Growth and survival of juvenile rainbow mussels, *Villosa iris* (Lea, 1829) (Bivalvia: Unionidae), reared on algal diets and sediment

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Abstract: To develop a suitable diet for rearing recently metamorphosed freshwater mussels, nine species of algae and one live bacterium were tested in various trialgal or bialgal and bacterium combinations. A substratum was included in the culture chambers with each treatment to facilitate pedal-feeding by juvenile mussels. Kaolin, an artificial substratum, and fine sediment were tested in combination with algae and without algae. An unfed (no algae or substratum) control treatment also was tested.

Juveniles fed a freshwater trialgal diet consisting of *Neochloris oleoabundans* Chantanachat and Bold, 1962, *Bracteacoccus grandis* Bischoff and Bold, 1963, *Phaeodactylum tricornutum* Bohlin, 1897, with fine sediment showed the best growth over time (140 d). These individuals achieved a mean shell length of 1747 µm, and had 30.0% survival after 140 d. Other trialgal mixtures containing *N. oleoabundans*, *Nitzschia acicularis* (Kützing) Wm. Smith, 1853, and *Cyclotella meneghiniana* Kützing, 1844, enhanced growth more than a green trialgal mixture of *Chlorella vulgaris* Beyernik, 1890, *Ankistrodesmus falcatus* (Corda) Ralfs, 1848, and *Chlamydomonas reinhardtii* Dangeard, 1888. A diet of fine sediment alone sustained juveniles through 140 d; however, an additional food source such as algae was necessary to increase survival. Bacteria did not contribute appreciably to juvenile growth and survival. Juvenile mussels reared on commercial yeast diets survived only 8 d; juveniles reared on kaolin and algae survived 60 d.

Key Words: juvenile freshwater mussels, algal diets, fine sediment, kaolin

With nearly 70% of native freshwater mussel species (Unionidae) in decline and another dozen commercial species threatened by the invasion of the zebra mussel, research aimed at the conservation of freshwater mussels has become a priority in the United States (Williams *et al.*, 1993). Culture and propagation of freshwater mussels for stock enhancement, preservation of endangered species, and creation of refugia for native populations from the invading zebra mussel, exemplify current conservation efforts. Research reported here is from an ongoing study on the culture and propagation of freshwater mussels. Previous studies in the United States and Europe on culturing freshwater mussels have reported difficulties in maintaining juveniles beyond 4 wk (Lefevre and Curtis, 1910; Howard, 1917, 1922). One reason could be that the food source used to rear juvenile mussels was inadequate. Unfortunately, little information is available on the suitability of various foods for unionids other than the experiments of Coker *et al.* (1921), Imlay and Paige (1972), and Hudson and Isom (1984) who showed that algae, detritus, and commercial fish food (as dissolved nutrients in the water) were potential food sources for freshwater mussels.

Numerous studies in aquaculture have shown that algae are the principal food of marine shellfish (Ukeles, 1971; Webb and Chu, 1983; Tan Tiu *et al.*, 1989), and more importantly, that polyunsaturated fatty acids (PUFA's) are essential to the early larval and juvenile life stages of most fish and shellfish (Ando, 1968; Castell, 1970; Ackman, 1983; Watanabe *et al.*, 1983; Webb and Chu, 1983; Napolitano *et al.*, 1988, 1990). It is generally thought that PUFA's are important functional components of cells and membranes (Ackman, 1983), and that lipid storage products provide a cheap energy source. In addition, mixed diets of different species of algae provided better growth in marine bivalves than did quantities of any single food tested (Davis and Guillard, 1958; Walne, 1970; Epifanio, 1979; Romberger and Epifanio, 1981; Enright *et al.*, 1986a). Resident bacteria in aquatic systems also have been implicated as an important ingredient in the juvenile bivalve diet (Urban and Langdon, 1984; Crosby *et al.*, 1990; Baldwin and Newell, 1991).

Feeding in the sediments with foot ciliation is common among bivalves, especially in the juvenile life stage, until their gills are fully developed for filter-feeding (Allen, 1961, 1985; Morton, 1976; Lasee, 1991; Yeager *et al.*, 1994). Thus, juvenile bivalves can derive nutrition from detrital material adhered to or adsorbed on the surface of sediment particles. In an earlier study, pedal-feeding

behavior by juveniles of two freshwater mussel species was reported by Gatenby *et al.* (1996). Specific research objectives of our study, therefore, were to identify a suitable diet for rearing juvenile freshwater mussels, to examine the nutritional role of riverine sediment and bacteria, and to compare growth and survival of juvenile mussels reared on different diets after metamorphosis.

MATERIALS AND METHODS

The rainbow mussel, *Villosa iris* (Lea, 1829), was selected for this study because it is common in Virginia, and like most endangered freshwater mussel species, is found in fast-moving riverine environments. Recently metamorphosed juveniles were fed various combinations of nine species of algae, including six green algae and three diatom species, one bacterium, yeast, kaolin, and riverine sediment (Table 1). Juvenile mussels can contain fatty reserves from their parasitic life stage that can allow them to live 2 wk post-metamorphosis without food supplementation (Lasee, 1991). We decided, therefore, that we would monitor growth and survival in mussels reared on our diets for at least 45 d post-metamorphosis. We were able, however, to maintain juvenile mussels in laboratory culture much longer than 45 d. Thus, three tests were performed on data collected at 60, 100, and 140 d post-metamorphosis. Gut contents of juveniles and river sediment were examined for algae and bacteria. Pedal-feeding behaviors also were observed, and the number of days post-metamorphosis that juveniles employed pedal-feeding was estimated.

