Carbohydrates are a primary energy source for both juvenile and adult oysters, with glycogen representing 40-50% of the total carbohydrate content of the oyster (Holland and Spencer 1973). Carbohydrates are used as a respiratory substrate in bivalves, and are normally high in healthy bivalves and depleted in starved ones (Gabbot and Bayne 1973, Mann 1979). Adult bivalves also accumulate carbohydrates during the fall as fuel for over-wintering and subsequent gametogenesis during winter (Gabbott 1976, 1983). Castell and Trider (1974) varied the carbohydrate and protein content of oyster diets and observed a higher glycogen production in adult eastern oysters (Crassostrea virginica) fed 60% carbohydrate than diets with 20% carbohydrate content. A high carbohydrate diet, however, was of little use to juvenile oysters (Ostrea edulis) when the protein content became limiting (Enright et al. 1986). Yet, CHO content was previously shown to enhance growth of young, post-set eastern oysters (C. virginica) (Wikfors et al. 1984). Thompson and Harrison (1992) noted higher growth rates in C. gigas larvae fed algae that were high in carbon per cell. Detailed analyses revealed that the observed difference between the low and high carbon diets was in their fatty acid content, suggesting that the C. gigas larvae were responding to the increased fatty acids rather than the increased carbohydrate. There is an apparent biochemical basis for differences in nutritional value of certain algal species for different species of bivalves

and for bivalves at different life stages. The carbohydrate requirements of freshwater mussels will need to be identified, focusing on differences between life stages. The CHO content of the algae examined in this study are in line with other bivalve diets, and thus, do not appear limiting to adult or juvenile bivalve energy maintenance requirements.

Lipids. Lipid content increased with age through stationary phase. It is well documented that stationary phase algae cultures have lipid levels significantly higher than that of exponential phase cultures (Taub and Dollar 1965, Shifrin and Chisholm 1981, Pillsbury 1985). Interestingly the lipid content (% algal dry wt) decreased from stationary to late stationary phase, especially in N. oleoabundans, or showed little change between growth phase as in S. quadricauda and P. tricornutum. Correspondingly, the carbohydrate levels showed a significant increase at late stationary phase in N. oleoabundans. When lipids were highest, carbohydrates were at their lowest levels for a given species. B. grandis lipid levels fluctuated with growth phase, and carbohydrate levels showed the inverse relationship to growth phase as lipids. The effect of age also was observed in the relative increase in saturated and unsaturated fatty acids; however, in N. oleoabundans, and B. grandis, the pattern of increase was not in parallel with total lipid levels. Total fatty acids (% lipid) showed a decrease from late log to stationary phase, and then increased to its highest TFA levels at late stationary phase when total lipid levels showed a decrease. TFA content in P. tricornutum, however, followed the same trend as lipid content with growth phase.

Dietary long-chain polyunsaturated fatty acids, such as 20:5 and 22:6, are essential for optimum growth of several marine fish, penaeid prawns, and marine

bivalves because they may have limited ability to synthesize higher unsaturated fatty acids (Langdon and Waldock 1981, Chu and Greaves 1991, Wikfors et al. 1996, Knauer and Southgate 1997, Takeuchi 1997). Freshwater fish, however, can biosynthesize long chain PUFA from shorter chain precursors and may be cultured successfully on diets containing C18 fatty acids (Mourente et al. 1995, Castell et al. 1994). Little is known on the lipid biosynthesis and lipid requirements of freshwater mussels. Recently, Vanderploeg et al. (1996) showed that the larvae of the freshwater bivalve, Dreissena polymorpha, developed at a faster rate when fed algae rich in long-chain (≥18 C) unsaturated fatty acids (3 double bonds), including C20:5 and C22:6. Similarly, Ahlgren et al. (1992) found that freshwater cladocerans grew fastest on PUFA-rich cryptophytes. Mullernavarra (1995) examined the seasonal patterns of long-chain PUFAs in natural lake seston and showed that growth of Daphnia was highly correlated with C20:5 concentrations. It appears that some freshwater invertebrates may need highly unsaturated fatty acids in their diet, especially at the earlier stages in life.

N. oleoabundans and P. tricornutum were abundant in the unsaturated fatty acids 18:1, 18:2, 18:3, 18:4, 20:1, 20:4, and 20:5, making these two alga's nutritionally beneficial food sources for the culture of freshwater mussels. Tornabene et al. (1983) reported a similar range of fatty acids from C14 to C20 in N. oleoabundans, including 20:1. Tornabene et al. (1983) cultured N. oleoabundans in nitrogen-deficient media, however, which favored biosynthesis of more saturated fatty acids (Pohl and Zurheide 1979) than observed in my cultures of N. oleoabundans. Arao et al. (1987) reported a range in fatty acids (14:0 to 20:5) for P. tricornutum similar to those I observed in my

cultures. Arao et al. (1987) also reported an increase in the unsaturation of the lipids from lag phase through logarithmic to stationary phase. I observed a greater percentage of unsaturated fatty acids at stationary phase in P. tricornutum, whereas N. oleoabundans and B. grandis showed a greater percentage of unsaturation at late stationary phase. In contrast, Dunstan et al. (1993) noted higher proportions of PUFA at logarithmic phase than at stationary phase cultures of several marine algae. The TFA of individual algae can vary considerably in different environments (Piorreck et al. 1984, Mayzaud et al. 1990, Thompson et al. 1990).

