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EVALUATION OF POTENTIAL ANESTHETICS FOR THE FRESHWATER MUSSEL *ELLIPTIO COMPLANATA*

WILLIAM A. LELLIS,[1] TIMOTHY A. PLERHOPLES,[2] AND KIMBERLY A. LELLIS[3]
U.S. Geological Survey
Biological Resources Division
Northern Appalachian Research Laboratory
R.D. 4, Box 63
Wellsboro, Pennsylvania 16901

ABSTRACT A series of experiments was conducted to develop a safe, rapid, and reliable method to relax and anesthetize freshwater mussels for collection of biological samples and assessment of reproductive status. Various concentrations and combinations of eight potential anesthetic agents were administered by bath, slow drip, or injection into the foot or incurrent aperture of 10–40 *Elliptio complanata* per treatment group. Mussels were considered relaxed when the foot extended 2 cm beyond the shell and anesthetized when the valves gaped, and the animal became impervious to touch. Buffered MS-222 (pH 7) produced relaxation within 60 min at concentrations greater than 50 ppm and anesthesia within 3 h at 500–1,000 ppm. Mussels exposed to unbuffered MS-222 at concentrations greater than 250 ppm (pH 3–4) ceased siphoning and closed tightly. Phenoxyethanol at 1.5–3.0% produced anesthesia within 20–50 min but had associated mortality. The effective dose of phenoxyethanol could be reduced to 0.25% with no mortality if mussels were first relaxed with MS-222. Injection of 0.5–5.0 mg succinylcholine chloride into the foot produced rapid immobilization that lasted 20–30 min. Dichlorvos at concentrations of 25–50 ppm induced anesthesia in 3–5 h, but mussels were extremely slow to recover. Clove oil at 0.25–1.00 mL/L anesthetized 65–95% of the mussels tested but proved difficult to work with in confined spaces. Magnesium chloride, potassium chloride, and menthol crystals had no apparent affect on *Elliptio complanata*. This study identified several anesthetic agents for freshwater mussels, each differing in induction time, duration of effect, and degree of foot relaxation. We recommend 500 ppm buffered MS-222 for general laboratory use on *Elliptio complanata* because of ease of handling and safety for both humans and animals.

KEY WORDS: freshwater mussels, *Elliptio complanata*, anesthetics, relaxants

INTRODUCTION

The introduction and rapid spread of the zebra mussel *Dreissena polymorpha* throughout North America has lead to extensive mortality among native unionid mussels in many freshwater systems (Schloesser et al. 1996). Death of the mussels is believed to be caused by either colonization of exposed valves by *Dreissena*, habitat alteration, or depletion of food resources from the water column (Strayer and Smith 1996). No effective means of control or eradication for zebra mussels has yet been devised. One conservation strategy being considered to preserve populations of the most threatened native species is to remove them from *Dreissena*-infested waters physically and place them into uninfected refugia (Cope and Waller 1995). Monitoring of the chosen refugia for adequacy in maintaining health, metabolic condition, and reproduction of the relocated mussels often requires visual examination of mussel soft body parts and collection of tissue and physiological fluids. Samples are usually obtained by mechanically prying the valves apart with a reversing plier, which can fracture the shell, damage the mantle, exhaust the mussel, and tear the adductor. The additional stress of sample collection potentially could reduce the mussels' tolerance to the refugia environment.

An alternate means of obtaining tissue samples is to relax or anesthetize the mussels with a chemical agent. This has been accomplished with such marine bivalves as oysters (Namba et al. 1995, Norton et al. 1996), scallops (Heasman et al. 1995), and giant clams (Rosewater 1963) using benzocaine, chloral hydrate, clove oil, magnesium chloride, menthol, MS-222, 2-phenoxyethanol, propylene phenoxetol, and sodium pentobarbitone. Other mollusks, including sea hares (Beeman 1968), land snails (Chung 1985), pond snails (Girdlestone et al. 1989), and abalone (White et al. 1996) have been successfully relaxed using enflurane, halothane, isoflurane, magnesium sulfate, and succinylcholine chloride. Although not intended as an anesthetic agent, the organophosphate dichlorvos, used for treatment of sea lice in Atlantic salmon net pens, has been reported to relax the adductor muscle of marine bivalves for as long as 42 h after the end of exposure (Le Bris et al. 1995).