Newly metamorphosed mussels were obtained from host-fish infestations in the laboratory (Zale and Neves, 1982; Gatenby *et al.*, 1996). Gravid *Villosa iris* were collected from Copper Creek, Clinch River drainage, Scott County, Virginia. The host fish [rock bass, *Ambloplites rupestris* (Rafinesque, 1817), and largemouth bass, *Micropterus salmoides* (Lacèpéde, 1802)], 15-30 cm in length (Table 1) were collected from Tom's Creek, New River drainage, Montgomery County, Virginia, treated for parasites, and acclimated in the laboratory prior to infestation with glochidia (Zale and Neves, 1982; Neves *et al.*, 1985).

River water and sediment were collected from the New River, Montgomery County, Virginia. Water hardness ($CaCO_3$) was 55.0 mg/l, and pH was 7.6. River water was filtered using a 4.25 cm diameter Whatman Glass Microfiber Filter to remove particles > 0.45 µm. Sediment was maintained in the laboratory under aeration, and when needed, it was sieved through a 130 µm mesh screen before being added to the juvenile culture chamber. Thus, the sieved sediment contained the natural assemblage of microorganisms and other organic material associated with riverine sediments.

Table 1. Summary of diets tested for rearing juvenile freshwater mussels of *Villosa iris*. (CAC = *Chlorella vulgaris, Ankistrodesmus falcatus,* and *Chlamydomonas reinhardtii*; NNiC = *Neochloris oleoabundans, Nitszchia acicularis,* and *Cyclotella meneghiniana*; NOC = *N. oleoabundans, Oocystis marsonii,* and *C. menenghiana*; NPB = *N. oleoabundans, Phaeodactylum tricornutum,* and *Bracteacoccus grandis*; NPE = *N. oleoabundans, P. tricornutum,* and *Enterobacter aerogenes*; Sed = fine sediment).

Treatment (replicates)	Juveniles per replicate
Unfed (1)	45
Kaolin-only (3)	50, 50, 50
Sed-only (2)	71, 70
Yeast/Sed (3)	100, 100, 100
CAC/Kaolin (2)	92, 100
CAC/Sed (3)	200, 200, 215
NOC/Sed (3)	100, 100, 100
NNiC/Sed (3)	111, 100, 103
NPB/Sed (3)	100, 101, 108
NPE/Sed (3)	100, 103, 100

Newly metamorphosed juveniles were transferred to replicated glassculture dishes, 8 cm in diameter, 5 cm in height, and filled with 175 ml of filtered river water. A slow stream of air was introduced into each of the static chambers by fixing an Eppendorf pipette to the end of vinyl air tubing. Approximately 25-40 ml (4 g dry wt) of fine sediment was added to the static chambers, with the exception of those treatments receiving kaolin. Fine sediment covered an area of 50 cm^2, was 1.0-4.0 mm in depth and loosely packed in the culture chamber. Sediment particles we term "fine" were defined as "fine sand to clay" by Wentworth (1922). Approximately 2-3 g of kaolin were added to the kaolin treatments at the onset of the experiment. Water temperature was monitored daily, water was changed weekly, and chambers received new sediment or kaolin weekly. About two-thirds of the substratum in any given culture dish was discarded and replaced with fresh substratum.

Algae were cultured in media ideal for growth of each species (Tables 2, 3, and 4), under continuous cool white fluorescent light of 60-100 µmol/m^2/s photon flux at 20 ± 1°C. All media were autoclaved at 121°C, >15 psi, for 20 min, then cooled prior to being innoculated with algae. Unialgal cultures were not axenic; however, the culture flasks were capped with cotton, cheese-cloth stoppers to prevent contamination and allow aeration through a sterile filter system. At near-maximum stationary phase, algal cells were counted in a hemacytometer. Cultures were then centrifuged at 7000-10,000 rpm for 25 min and the medium decanted. Algae were resuspended in tap water and kept under dark refrigeration for up to 2 wk.

Approximately 1 g of a manipulated yeast diet (Artemia Reference Center, Ghent, Belgium) was mixed in

Table 2. Algae and bacteria species tested in feeding experiment, species sources, and growth medium used to culture species. (BMB = Bold's Modified Bristol's; OCM = Our *Chlorella* Medium).

Species tested	Species source	Growth medium[1]
Neochloris oleoabundans Chantanachat and Bold, 1962	Martek BioSciences Corp., Columbia, Maryland	*Neochloris*[2]
Bracteacoccus grandis Bischoff and Bold, 1963	University of Texas, Austin, Texas	OCM[3]
Ankistrodesmus falcatus (Corda) Ralfs, 1848	Carolina Biological Supply, Burlington, North Carolina	BMB[4]
Chlorella vulgaris Beyernik, 1890	Carolina Biological Supply	BMB[4]
Oocystis marsonii Lemmermann, 1898	University of Texas	OCM[3]
Chlamydomonas reinhardtii Dangeard, 1888	University of Texas	BMB[4]
Cyclotella meneghiniana Kützing, 1844	Loras College, Dubuque, Iowa	Bi-phasic/BMB[4]
Nitzschia acicularis (Kützing) Wm. Smith, 1853	Loras College	Bi-phasic/BMB[4]
Phaeodactylum tricornutum Bohlin, 1897	University of Texas	Soil extract/BMB[4]
Enterobacter aerogenes Kruse, 1896	Biology Dept., Virginia Tech	Nutrient Broth[5]

[1]Media formulations given in Table 3.
[2]McArdle *et al.*, 1994.
[3]Behrens *et al.*, 1989.
[4]Nichols, 1973.
[5]DIFCO, Detroit, Michigan.