Other than this study, the lipid content and fatty acid composition has not been reported on B. grandis. Several studies on the fatty acid composition of Scenedesmus sp. have been conducted; however, the species was either unknown or varied between studies (Choi et al. 1988, Cranwell et al. 1990). In one freshwater study, S. quadricauda consisted mainly of n-3 polyenoic fatty acids, having no more than 18 carbon atoms (Cranwell et al. 1990). Similarly, Choi et al. (1988) reported that S. obliquus contained the fatty acids 16:0, 16:1, 16:2, 16:3, 16:4, 18:1, 18:2, and 18:3. In the cited studies, the authors reported a greater diversity of fatty acids than I observed in my S. quadricauda cultures; this could be attributed to differences in culture conditions.

The absence of polyunsaturated, long-chain fatty acids in the green algae was not unexpected. Freshwater green algae generally do not synthesize polyunsaturated fatty acids commonly found in marine species (Pohl and Zurheide 1979, Ahlgren et al. 1992, Zhukova and Aizdaicher 1995). N. oleoabundans contained small amounts of 20:1; it is possible, therefore, that specific alterations to the culture conditions such as varying the

irradiance, temperature, and phosphorus content could lead to a higher percentage of polyunsaturated fatty acids (Shifrin and Chisholm 1981, Enright et al 1986, Thompson and Harrison 1992, Reitan et al. 1994). For example, Orcutt and Patterson (1974) showed that saturating light resulted in more short-chain saturated fatty acids. Algae proposed as diets for freshwater mussels must be examined under various culture conditions to determine what biochemical composition will promote growth and survival in cultured mussels.

Research on the sterol metabolism in bivalves is very incomplete, considering its importance in embryonic development, hormone synthesis and regulation, and involvement in spermatogenesis (Pollero et al. 1983, Napolitano et al. 1990). For the most part, bivalves have limited abilities to synthesize sterols (Teshima and Kanazawa 1972, Teshima and Kanazawa 1973, Teshima and Patterson 1981, Holden and Patterson 1991). Idler and Wiseman (1972) found cholesterol to be the major sterol in oysters and that it could be synthesized from squalene. Holden and Patterson (1991), however, reported the absence of sterol synthesis. Other reports on oysters have shown that their sterols were dietary in origin (Tamura et al. 1964). It is well known that molluscs contain large amounts of sterols, although their origin is unclear (Kanazawa et al. 1979, Napolitano et al. 1993). Many researchers assume that C28 and C29 sterols are derived from dietary sources. Napolitano et al. (1993) found the three major sterols (brassicasterol, cholesterol, and β-sitosterol) in the algal diet fed to scallops (Placopecten magellanicus) significantly increased in proportions in the scallop.

For lack of information on freshwater mussel biochemical composition, it is not possible to predict which algae might provide the necessary sterol components required by freshwater mussels. However, in a review of sterol metabolism, Yasuda (1971), Voogt (1972), and Idler and Wiseman (1972) reported similarities in sterol composition between marine and freshwater bivalves. All of the algae in this study contained sterols that might be used as precursors for the synthesis of cholesterol. The green algae, B. grandis and S. quadricauda, however, contained a higher percentage of sterol per / mg of lipid than N. oleoabundans and P. tricornutum. The most abundant sterol component S. quadricauda was Δ 5,7-ergostadienol followed by Δ 5,7,22 ergostatrienol (ergosterol). Rezanka et al. (1986) found Δ 5,7 sterols in addition to ergosterol in Scenedesmus sp. Earlier studies have shown a considerably high level of Δ 5,7 –sterols in oysters (Idler and Wiseman 1972, Voogt 1972, Teshima and Patterson 1981). Ergosterol also was the largest sterol component in N. oleoabundans. Teshima and Patterson (1991) identified mixtures of ergosterol and epiergosterol in the eastern oyster, C. virginica, which they inferred were dietary in origin. Similarly, incorporation of dietary sterols, such as brassicasterol, appeared to be an important process in the sea scallop (Napolitano et al. 1993). In this study, P. tricornutum contained significant amounts of brassicasterol. Rzama et al. (1994) reported that chondrillasterol was the dominant sterol in Scenedesmus sp. Interestingly, I found Δ 7-chondrillasterol in B. grandis. If the sterol composition of marine and freshwater molluscs is similar, then freshwater mussels also may utilize and possibly require sterols such as ergosterol, cholesterol, brassicasterol, and Δ 5,7 –sterols in their diet.

Protein. Protein is typically the major biochemical component of algae (Brown and Jeffrey 1992, Wikfors et al. 1992), although growth media will affect gross biochemical composition (Wikfors et al. 1984). In my study, I observed very high protein levels of 70% algal dry wt. Wikfors et al. (1984) found that protein levels ranging from 15.6 % to 57.4% of algal dry wt had no effect on clam growth, indicating that protein was not limiting to juvenile oysters (C. virginica). Enright et al. (1986) found that when lipids supplied energy, protein could be directed towards growth in juvenile oysters (Ostrea edulis). Similarly, Langton et al. (1977) found the gain in total weight and protein of juvenile clams correlated with the total amount of algal protein in the diet. Wikfors et al. (1992) also found protein had the greatest effect on growth in juvenile hard clams (Mercenaria mercenaria); however, the lipid composition and lipid quantities must be sufficient to support rapid growth of hard clams. Conversely, Whyte et al. (1989) suggested that a balance between dietary protein for tissue production and carbohydrate to fuel metabolic processes was needed by scallop larvae (Patinopecten yessoensis). As has already been stated, the dietary demand for gross biochemical constituents will likely vary between species and life stages. Kreeger (1993) showed that adult Mytilus trossulus utilized dietary protein with different efficiencies during the year. Similarly, the mussel M. edulis showed seasonal variation in the protein assimilation rates with rates of protein synthesis greatest in July; dietary protein was primarily used as a substrate for tissue synthesis rather than for energy (Hawkins and Bayne 1985, Kreeger et al. 1995). This variability was related to seasonal changes in the digestive physiology and reproductive

condition of mussels. The protein content of the algae examined in this study would not be considered limiting to tissue production (Kreeger 1993).