Studies with freshwater unionids indicate that combinations of pentobarbitol, urethane, clove oil, MS-222, nembutal, and menthol can induce muscle relaxation or general anesthesia (Araujo et al. 1995, Coney 1993, Smith 1996). These techniques, however, were developed to relax mussels into a lifelike position before death for subsequent dissection or fixation, and thus recovery potential was not considered. The objective of our study was to identify a method to anesthetize freshwater mussels in a relaxed position (i.e., foot extended, shell gaped, unresponsive to touch) in a minimum of time (less than 4 h) using agents that were safe to handle, obtainable without a permit, inexpensive, and provided full and unharmed recovery of the subject animal.

MATERIALS AND METHODS

Twenty-nine separate trials were conducted between July 1995 and June 1997 to evaluate the potential of eight chemical agents to produce nonlethal anesthesia in the freshwater mussel *Elliptio complanata* (Lightfoot 1786). Mature mussels ranging in size from

[1]Corresponding author. E-mail: lelliswm@usgs.gov
[2]Current address: 234 Delevan Avenue, Corning, NY 14830.
[3]Current address: Box 1381, Gettysburg College, Gettysburg, PA 17325.

63 to 115 mm and 26 to 165 g (mean 95 mm, 100 g) were collected from Pine Creek, Tioga County, Pennsylvania (41°44.408′ N, 077°25.777′ W) and transported in coolers without water to the U.S. Geological Survey (USGS) Northern Appalachian Research Laboratory in Wellsboro, Pennsylvania for experimentation. At the laboratory, mussels were cleaned and measured then sorted into groups of 10 mussels of equal total mass. Each group of 10 mussels was then randomly allocated into a 132-L glass culture aquarium containing 8 cm of white sand substrate and assigned a treatment. The aquaria were each supplied with 1 L/min of 15–17 °C well water and illuminated with overhead fluorescent lamps set to 14 h light:10 h dark photoperiod. Water was circulated within each aquarium by aeration from a 29-cm air diffuser set at the drain end of the tank. Mussels were fed twice daily a mixture of cultured *Nanochloropsis* sp. (Florida Aqua Farms, Inc., Dade City, Florida) and benthic detritus vacuumed from a concrete fish pond. Tanks were cleaned weekly by scrubbing glass, stirring sand, and draining.

Chemicals tested included MS-222 (Finquel; tricaine methanesulfonate, Argent Chemical Laboratories, Redmond, Washington) with or without Tris buffer (Sigma 7–9; Sigma Chemical Company, St. Louis, Missouri), magnesium chloride ($MgCl_2$, Fisher Scientific Company, Fair Lawn, New Jersey), potassium chloride (KCl, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin), succinylcholine chloride, 2-phenoxyethanol, clove oil, menthol (Sigma), and dichlorvos (DDVP; *O,O*-dimethyl-2,2-dichlorovinyl phosphate, AMVAC Chemical Corporation, Los Angeles, California). MS-222, $MgCl_2$, and KCl were dissolved in well water before addition to test tanks. Succinylcholine chloride was dissolved in distilled water then either added directly to the test tanks or injected into the mussel using a 26-G 12.7-mm hypodermic needle. Clove oil and 2-phenoxyethanol were shaken vigorously with 250 mL well water before addition to the tanks. Menthol crystals were powdered and mixed in 100 mL well water before application. Dichlorvos was dissolved in distilled water to form a 6 mg/mL stock solution, and the appropriate volume was added to the tanks.