500 ml of tap water, the equivalent of 5-10 l of live algae or 1.0×10^7 - 2.0×10^7 cells/ml.

The bacterial culture was axenic and grown in nutrient broth media (Table 2), under continuous 60-100 µmol/m²/s photon flux of white fluorescent light. The air temperature was approximately 20 ± 1°C. Bacterial cell concentration was determined by plate counts. Bacteria were concentrated by centrifugation at 7000 rpm for 20 min; cells were separated from culture media, resuspended in tap water, and then kept alive under refrigeration for the duration of the experiment, with growth arrested at a concentration of 1.0×10^8 cells/ml. Concentrated algae were administered daily to achieve treatment diets of 3.0×10^5 to 5.0×10^5 cells/ml in juvenile culture dishes.

Nine treatments tested whether riverine sediment with associated native bacteria and other organic material provided nutritional value to juvenile mussels, whether bacteria added to the culture diet enhanced growth and survival, and whether various algae or a commercial yeast diet known to be high in lipids, could enhance growth and survival of juvenile mussels. Three replicates of most treatments were tested; however, due to constraints on availability of juveniles, fewer replicates of some treatments were necessary (Table 1). The treatments included an unfed control treatment (Unfed), Kaolin-only (USP K2-500, a fine clay product of Fisher Chemical), fine sediment (Sed-only), yeast with fine sediment (Yeast/Sed), *Chlorella vulgaris, Ankistrodesmus falcatus, Chlamydomonas reinhardtii* with kaolin (CAC/Kaolin), CAC with fine sediment (CAC/Sed), *Neochloris oleoabundans, Oocystis marsonii, C. meneghiniana* with fine sediment (NOC/Sed), *N. oleoabundans, Nitzschia acicularis, Cyclotella meneghiniana* with fine sediment (NNiC/Sed), *N. oleoabundans, Bracteacoccus grandis, Phaeodactylum tricornutum* with fine sediment (NPB/Sed), and *N. oleoabundans, P. tricornutum, Enterobacter aerogenes* with fine sediment (NPE/Sed) (Table 1). Neither sediment nor kaolin was included in the particle concentration calculation; however, the bacterium *E. aerogenes* was included in the particle concentration for the diet NPE/Sed. All experiments were conducted on a 12:12 hr light:dark cycle, and each chamber contained 50-100 juveniles (Table 1).

Table 3. *Neochloris* (McArdle *et al.*, 1994) and OCM (Our *Chlorella* Medium) (Behrens *et al.*, 1989) growth medium formulations.

Neochloris		OCM	
Component	Concentration	Component	Concentration
NaCl	5.82 g/l	KNO_3	1.0 g/l
$MgSO_4 \cdot 7H_2O$	2.47 g/l	$MsSO_4 \cdot 7H_2O$	5.0 ml, 100 g/l
KNO_3	1.0 g/l	$Ca(NO_3)_2 \cdot 4H_2O$	2.5 ml, 25 g/l
KCL	0.75 g/l	K_2HOP_4	2.0 ml, 50 g/l
$FeCl_3$	1.0 ml, 0.81 g/l	K_2HOP_4	1.5 ml, 50 g/l
$CaCl_2$	2.0 ml, 43.9 g/l	Fe - versenate*	2.0 ml/l
Na_2EDTA	2.0 ml, 11.17 g/l	*Na_2EDTA	4 g/l
H_3BO_3	1.0 ml, 12.36 g/l	*$FeSO_4 \cdot 7H_2O$	5 g/l
Metals*	10.0 ml/l	*Distilled H_2O	to 1 l
*$FeCl_3$	20 ml, 33 g/l	Metals*	1.0 ml/l
*$ZnCl_2$	20 ml, 1.1 g/l	**$ZnSO_4 \cdot 7H_2O$	222 mg/ml
*$CuSO_4 \cdot 5H_2O$	2.0 ml, 5 g/l	**$CuSO_4 \cdot 5H_2O$	79 mg/ml
*H_3BO_3	20 ml, 6 g/l	**H_3BO_3	2.86 g/l
*$MnCl_2 \cdot 4H_2O$	20 ml, 16 g/l	**$MnCl_2 \cdot 4H_2O$	1.81 g/l
*$Na_2MoO_4 \cdot 2H_2O$	20 ml, 1.2 g/l	**$Na_2MoO_4 \cdot 2H_2O$	390 mg/ml
*$CoCl_2 \cdot 6H_2O$	2.0 ml, 4 g/l	**$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg/ml
*Na_2EDTA	4.0 g/l	**Distilled H_2O	to 1 l
*Distilled H_2O	to 1 l	Distilled H_2O	to 1 l
Vitamins** (add 50 ml to 1 l media after autoclaving)			
**KH_2PO_4	2.0 ml, 54.4 g/l		
**Thiamine•HCl	0.1 ml, 1 mg/ml		
**Biotin	2.5 ml, 0.2 mg/ml		
**Vitamin B12	2.5 ml, 0.2 mg/ml		
Distilled H_2O	to 1 l		

We examined growth and survival at 60 d and 100 d, and growth over 140 d post-metamorphosis. Juveniles in a culture dish were sieved out and removed to a Petri dish for measuring and assessing survival, weekly. Shell lengths of a random sample of 15 to 25 juveniles in each dish were measured using a calibrated ocular micrometer on a dissecting microscope. Randomness was achieved by moving the Petri dish on the dissecting stage and measuring the first animal that came into view. Juveniles were measured, counted, and returned to their culture dish. Thus, no juvenile was measured twice, nor counted twice.