CONCLUSIONS

The percentages of protein and carbohydrate showed only minor differences among the species of algae examined. On the basis of providing adequate protein for biosynthesis and tissue regeneration, and carbohydrate for energy storage and energy maintenance, any of the algae examined in this study would be a suitably nutritious food source for freshwater mussels. The carbohydrate requirements of freshwater mussels will need to be identified, focusing on differences between life stages. Because little is known on the lipid biosynthesis capabilities of molluscs, and even less on the lipid requirements of freshwater mussels, we assume that dietary lipids (unsaturated and saturated fatty acids and sterols) will be required to provide precursors for chain elongation of essential highly unsaturated fatty acids and synthesis of hormones (Nes 1974, Kanazawa et al. 1979, Waldock and Holland 1984). Sterols and fatty acids also will be required to provide metabolic reserves for developing larvae or juvenile mussels as well as an energy source in subsequent developing embryos (Wikfors et al. 1984, Cohen et al. 1988, Napolitano et al. 1993).

Numerous studies have shown that a mixed algal diet enhanced growth and survival in cultured bivalves and zooplankton more than any uni-algal diets (Ukeles 1976, Epifanio 1979, Chu and Dupuy 1981, DePauw and Pruder 1986, Enright et al. 1986, Napolitano et al. 1990, Wikfors et al.1992). It is believed that an optimal balance

of macro- and micro-nutrients is provided by a mixed diet that is not provided by a uni-algal diet. The green alga, N. oleoabundans and the diatom, P. tricornutum contained a variety of saturated and unsaturated fatty acids including long-chain C18's and C20's. B. grandis contained a greater percentage of lipid sterols than the other algae, including precursors for the synthesis of cholesterol. Freshwater mussels (adults and juveniles) in culture would benefit, therefore, from a mixed diet of B. grandis, N. oleoabundans, and P. tricornutum because an abundance (concentration and composition) of sterols, fatty acids, carbohydrate, and protein is available.

Juvenile mussels undergo significant developmental changes from the glochidial stage with internal food reserves, to post-set pedal-feeders with a primitive digestive system, capturing food by a highly ciliated foot and mantle, and then finally filter-feeding with fully developed gills and a digestive system. Algae harvested at stationary phase when total lipid content is highest would support rapid growth and development in juvenile mussels. If we assume that carbohydrates are more important as an energy reserve for maintaining condition in adult bivalves, then late stationary phase cultures of B. grandis, N. oleoabundans, and P. tricornutum would be a suitable diet for feeding adult mussels. Future research should determine the digestibility of these algae, and identify the nutritional requirements for specific fatty acids and the importance of dietary sterols. In addition, the seasonal variation in nutritional demands for protein, carbohydrate, and lipid should be identified in order to provide complete and balanced diets that promote reproductive viability, growth, and development. Varying the algal growth media in

future studies could achieve different carbohydrate and lipid levels to meet demands of juveniles and adults.

ACKNOWLEDGEMENTS

I would like to thank Vanessa Jones for her tireless assistance with the lipid extractions, TLC, and for conducting the greater part of the GC work. I would like to thank Nina Hopkins for teaching me the fine art of lipid analysis, and for her fabulous humor and endless support throughout my trials in the lab. I also must thank Dr. D. M. Orcutt for allowing me into his lab, sharing his lipid extraction protocol, and advising me throughout this project. I wish to thank Kim Harrick for conducting the MS work. Finally, I wish to thank Dr. B. C. Parker and Dr. R. J. Neves for acquiring the extra funding needed to complete this experiment. This project was funded by the Biological Resources Division of the United States Geological Survey.

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Appendix 1. Algae growth media composition.

Neochloris Growth Media

Chemical	Stock Concentration	Amount per Liter
NaCl	Dry chemical	5.82 g
$MgSO_4 \cdot 7H_2O$	Dry chemical	2.47 g
KNO_3	Dry chemical	1.0 g (varies)
KCL	Dry chemical	0.75 g
$CaCl_2$	0.3M, 43.9 $g \cdot L^{-1}$	2.0 ml
H_3BO_3	0.2M, 12.36 $g \cdot L^{-1}$	1.0 ml
Na_2EDTA	30mM, 11.17 $g \cdot L^{-1}$	2.0 ml
$FeCl_3$	3mM, 0.81 $g \cdot L^{-1}$	1.0 ml
Metals (2x)	see metals recipe	10.0 ml
Distilled Water	----------	to one liter

Autoclave at 121 °C, >15 psi, for 20 min.

After autoclaving:

KH_2PO4	0.4M	2.0 ml
Thiamine·HCl	1 $mg \cdot mL^{-1}$	0.1 ml
Biotin	0.2 $mg \cdot mL^{-1}$	2.5 ml
Vitamin B_{12}	0.2 $mg \cdot mL^{-1}$	2.5 ml

Metals (2x) for Neochloris media:

Chemical	Stock Concentration	Amount per Liter
$Na_2MoO_4 \cdot 2H_2O$	1.2 $g \cdot L^{-1}$	20 ml
$FeCl_3$	33 $g \cdot L^{-1}$	20 ml
$CuSO_4 \cdot 5H_2O$	5 $g \cdot L^{-1}$	2.0 ml
$CoCl_2 \cdot 6H_2O$	4 $g \cdot L^{-1}$	2.0 ml
H_3BO_3	6 $g \cdot L^{-1}$	20 ml
$MnCl_2 \cdot 4H_2O$	16 $g \cdot L^{-1}$	20 ml
$ZnCl_2$	1.1 $g \cdot L^{-1}$	20 ml
Na_2EDTA	dry chemical	4.0 g
Distilled Water	----------	to one L