Tests were conducted by transferring mussels from their culture aquaria into separate treatment aquaria (no substrate) containing 2–30 L well water at the same temperature as the culture tanks. Mussels were arranged within each treatment aquarium in two rows of five and numbered 1–10 according to position. Water was circulated within each aquarium by aeration from a 29-cm air diffuser set in the center of the tank. After a 60 min acclimation, treatments were administered to each aquarium as either a bath (entire dosage applied at one time), by slow drip over a predetermined period, or by injection. Drip treatments were administered from a 1,000-mL separation flask suspended over each tank. Number of replicate tanks of 10 mussels varied from 1–4, depending upon treatment (Tables 1–3). Time to relaxation and/or anesthesia was recorded for each mussel from the moment of treatment application, or from the start of application in the case of drip delivery. Some treatments included a pre-application of unbuffered MS-222 before the primary chemical in an attempt to improve the effectiveness or lower the required dosage of the primary chemical agent. In these cases, time to relaxation was recorded from the MS-222 pretreatment; whereas, time to anesthesia was recorded from application of the primary treatment. A mussel was considered relaxed when the foot extended 2 cm beyond the valve and anesthetized when the valves gaped, and the animal became impervious to touch. These two events are independent phenomena, with the goal being to identify a treatment that produced an anesthetized animal in a relaxed state.

Mussels were removed from treatment tanks either immediately upon detection of anesthesia or after a predetermined period of 1–6 h. Mussels were rinsed in clean water, returned to culture tanks, and arranged in the same order as when in the treatment tank.

Recovery time, defined as the ability to maintain valve closure, was individually recorded from moment of removal from the treatment tank. Mussels were checked 24 h post-treatment for activity (movement or burrowing) and at 7 days for mortality. Mussels subjected to 2-phenoxyethanol, clove oil, menthol, and dichlorvos were also checked for activity 7 days post-treatment (Table 3). Data were analyzed using the general linear models procedure of the Statistical Analysis System (SAS 1988). Any variable expressed as a percentage was arcsine transformed before analysis (Rohlf and Sokal 1981). Differences in treatment means were detected using the Waller/Duncan multiple range test. Orthogonal polynomials were used to make linear, quadratic, and cubic contrasts among treatment effects in the analysis of variance (Rohlf and Sokal 1981).

RESULTS

Bath solutions of 50–250 ppm unbuffered MS-222 produced relaxation in 50–85% of test *Elliptio complanata* within 39–54 min of treatment application (Table 1). Mussels exposed to MS-222 concentrations below 50 ppm siphoned normally but did not respond to treatment; whereas, those exposed to concentrations above 250 ppm stopped siphoning and closed valves tightly within 30 min of treatment application. Water pH decreased within the treatment tanks from pH 7.0 at 50 ppm MS-222 to pH 3.4 at 1,000 ppm MS-222. No bath treatments of unbuffered MS-222 produced anesthesia within the 4-h trial period. Activity at 24 h varied greatly within each group of mussels unrelated to specific treatment. There were no mortalities among the 320 mussels exposed to unbuffered MS-222 bath.

Dripping unbuffered MS-222 to final concentrations of 25–1,000 ppm over 20–120 min produced relaxation in 0–100% of the test animals in times ranging from 24–85 min, depending upon treatment combination (Table 1). As with the bath treatments, MS-222 drip did not produce anesthesia at any concentration tested within the time allowed, and 24-h activity varied greatly among groups. Most mussels exposed to MS-222 concentrations above 250 ppm were tightly closed by the end of the drip period. One mortality occurred among the 320 mussels subjected to unbuffered MS-222 drip, that being at 750 ppm. Injection of 1.2 cc of 1,000 ppm unbuffered MS-222 into the incurrent aperture after 60 min of 100 ppm MS-222 bath produced relaxation similar to bath and drip techniques, but no anesthesia.

MS-222 buffered to pH 7.0 with Tris produced relaxation in 65–95% of the mussels tested within 31–66 min of exposure to concentrations of 50–1,000 ppm (Table 1). Unlike unbuffered MS-222, mussels exposed to buffered MS-222 at concentrations above 250 ppm continued siphoning normally and reached a state of relaxed anesthesia within 126–194 min at concentrations between 500–1,000 ppm (Table 1, Figure 1). Time to anesthesia decreased ($P < 0.01$), and time to recovery increased ($P < 0.01$) with increasing dosage above 500 ppm. Activity at 24 h was lower ($P = 0.03$) among anesthetized mussels than those that were treated but did not reach anesthesia. There were no mortalities among the 260 mussels exposed to buffered MS-222.