Shell lengths at 60 and 100 d were compared using one-way analysis of variance (ANOVA) with nested replicates, followed by multiple contrast tests (Sokal and Rohlf, 1981). The survival data were compared by ANOVA (without nested replicates), followed by Tukey-Kramer multiple comparison. Arcsine transformation was applied to the percent survival data to satisfy the normality assumption. Multiple comparisons and contrast tests were evaluated at $\alpha < 0.01$ (0.003 and 0.005 for the 60 and 100 d nested ANOVA's) to control the overall experiment-wise error of $\alpha = 0.05$ (Zar 1974; Sokal and Rohlf, 1981). Growth rates at 140 d post-metamorphosis were compared using analysis of covariance (ANCOVA), followed by a sequential analysis of the slopes to determine differences among treatments. Multiple comparisons were evaluated at $\alpha = 0.01$, so that the overall experimental error also was controlled at $\alpha = 0.05$. Treatments without survivors were not included in any of the analyses. All statistical tests were calculated using JMP 2.0 (SAS Institute, Inc., 1991).

Fluorescence microscopy, which induces a distinctive red fluorescence of chlorophyll under blue light, was used to verify that juveniles were ingesting algae, and that the algae were well distributed throughout the substratum. Juveniles were placed on microscope slides and squashed under cover slips for examination.

Locomotory and feeding behaviors of *Villosa iris* were observed using a dissecting microscope at the time of shell measurement and survival tabulation. Approximately 20 juveniles from each culture dish were observed for about 20 min each week.

Table 4. Bold's Modified Bristol's (BMB) (Nichols, 1973) and bi-phasic growth media formulations, and soil extract preparation.

Component	Concentration
NaCl	10 ml, 1 g/400 ml
$MgSO_4 \cdot 7H_2O$	10 ml, 3 g/400 ml
$NaNO_3$	10 ml, 3 g/400 ml
K_2HPO_4	10 ml, 3 g/400 ml
KH_2PO_4	10 ml, 7 g/400 ml
$CaCl_2 \cdot 2H_2O$	10 ml, 1 g/400 ml
$Na_2EDTA \cdot 2H_2O$	1 ml, 50 g/l
KOH	1 ml, 31 g/l
$FeSO_4 \cdot 7H_2O$	1 ml, 5 g/l
H_2SO_4	1 ml, 1 ml/l
H_3BO_3	1 ml, 11.42 g/l
$ZnSO_4 \cdot 7H_2O$	1 ml, 8.82 g/l
$MnCl_2 \cdot 4H_2O$	1 ml, 1.44 g/l
MoO_3	1 ml, 0.71 g/l
$CuSO_4 \cdot 5H_2O$	1 ml, 1.57 g/l
$Co(NO_3)_2 \cdot 6H_2O$	1 ml, 0.49 g/l
Soil extract	40 ml/l
Autoclave soil (with water overlay) two times, at 121°C, > 15 psi, for 20 min.	
Decant soil water; preferably using Whatman 1 Filter.	
If still turbid, centrifuge to achieve a clear extract.	
Biphasic	
Place autoclaved river soil to a depth of about 3 cm, with Bold's Basal media overlay.	

RESULTS

The Unfed, Kaolin-only, Yeast/Sed, and NOC/Sed diets did not support growth beyond 35-40 d, and thus, were not included in the statistical analyses. Shell lengths of juveniles reared on all other diets were not significantly different ($p = 0.4104$) at 60 d. Shell lengths of juveniles at 100 d also were not significantly different (0.0830); however, all juveniles reared on CAC/Kaolin were dead. Subsequent contrast t-tests at 100 d ($\alpha = 0.005$) indicated that mean shell lengths of juveniles fed NPB/Sed (1354 µm) was significantly greater ($p < 0.0018$) than those of juveniles fed on all other treatments. At 100 d, mean shell lengths of juveniles fed CAC/Sed (874 µm) and Sed-only (828 µm) were similar ($p = 0.6183$), and mean lengths of juveniles fed CAC/Sed, NPE/Sed (978 µm) and NNiC/Sed (1009 µm) also were similar (Table 5). Juveniles fed NNiC/Sed and NPE/Sed, however, exhibited more growth at 100 d than juveniles in Sed-only ($p = 0.0661$ and 0.0556, respectively), although not significant at the contrast test level of $\alpha = 0.005$. Individuals fed the better diet (NPB/Sed) achieved a maximum shell length of 1077 µm at 60 d, and a maximum shell length of 1795 µm at 100 d (Table 5).