Phaeodactylum tricornutum Growth Media (Bold's Basal Medium with Soil Extract)

Chemical	Stock Concentration	Amount per Liter
$NaNO_3$	10 g · 400 ml^{-1}	10 ml
$CaCl_2 \cdot 2H_2O$	1 g · 400 ml^{-1}	10 ml
$MgSO_4 \cdot 7H_2O$	3 g · 400 ml^{-1}	10 ml
K_2HPO_4	3 g · 400 ml^{-1}	10 ml
KH_2PO4	7 g · 400 ml^{-1}	10 ml
NaCl	1 g · 400 ml^{-1}	10 ml
$Na_2EDTA \cdot 2H_2O$	50 g · L^{-1}	1 ml
KOH	31 g · L^{-1}	1 ml
$FeSO_4 \cdot 7H_2O$	4.98 g · L^{-1}	1 ml
H_2SO_4	1.0 ml · L^{-1}	1 ml
H_3BO_3	11.42 g · L^{-1}	1 ml
$ZnSO_4 \cdot 7H_2O$	8.82 g · L^{-1}	1 ml
$MnCl_2 \cdot 4H_2O$	1.44 g · L^{-1}	1 ml
MoO_3	0.71 g · L^{-1}	1 ml
$CuSO_4 \cdot 5H_2O$	1.57 g · L^{-1}	1 ml
$Co(NO_3)_2 \cdot 6H_2O$	0.49 g · L^{-1}	1 ml
Distilled Water		940 ml

Soil Extract Preparation: 1) Autoclave soil (with water overlay) two times, at 121 °C, >15 psi, for 20 min.. 2) Decant soil water; preferably using Whatman #1 Filter. 3) If still turbid, centrifuge to achieve a clear extract.

Bracteacoccus grandis and Scenedesmus Growth Media (Our Chlorella Media (OCM), Martek, Inc.)

Chemical	Stock Concentration	Amount per Liter
K_2HPO_4	50 g/L	2.0 ml
KH_2PO_4	50 g/L	1.5 ml
$MgSO_4 \cdot 7H_2O$	100 g/L	5.0 ml
$Ca(NO_3)_2 \cdot 4H_2O$	25 g/L	2.5 ml
KNO_3	dry chemical	1.0g
A5 + Co metals	see A5 recipe	1.0 ml
Fe-versenate	see Fe-versenate	2.0 ml

A5 + Co Metals:

Chemical	Amount per Liter
H_3BO_3	2860 mg
$MnCl_2 \cdot 4H_2O$	1810 mg
$ZnSO_4 \cdot 7H_2O$	222 mg
$Na_2MoO_4 \cdot 2H_2O$	390 mg
$CuSO_4 \cdot 5H_2O$	79 mg
$Co(NO_3)_2 \cdot 6H_2O$	49 mg

Fe - versenate

Chemical	Stock Concentration	Amount per Liter
$FeSO_4 \cdot 7H_2O$	Dry Chemical	5g
Na_2EDTA	Dry Chemical	4g
Distilled Water	- - - - - -	to 1 L

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Table 1. Carbohydrate content (mean ± SD % of algal dry weight) of four algae at different growth phases. Values followed by the same lower-case letter within a column were not significantly different ($p > 0.05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Table 2. Lipid content (mean ± SD % of algal dry weight) of four algae at different growth phases. Values followed by the same lower-case letter within a column for a given species were not significantly different ($p > 0.05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Table 3. Mean fatty acid concentration (μg TFA·mg lipid^{-1}) of four algae at different growth phases, the respective total mean concentration of TFA, and the total mean TFA content (% lipid wt). Values followed by the same lower-case letter within a column for a given species were not significantly different ($p > 0.05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Table 4. Mean saturated fatty acid (SAFA) concentration (ug·mg lipid $^{-1}$) of four algae at different growth phases, and the respective total mean SAFA concentration (ug·mg lipid $^{-1}$). Values followed by the same lower-case letter within a column for a given species were not significantly different ($p > 0.05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Table 5. Mean unsaturated fatty acid (UFA) concentration (ug·mg lipid $^{-1}$) of four algae

at different growth phases, and the respective total mean UFA concentration (ug·mg lipid^{-1}). Values followed by the same lower-case letter within a column for a given species were not significantly different ($p > 0.05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Table 6. Fatty acid composition of four algae.

Table 7. Sterol composition and concentration (µg·mg lipid^{-1}), mean sterol content (% lipid wt and % algal dry wt) of four algae. Means followed by the same upper case letter were not significantly different ($p > 0.05$).

Table 8. Protein content (mean $\pm$ SD % of algal dry weight) of four algae at different growth phases. Values followed by the same lower-case letter within a column for a given species were not significantly different ($p > 0.05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Table 1. Carbohydrate content (mean ± SD % of algal dry weight) of four algae at different growth phases. Values followed by the same lower-case letter within a column were not significantly different ($p > 0.05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Growth Phase	B. grandis	N. oleoabundans	P. tricornutum	Scenedesmus sp.
Early Log/Log	24.84 ± 6.91 a,A	5.93 ± 0.15 a,B	19.58± 6.33 b,A,C	15.44 ± 0.51 a,C
Late Log	17.1 ± 2.8 b, A	14.48± 11.10 b, A	18.52± 7.5 b, ,A	38.05 ± 13.22 b, B
Stationary	26.4± 1.6 a, A	20.57± 1.06 c, B		25.44± 11.37 c,A
Late Stationary	43.4 ± 5.5c, A	33.07± 6.89 d, B	39.37± 3.07a, A	
Mean CHO content (% algal dry wt)	27.94 ± 4.17 A	18.52± 4.8 B	25.82± 5.63 A	26.31± 8.37 A
Sample Size	N = 96	N = 90	N = 48	N = 42