TABLE 1.

Summary of trials conducted to evaluate MS-222 as a potential anesthetic for *Elliptio complanata*.

Treatment	Level	Application Method	Mussels #	Mussels Relaxed (%)	Time to Relax (min)	Mussels Anesthetized (%)	Time to Anesthesia (min)	Time to Recover (min)	Active at 24 h (%)	Mortality at 7 days (%)	Notes
MS-222	0 ppm	Bath	20	5	120	0	———	———	40	0	pH 7.1; Trial maxima 4 h
MS-222	5 ppm	Bath	20	15	42	0	———	———	90	0	pH 7.1
MS-222	10 ppm	Bath	20	0	———	0	———	———	75	0	pH 7.1
MS-222	15 ppm	Bath	20	0	———	0	———	———	15	0	pH 7.1
MS-222	20 ppm	Bath	20	0	———	0	———	———	45	0	pH 7.0
MS-222	50 ppm	Bath	40	50	40	0	———	———	32	0	pH 7.0
MS-222	75 ppm	Bath	20	85	54	0	———	———	10	0	pH 6.7
MS-222	100 ppm	Bath	40	85	44	0	———	———	32	0	pH 6.6
MS-222	250 ppm	Bath	40	82	39	0	———	———	48	0	pH 4.5
MS-222	500 ppm	Bath	40	2	205	0	———	———	70	0	pH 3.8
MS-222	750 ppm	Bath	20	0	———	0	———	———	35	0	pH 3.6
MS-222	1,000 ppm	Bath	20	0	———	0	———	———	30	0	pH 3.4
MS-222	0 ppm	Drip—20 min	20	45	112	0	———	———	75	0	Trial maxima 4 h
MS-222	25 ppm	Drip—20 min	20	45	85	0	———	———	90	0	———
MS-222	50 ppm	Drip—20 min	20	85	62	0	———	———	55	0	———
MS-222	75 ppm	Drip—20 min	20	80	46	0	———	———	70	0	———
MS-222	100 ppm	Drip—120 min	20	85	26	0	———	———	50	0	———
MS-222	250 ppm	Drip—30 min	20	30	25	0	———	———	———	0	———
MS-222	400 ppm	Drip—120 min	20	100	30	0	———	———	100	0	———
MS-222	500 ppm	Drip—30 min	20	30	26	0	———	———	———	0	———
MS-222	700 ppm	Drip—120 min	20	0	———	0	———	———	100	0	———
MS-222	750 ppm	Drip—30 min	20	35	24	0	———	———	———	0	———
MS-222	750 ppm	Drip—45 min	20	70	26	0	———	———	30	5	———
MS-222	750 ppm	Drip—60 min	20	90	36	0	———	———	80	0	———
MS-222	750 ppm	Drip—90 min	20	70	42	0	———	———	40	0	———
MS-222	750 ppm	Drip—120 min	20	95	56	0	———	———	60	0	———
MS-222	1,000 ppm	Drip—30 min	20	55	24	0	———	———	———	0	———
MS-222	1,000 ppm	Drip—120 min	20	65	28	0	———	———	100	0	———
MS-222/MS-222	100/1,000 ppm	Bath/inject	20	75	39	0	———	———	40	0	Inject 1.2 cc of 1,000 ppm MS-222 into aperture after 60-min bath
MS-222 Buffered	0 ppm	Bath	20	5	19	0	———	———	70	0	Trial maxima 5 h
MS-222 Buffered	50 ppm	Bath	20	65	50	0	———	———	30	0	All treatments buffered to pH 7.0 with Tris
MS-222 Buffered	75 ppm	Bath	20	70	44	0	———	———	25	0	———
MS-222 Buffered	100 ppm	Bath	40	85	43	0	———	———	30	0	———
MS-222 Buffered	250 ppm	Bath	40	95	31	0	———	———	8	0	———
MS-222 Buffered	500 ppm	Bath	40	80	58	70	194	22	18	0	———
MS-222 Buffered	750 ppm	Bath	40	85	66	85	149	35	5	0	———
MS-222 Buffered	1,000 ppm	Bath	40	88	50	85	126	49	10	0	———