An analysis of covariance after 140 d confirmed that juveniles fed various diets exhibited significantly different growth rates over time ($p < 0.001$) (Fig. 1). A sequential analysis of estimated slopes indicated that the growth rate of juveniles fed NPB/Sed was significantly greater than the growth rates recorded for all other treatments, and that all algal diets produced greater growth than a diet of fine sediment only (Table 6). Growth over time was similar for juveniles fed NPE/Sed and NNiC/Sed ($p = 0.7626$), and both diets marginally improved growth over the green algal diet, CAC/Sed ($p = 0.0656$ for NPE/Sed versus CAC/Sed and $p = 0.0177$ for NNiC/Sed versus

Table 5. Mean shell length (± SD, with minimum-maximum in parentheses) and percent survival of *Villosa iris* juveniles fed various diets for 60 and 100 d post-metamorhosis. Initial lengths of juveniles were 263 ± 37 µm. p-values are given for the ANOVA test for treatment effect ($\alpha = 0.05$). Means with the same superscripts (a, b) were similar according to contrast t-tests and Tukey-Kramer test; however, due to high variability within treatments in survival data, the ANOVA test at 60 d was sufficiently robust to detect differences among treatments. (Abbreviations as in Table 1).

	60 Days		100 Days	
Diet	Length (µm)	Survival (%)	Length (µm)	Survival (%)
Unfed	total mortality		total mortality	
Kaolin-only	total mortality		total mortality	
Sed-only	710^a ± 108 (462-897)	50.8	828^b ± 75 (667-949)	31.1^a
Yeast/Sed	total mortality		total mortality	
CAC/Kaolin	744^a ± 69 (641-795)	4.3	total mortality	
CAC/Sed	658^a ±108 (500-886)	51.3	874^b ± 138 (643-1143)	36.0^a
NOC/Sed	total mortality		total mortality	
NNiC/Sed	638^a ± 69 (513-769)	13.6	1009^b ± 155 (769-1281)	20.7^a
NPB/Sed	734^a ± 153 (564-1077)	33.7	1354^a ± 296 (1026-1795)	32.5^a
NPE/Sed	655^a ± 105 (513-923)	48.6	978^b ± 231 (564-1795)	34.2^a
p-value	0.4104	0.4591	0.0830	0.7768

CAC/Sed) (Table 6). After 140 d, individuals fed NPB/Sed achieved a mean length of 1747 µm and maximum length of 2359 µm (Table 7).

Survival at 60 d ranged from 4.4% for CAC/Kaolin to 51.3% for CAC/Sed, and variability within a treatment was high (Table 5). Although 4.4% survival is drastically different from 51.3% survival and is probably different from survival for all other diets, except perhaps NNiC/Sed (13.6%), the ANOVA was of insufficient robustness to detect differences among treatments. Mean percent survival at 100 d for all treatments ranged from 20.7-36.0%, and an ANOVA indicated no difference in percent survival among treatments ($p = 0.7768$) (Table 5). However, one to two replicates within each treatment showed 0% survival; thus, with so few experimental units, no statistical test was sufficiently robust to adequately analyze our survival data. After 140 d, algae in combination with sediment appeared to gain significance in enhancing survival. Mean survival at 140 d for individuals reared on algae with fine sediment was 24.5%, whereas mean survival for individuals in fine sediment-only was 10% (Table 7).

The Unfed treatment was unable to maintain juveniles beyond 37 d, and the Kaolin-only treatment had no survivors at 38 d. The Yeast/Sed diet did not support juveniles beyond 8 d, and all individuals fed NOC/Sed were dead by 46 d, with replicates 1 and 2 dead at 24 d. Almost all juveniles died in two replicates of NNiC/Sed between 49 and 65 d. Chemical analysis of the prepared diets indicated that NOC and NNiC contained 89.2 and 55.9 mg/l of potassium, respectively, and these levels could have been toxic to mussels (Imlay, 1973). Two tanks of NPB/Sed were lost at 80 d due to a waterline break.

Juveniles were very active during the 20 min they were observed each week. By protracting and retracting the foot, the juveniles moved through the substratum in a "tumble-like" fashion. While lying valve-side down, juveniles continually used a foot-sweeping movement which carried a current of particles toward the pedal gape. The foot was highly ciliated, and some particles adhered to it, while larger particles were lost as the juvenile retracted its foot inside the valves. These behaviors have previously been described by Reid *et al.* (1992) and Yeager *et al.* (1994) for several marine and freshwater bivalves. Fluorescence microscopy indicated that juveniles reared on sediment/algae and kaolin/algae had ingested algae (Fig. 2A), and that the algae were well distributed throughout the substratum (Fig. 2B). Subsequent gut squashes showed par-

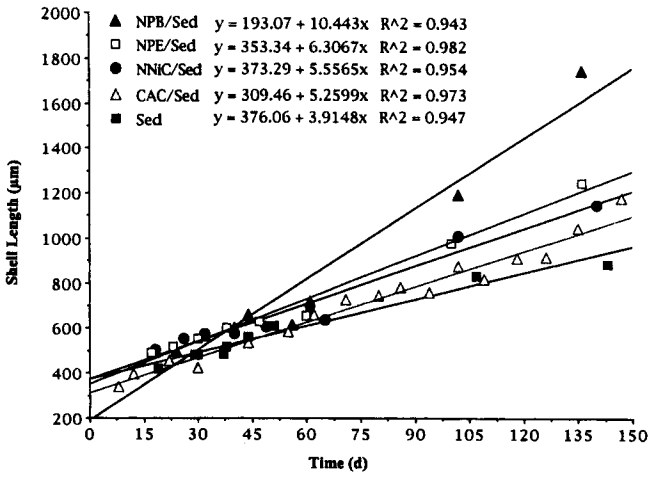

Fig. 1. Comparison of growth equations of juvenile *Villosa iris* fed various diets for 140 d post-metamorphosis. (Abbreviations as in Table 1).