Table 2. Lipid content (mean ± SD % of algal dry weight) of four algae at different growth phases. Values followed by the same lower-case letter within a column for a given species were not significantly different ($p > 0.05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Growth Phase	B. grandis	N. oleoabundans	P. tricornutum	Scenedesmus sp.
Early Log/Log	15.0 ± 4.4 a, A	13.7 ± 3.1 a, A	18.3± 2.5 a, A	27.5 ± 18.1 a, B
Late Log	7.8 ± 11.2 b, A	25.4 ± 11.2 a, B	22.6 ± 3.2 a, B	22.6 ± 30.6 a, B
Stationary	13.7± 3. a, A	58.3 ± 8.3 b, B	23.3 ± 11.4 a, A	23.1 ± 1.8B a, A
Late Stationary	6.1 ± 0.1 b, A	30.0 ± 15.0 a, B	23.0 ± 0.0 a, B	
Mean Lipid Content (% algal dry wt)	11.6 ± 5.1 A	29.7 ± 17.1 B	21.8 ± 5.7 B	24.6 ± 15.3 B
Sample Size	N = 20	N = 19	N = 12	N = 8

Table 3. Mean fatty acid concentration (μg TFA mg lipid^{-1}) of four algae at different growth phases, the respective total mean concentration of TFA, and the total mean TFA content (% lipid wt). Values followed by the same lower-case letter within a column for a given species were not significantly different ($p > .05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Growth Phase	B. grandis	N. oleoabundans	P. tricornutum	S. quadricauda
Early Log/Log Phase	397.4 ± 225.1 a, A	316.3 ± 81.5 a, b, A	220.5± 50.7 a, A	160.9 ± 61.7d a A
Late Log Phase	360.2 ± 130.0 a, A	451.9 ± 181.9 b, A	345.5 ± 165.2 a, A	211.8 ± 31.5 a, A
Stationary Phase	288.2 ± 221.8 a, A	145.4 ± 9.2 a, A	632.2 ± 120.3 b, B	163.2 ± 46.7d a, A
Late Stationary Phase	478.7 + 132.8 a, A	541.3 + 152.2 b, A	133.1 + 0 a, B	
Total Mean TFA Concentration	376.4 ± 195.8 A	400.9 ± 192.5 A	368.2 ± 208 A	174.5 ± 48.8 B
Sample size	N = 20	N = 19	N = 12	N = 8
Mean TFA Content (% lipid wt)	37.6 ± 16.5 A	40.0 ± 3.0 A	37.0 ± 20.8 A	15.5 ± 7.3 B
Sample Size	N = 20	N = 19	N = 12	N = 8
Mean TFA Content (% algal dry wt)	3.4 ± 2.0 A	10.6 ± 0.8 B	8.2 ± 6.7 B	3.9 ± 2.3 A
Sample Size	N = 20	N = 19	N = 12	N = 8

Table 4. Mean saturated fatty acid (SAFA) concentration (ug· mg lipid $^{-1}$) of four algae at different growth phases, and the respective total mean SAFA concentration (ug· mg lipid $^{-1}$). Values followed by the same lower-case letter within a column for a given species were not significantly different ($p > 0.05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Growth Phase	B. grandis	N. oleoabundans	P. tricornutum	S. quadricauda
Early Log/Log Phase	104.1 ± 97.1 a, A	36.5 ± 20.8 a, B	77.6± 11.3 a, A	81.1 ± 28.2 a A
Late Log Phase	89.1 ± 9.1a, A	61.6 ± 28.5 a, A	97.6 ± 58.0 a, A	84.1 ± 6.9 a, A
Stationary Phase	82.5 ± 9 a, A	14.7 ± 6.4 a, B	197.5 ± 11.5 b, C	61.0 ± 21.0 a, A
Late Stationary Phase	109.6 + 21.7 a, A	150.2 + 110.6 b, ,A	59.4 + 0 a, B	
Total Mean SAFA Concentration	99.0 ± 64.5 A	60.3 ± 73.0 A	114.4 ± 62.7 A	74.7 ± 22.0 A
Sample Size	N = 20	N = 19	N = 12	N = 8

Table 5. Mean unsaturated fatty acid (UFA) concentration (ug· mg lipid $^{-1}$) of four algae at different growth phases, and the respective total mean UFA concentration (ug· mg lipid $^{-1}$). Values followed by the same lower-case letter within a column for a given species were not significantly different ($p > 0.05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Growth Phase	B. grandis	N. oleoabundans	P. tricornutum	Scenedesmus sp.
Early Log/Log	255.9 ± 174.0 a, A	276.85 ± 61.4 a, A	142.77± 39.4 a, A	79.82 ± 61.7 a, A
Late Log	240.5± 116.3 a, A	390.3 ± 171.6 a,b, B	247.94 ± 107.5 a, A	127.73 ± 31.5 a, A
Stationary	302.0 ± 63.6 a, A	128.6 ± 4.7 a, B, D	434.74 ± 111.0 b, C	100.41 ± 46.7 a, D
Late Stationary	344.48 ± 107.3 a, A	433.1 ± 114.1b, A	73.72 ± 0 a, B	
Total Mean UFA Concentration	277.4 ± 115.3 A, B	340.6 ± 232.5 B, C	253.8 ± 148.3 A	99.5 ± 32.3 D
Sample Size	N = 20	N = 19	N = 12	N = 8

Table 6. Fatty acid composition of four algae.

Fatty Acid	Bracteacoccus grandis	Neochloris oleoabundans	Phaeodactylum tricornutum	Scenedesmus quadricauda
	Concentration ($\mu g \cdot mg\ lipid^{-1}$)	Concentration ($\mu g \cdot mg\ lipid^{-1}$)	Concentration ($\mu g \cdot mg\ lipid^{-1}$)	Concentration ($\mu g \cdot mg\ lipid^{-1}$)
C14:0	0	6.2	12.5	
C15:0	0	8.3		
C16:0	82.9	20.4	81.8	59.7
C16:1	14.45	65.2	105.3	5.3
C16:2	20.5	27.2	9.2	
C16:3		13.0		
C18:0	16.1	20.5	9.7	10.3
C18:1	83.4	83.2	8.2	37.1
C18:2	78.7	77.9	15.7	18.7
C18:3	80.5	66.3	14.3	38.4
C18:4			11.4	
C20:0				4.7
C20:1		7.3		
C20:4			17.9	
C20:5			71.6	
C24:0		4.9	10.4	
SAFA	99.0	60.3	114.4	74.7
UFA	277.4	340.6	253.8	99.5
Mean TFA	376.4	400.9	368.2	174.2
Sample Size	N = 20	N = 19	N = 12	N = 8

Table 7. Sterol composition and concentration (μg mg lipid^{-1}), mean sterol content (% lipid wt and % algal dry wt) of four algae. Means followed by the same upper case letter were not significantly different ($p > 0.05$).

Sterols	B. grandis	N. oleoabundans	P. tricornutum	S. quadricauda
Δ 5, 22 ergostadienol (brassicasterol)			6.8	
Δ 5, 7, 22 ergostatrienol (ergosterol)		2.9		6.14
Δ 5-ergostenol	4.5			
Δ 5, 22 stigmastenol (poriferasterol)	8.5			
Δ 5,7-ergostadienol		1.8		13.0
Δ 7 ergostenol	1.8	1.7		3.7
Δ 5 stigmastenol (clionasterol)	7.6			
Δ 7, 22-ergostadienol			0.77	
Δ 5 cholestenol (cholesterol)			Minor	
Δ 8,9-stigmastenol	1.4			
Δ7-chondrillasterol	0.6			
Total Mean Sterol Concentration (μg mg lipid^{-1}) Sample Size	19.3 ± 12.6 A N = 20	5.9 ± 0.01 B N = 19	7.4 ± 3.8 B N = 12	19.5 ± 17.7 A N = 8
Total Mean Sterol Content (% lipid wt); Sample Size	1.7 ± 1.2 N = 20	1 ± 1.0 N = 19	1.0 ± 0.4 N = 12	1.8 ± 1.8 N = 8
Total Mean Sterol Content (% algal dry wt); Sample Size	0.2 ± 0.2 N = 20	0.02 ± 0.03 N = 19	0.2 ± 0.09 N = 12	0.44 ± 0.0 N = 8

Table 8. Protein content (mean ± SD % of algal dry weight) of four algae at different growth phases. Values followed by the same lower-case letter within a column for a given species were not significantly different ($p > 0.05$). Values followed by the same upper case letter within a row were not significantly different ($p > 0.05$).

Phase	B. grandis	N. oleoabundans	P. tricornutum	S. quadricauda
Early Log/Log	63.6 ± 11.6 a	69.1 ± 3.1 a	44.2 ± 11.2 a	66.5 ± 12.7a
Late Log	77.0 ± 7.3 a	78.6 ± 0 a	62.4 ± 11.0 a	
Stationary	57.5 ± 17.5 a	48.7 ± 2.2 a	74.8 ± 20.4 a	67.2± 8.9
Late Stationary	79.1 ± 0.9 a	63.8 ± 16.1 a		75.4± 1.7
Mean Protein Content (% algal dry wt)	67.8 ± 13.4 a, A	61.8 ± 13.8 a, A	60.7 ± 17.0 a, A	70.3 ± 9.3 a, A
Sample Size	N = 13	N = 10	N = 7	N = 12

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Figure 1. Growth of Neochloris oleoabundans over time with growth phases identified.

Figure 2. Ratio of saturated to unsaturated fatty acids in four algae at different growth phases.

Figure 3. Sterol content (ug·mg lipid^{-1}) of four algae at different phases of growth. BRAC = Bracteacoccus grandis, NEO = Neochloris oleoabundans, PHAEO = Phaeodactylum tricornutum, SCEN = Scenedesmus quadricauda.