Table 6. Comparison of growth equations of *Villosa iris* juveniles fed various diets for 140 d post-metamorphosis. The diet with the largest slope (NPB) was compared to all other diets first. Diets found to be significantly different from the diet in question were then excluded sequentially from each test until all desired comparisons were made. p-values are given for the multiple comparisons of slope estimates from a sequential analysis of covariance. Slopes are signficantly different at $\alpha < 0.01$, thus, the overall experimental error is controlled at $\alpha = 0.05$. (* = the slope of this diet is significantly different from the diet to which all other diets are being compared in that particular test; na = not applicable; other abbreviations as in Table 1).

		Sequential Comparison of Slopes				
	Growth Equation	NPB/Sed with all other diets	NPB/Sed with NNic/Sed, NPE/Sed	NPE/Sed with all except NPB/Sed	NPE/Sed with NNic/Sed, CAC/Sed	NNic/Sed with, CAC/Sed, Sed-only
NPB/Sed	$y = 193.07 \pm 10.443x$	na	na	na	na	na
NPE/Sed	$y = 353.34 \pm 6.3067x$	0.1729	0.0040*	na	0.7626	na
NNiC/Sed	$y = 373.29 \pm 5.5565x$	0.4952	0.0080*	0.6925	na	na
CAC/Sed	$y = 309.46 \pm 5.2599x$	0.0000*	na	0.1431	0.0656	0.0177
Sed-only	$y = 376.06 \pm 3.9148x$	0.0000*	na	0.0007*	na	0.0045*

Table 7. Mean shell length (± SD, with minimum-maximum in parentheses) and percent survival of *Villosa iris* juveniles in each culture chamber, and pooled survival for all juveniles reared on algae and sediment or sediment-only for 140 d post-metamorphosis. [nd = no data (not considered in the pooled survival calculation); other abbreviations as in Table 1).

Diet	Length (μm)	Survival (%)
Sed-only 1	914 ± 120 (769-1180)	15.5
Sed-only 2	855 ± 74 (769-897)	4.3
Pooled survival for Sed-only		10.0
CAC/Sed 1	nd[3]	nd
CAC/Sed 2	1146 ± 248 (718-1590)	24.5
CAC/Sed 3	nd[3]	nd[3]
NNic/Sed 1	1149 ± 283 (564-1667)	15.3
NNic/Sed 2	nd[1]	nd[1]
NNic/Sed 3	nd[1]	nd[1]
NPB/Sed 1	nd[2]	nd[2]
NPB/Sed 2	nd[2]	nd[2]
NPB/Sed 3	1747 ± 301 (1282-2359)	29.6
NPE/Sed 1	954 ± 249 (610-1439)	30.0
NPE/Sed 2	1369 ± 436 (897-2051)	9.7
NPE/Sed 3	1410 ± 308 (897-2051)	38.0
Pooled survival for algae and sediment		24.5

[1]NNic could have contained toxic levels of potassium (from algal medium).
[2]Burst water line caused mortality.
[3]Chironomid larvae might have preyed upon juveniles.

tially digested algal cells, or ghost cells lacking chloroplasts, as well as colloidal particles, indicating that algae associated with the fine sediment were utilized as food by the juvenile mussels. *Villosa iris* exhibited locomotory and pedal-sweep movements for approximately 140 d. After 272 d, *V. iris* were observed positioned anterior-end in the sediment; apertures were visible and siphoning.

DISCUSSION

The fatty acid composition of many algae has been characterized, although most of the work has focused on marine algae (Ben-Amotz *et al.*, 1985; Cranwell *et al.*, 1988; Ahlgren *et al.*, 1992). Thus, although we did not perform lipid analyses on our diets, it was anticipated that certain freshwater algae high in lipids, especially PUFA's, would enhance growth in juvenile freshwater mussels. Because the culture environment affects the lipid content of algae (Spoehr and Milner, 1949; Fogg, 1959; Shifrin and Chisholm, 1981; Enright *et al.*, 1986b), we harvested our algal cultures at the late-logarithmic, early-stationary phase when algae tend to produce more unsaturated than saturated fatty acids (Chu and Dupuy, 1980; Ahlgren *et al.*, 1992; Dunstan *et al.*, 1993).

Freshwater Chlorophyceae generally lack substantial amounts of long-chained PUFA's and, unlike other marine algae, are able to synthesize only minimal amounts of polyunsaturated fatty acids (Pohl and Zurheide, 1979; Ahlgren *et al.*, 1992). It was not surprising, therefore, that the entirely green algal diet of *Chlorella, Ankistrodesmus,* and *Chlamydomonas* produced poorer growth than the other algal diets tested. It should be noted, however, that these algal species are not without some nutritional value. Total lipids (as percent dry weight) reported for *C. vulgaris, Chlamydomonas* sp., and *Ankistrodesmus* sp. were 13, 20, and 18%, respectively (Shifrin and Chisholm, 1981; Ben-Amotz *et al.*, 1985), and this trialgal mixture produced better growth than a diet of fine sediment only.