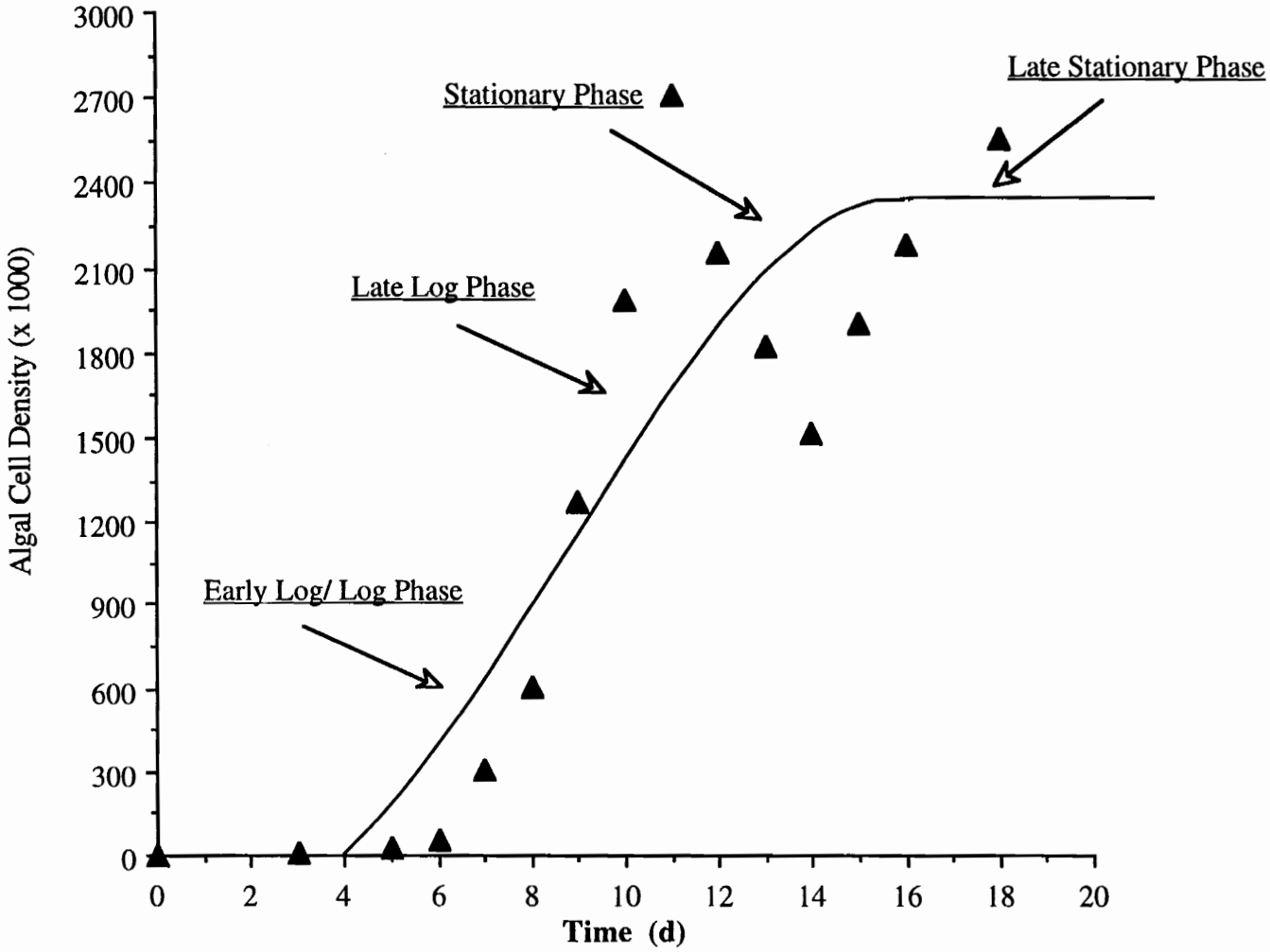

Figure 1. Growth of Neochloris oleoabundans over time with growth phases identified.