We suspected that juvenile mussels fed diets containing the diatoms *Nitzschia, Cyclotella,* and *Phaeodactylum* would show the best growth because

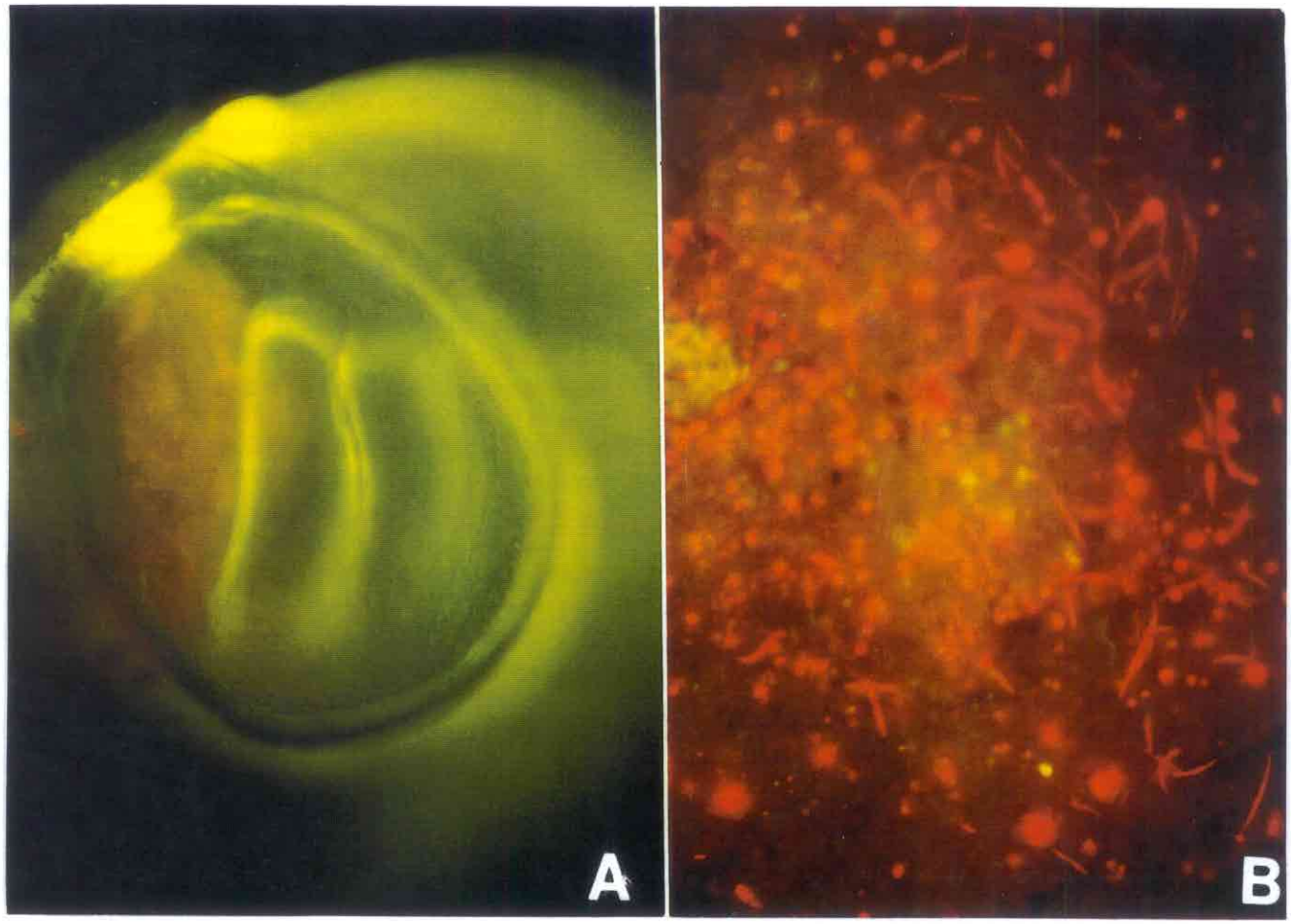

Fig. 2. A. Red fluorescing chlorophyll from ingested algae as seen in gut area of live 30 d juvenile *Villosa iris*. B. Red fluorescing chlorophyll from algae well-distributed in kaolin substratum.

diatoms are characterized by a high percentage of unsaturated fatty acids and generally store oils when nutrients are limited (Erwin, 1973; Werner, 1978; Pohl and Zurheide, 1979; Ahlgren *et al.*, 1992). In addition, species of *Nitzschia* and *Cyclotella* contain more lipids and have a significantly greater proportion of unsaturated fatty acids than green algae (Shifrin and Chisholm, 1981). *P. tricornutum* is abundant in PUFA's (Reitan *et al.*, 1994), and its delicate, siliceous walls are probably loosened easily by grinding or enzymatic action of the digestive system of juvenile mussels. Unlike most green algae, the oil-producing potential of *Neochloris oleoabundans* has been well described (Lien, 1981), and it could also contain PUFA's (Tornabene *et al.*, 1983). *Bracteacoccus grandis*, another green alga, is abundant in lipids (Bishoff and Bold, 1963). Thus, juveniles reared on *Neochloris*, *Bracteacoccus*, and *Phaeodactylum* showed the best growth over time, and juveniles fed the *Neochloris*, *Nitzschia*, and *Cyclotella* diet also showed very good growth when compared to those reared under the other diets tested.

Unfortunately, almost all juveniles died in two replicates of NNiC/Sed between 49 and 65 d. This sudden mortality likely resulted from the laboratory culture environment, and not the nutritional quality of the diet. Imlay (1973) reported that exposure of freshwater mussels to potassium levels of 4-7 mg/l over 7 d was lethal. Chemical analysis of the NNiC diet showed high levels of potassium (55.9 mg/l). The algal medium contained high levels of potassium (Tables 3 and 4). Due to the bi-phasic culture methods used to culture *Cyclotella* and *Nitzschia*, soil particles were often extracted along with the algal cells when preparing diets that included these two diatoms. Even though centrifuged cells were rinsed several times, soil particles could have retained more of the algal medium than expected. As judged by the volume of the culture chamber and the volume of algae administered, potassium levels could have reached 6.7 mg/l after 7 d and 9.6 mg/l after 10 d of administering the diet.

All individuals fed NOC/Sed were dead by 46 d, with replicates 1 and 2 dead by 24 d. A chemical analysis of the NOC diet also showed high potassium levels (89.2 mg/l). The water and sediment in each juvenile culture dish was usually changed every 7-10 d. The algae (NOC) were administered daily to the juvenile cultures; however, the water and sediment was not changed during the first 14 d. Thus, the level of potassium in the mussel culture dishes could have reached 21.4 mg/l after 14 d. The causes for high levels of potassium in the diet are possibly two-fold. First, *Oocystis marsonii* is characterized by thick cell walls with large cellulose microfibrils and embedded with hemicelluloses (Preston, 1974). As this was the only alga used in our studies that had such a thick wall, it is possible that more of the algal medium (and excess potassium) was retained by the cells than was desirable. Secondly, soil particles associated with the preparation of this diet also could have retained excessive amounts of the algal medium.

The addition of a cultured live bacterium, *Enterobacter aerogenes,* in combination with the algae *Neochloris* and *Phaeodactylum* and fine sediment, did not enhance growth over the trialgal diets NPB/Sed and NNiC/Sed. This was not surprising because bacteria are generally considered "poor food" for zooplankton due to a lack of PUFA (Wood, 1974). Crosby *et al.* (1990) reported that bacterial mediation enabled the eastern oyster, *Crassostrea virginica* (Gmelin, 1791), to make more efficient use of refractory detrital carbon and nitrogen. Thus, bacteria can break down previously unavailable food sources, but they are not a primary source of nutrition. Juveniles reared in fine sediment only, however, showed a 330% increase in shell length after 140 d. The success of a sediment only diet could be attributed to microbial and organic content because fine sediments contain an array of digestible material (Swain, 1970). Essential minerals, amino acids, and vitamins are also adsorbed onto soil particles creating a storehouse of nutrients (Kelley, 1942; Weiss, 1969; Swain, 1970; Baldwin and Newell, 1991).

Juveniles fed algae with kaolin exhibited growth similar to juveniles fed algae with sediment at 60 d. Thus, resident bacteria in sediments did not enhance growth and are likely not essential to digestion or superior in nutrition to algae. However, survival of juveniles fed algae with kaolin was very low. We attribute this mortality to the fine particle size (1-2 μm) of kaolin. The kaolin became paste-like over time, and we suspect this could have inhibited pedal-feeding or suffocated the juveniles. The fine sediment in our static chambers remained loose in composition and survival of juveniles was high. Yeager *et al.* (1994) observed suspension feeding by juvenile *Villosa iris* in the interstitial spaces of the sediment. More recently, research on the development of gills in juvenile mussels indicates that juveniles filter-feed in combination with pedal-feeding, until gill development is complete (R. Tankersley, pers. comm.). Particle size and composition of the substratum, therefore, seems important for effective pedal-feeding.

The Yeast/Sed diet did not support juveniles beyond 8 d. Preliminary trials using other commercial yeast-based diets also did not support juvenile mussels. These yeast-based diets were developed for mariculture of oysters, clams, and other invertebrates (Coutteau and Sorgeloos, 1992). Because juvenile mussels were short-lived in this treatment, when compared to the sediment only treatment, we believe that this diet could contain a toxic component that is otherwise not toxic to marine organisms. Perhaps the commercial yeast diet will be suitable if used in concentrations lower than that used in our experiments.

Although sediment provides minerals and some organic food material, an additional food source such as algae increased survival and growth. No specific algal diet produced statistically better survival over another. Thus, many algal diets administered with fine sediment could support juvenile mussels for at least several months. For long-term propagation, the more nutritious algae should produce the best growth and survival.

Information on the early life history requirements of freshwater mussels is minimal, with their nutritional requirements probably the least understood. Early investigators believed that "finding suitable nutrition for the first month or so of free life" was critical to the success of rearing mussels (Lefevre and Curtis, 1910, 1912; Coker *et al.*, 1921; Howard, 1922). This study has shown that the trialgal mixture of *Neochloris oleoabundans, Phaeodactylum tricornutum,* and *Bracteacoccus grandis* was a suitable diet for rearing juvenile mussels, and provided adequate nutrition for growth and survival through 140 d post-metamorphosis. The algae *Nitszchia acicularis,* and *Cyclotella meneghiniana* in combination with *N. oleoabundans* also was suitable for rearing juvenile mussels to 140 d post-metamorphosis. However, juveniles reared on *N. oleabundans, P. tricornutum,* and *B. grandis* showed the best growth over time. In addition, adult broodstock of various freshwater species have been maintained on *N. oleoabundans* to 10 mo at Virginia Tech's Aquaculture Center (F. O'Beirn, pers. comm.).

Research in aquaculture indicates that fish and marine bivalves have different requirements for protein, carbohydrates, and polyunsaturated fatty acids as they mature (Walne, 1973; Watanabe *et al.,* 1983; Hawkins and Bayne, 1991). It is likely, therefore, that certain algae might be more suitable to enhance growth in juvenile mussels, and other algae are more suitable for growth of adult mussels. The future success of propagating freshwater mussels will depend on a better understanding of the feeding ecology and environmental requirements of the juvenile life stage.

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