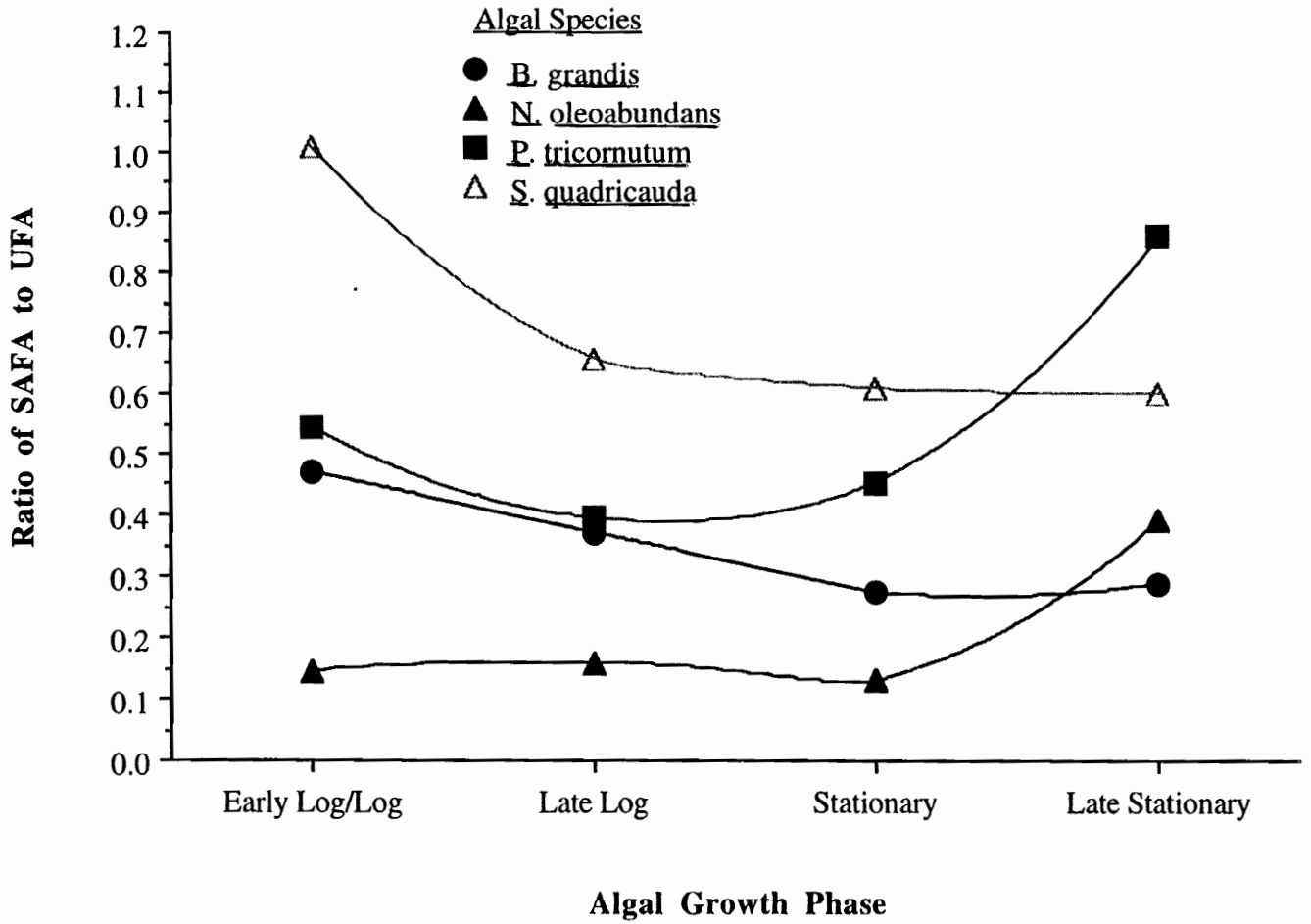

Figure 2. Ratio of saturated to unsaturated fatty acids in four algae at different growth phases. SAFA = saturated fatty acids, UFA = unsaturated fatty acids.

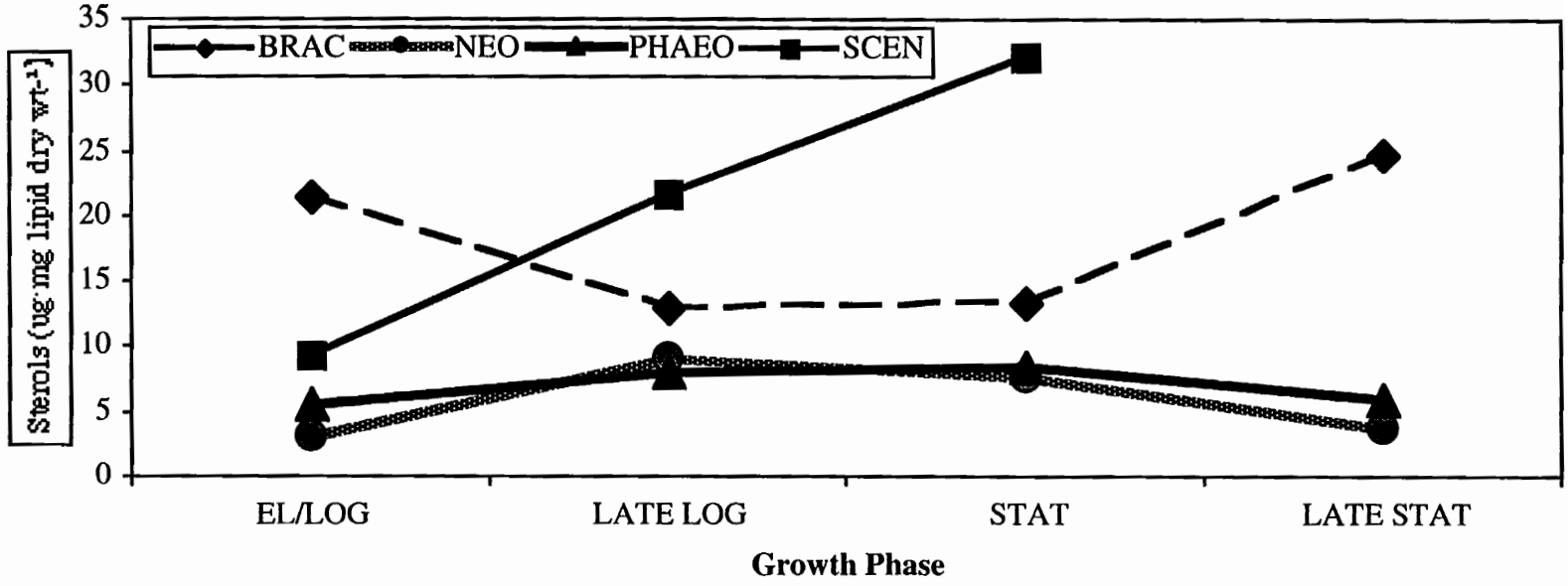

Figure 3. Sterol content of four algae at different phases of growth. BRAC = Bracteacoccus grandis, NEO = Neochloris oleoabundans, PHAEO = Phaeodactylum tricornutum, SCEN = Scenedesmus quadricauda.

VITA

Catherine M. Gatenby was born in Aurora, Illinois on September 19, 1959. In 1974, her family moved to Wayzata, Minnesota where she graduated from high school in 1977. After spending two years (1977-1979) at St. Cloud State University, in St. Cloud Minnesota, she decided to go to work full-time with the intention of returning to college at a later date. She worked for the Prudential Insurance Company of America for 7 years. In January 1987, she quit the secure and insured life to pursue a Bachelor's of Science at the University of Minnesota. She graduated from the U of MN in June 1991, and moved to Blacksburg, Virginia to pursue a Master's of Science in Fisheries at Virginia Tech, with Dr. Richard J. Neves. Following a year sabbatical from studies, she returned to Virginia Tech in 1995 to pursue a Ph. D. in Biology. Drs. Bruce C. Parker, Biology and Dr. Richard J. Neves, Fisheries and Wildlife Sciences were co-chairs of her doctoral committee. She will be moving to Philadelphia, Pennsylvania to a post-doctoral position with Dr. Daniel A. Kreeger, at the Academy of Natural Sciences in Philadelphia where she will continue studying the feeding ecology and natural history of freshwater mussels.

Her avocational interests involve the production of public education programs on environmental issues and ecological principles which combine the arts: theatre, dance, music, and photography, and traditional science education techniques. In the meantime, you can expect to find her in the dance studio studying tap, jazz, and folk dancing when she isn't in the lab or office.