Bath and drip solutions of 10–40 g/L $MgCl_2$ produced some relaxation but no appreciable anesthesia in *Elliptio complanata* (Table 2). Mortality occurred at the higher $MgCl_2$ doses. Mussels subjected to $MgCl_2$ generally closed very tightly within 60 min of exposure and produced copious mucus discharge. Relaxation of the mussels with 100 ppm MS-222 before $MgCl_2$ drip did not help to induce anesthesia. Injection of 30–60 mg of $MgCl_2$ into the incurrent aperture produced an anesthetic state in 10–20% of the mussels, but with 10% associated mortality. The anesthetized mussels, however, were not the same individuals that subsequently died.

Bath solutions of 10–40 g/L KCl had no relaxing or anesthetic effects on *Elliptio complanata* (Table 2). Mussels stopped siphoning upon exposure to KCl and remained tightly closed throughout the 2-h trial. Mortality (5–20%) occurred at all levels of KCl above 10 g/L.

Bath solutions of 250–1,000 ppm succinylcholine chloride had little effect on *Elliptio complanata* (Table 2). Mussels exposed to bath concentrations above 500 ppm became sluggish in response to touch, but only one mussel at 750 ppm reached an anesthetic state. One mussel exposed to 1,000 ppm died within 7 days of treatment. Injection of 0.5–5.0 mg succinylcholine chloride into the foot produced anesthesia in 100% of the mussels within 4–5 min of injection, with recovery in 23–30 min. Mussels gaped and were unresponsive to touch after injection, but feet remained in a constricted unrelaxed state. Mussels injected with 0.5 mg showed some sensitivity to stimulation, but could not sustain valve closure. Activity at 24 h decreased ($P = 0.02$) with increasing dosage. There were no mortalities among the 160 mussels injected with succinylcholine chloride.

Bath solutions of 0.25–3.0% 2-phenoxyethanol induced anesthesia but no foot relaxation (Table 3). Percentage of mussels

TABLE 2.

Summary of trials conducted to evaluate $MgCl_2$, KCl, and succinylcholine-Cl as potential anesthetics for *Elliptio complanata*.

Treatment	Level	Application Method	Mussels #	Mussels Relaxed (%)	Time to Relax (min)	Mussels Anesthetized (%)	Time to Anesthesia (min)	Time to Recover (min)	Active at 24 h (%)	Mortality at 7 days (%)	Notes
$MgCl_2$	0 g/L	Bath	20	10	91	0	——	——	40	0	Trial maxima 2 h
$MgCl_2$	10 g/L	Bath	20	30	11	0	——	——	45	0	——
$MgCl_2$	20 g/L	Bath	20	20	6	0	——	——	25	0	——
$MgCl_2$	30 g/L	Bath	20	0	——	10	29	16	30	5	——
$MgCl_2$	10 g/L	Drip—30 min	10	0	——	0	——	——	60	0	Trial maxima 2 h
$MgCl_2$	20 g/L	Drip—30 min	10	20	14	0	——	——	70	0	——
$MgCl_2$	30 g/L	Drip—30 min	10	20	9	0	——	——	80	0	——
$MgCl_2$	40 g/L	Drip—30 min	10	50	14	0	——	——	30	10	——
MS-222/$MgCl_2$	100 ppm/0 g/L	Bath/drip—90 min	10	80	44	0	——	——	40	0	Trial maxima 4 h
MS-222/$MgCl_2$	100 ppm/10 g/L	Bath/drip—90 min	10	90	37	0	——	——	30	0	MS-222 bath for 60 min before $MgCl_2$ drip
MS-222/$MgCl_2$	100 ppm/20 g/L	Bath/drip—90 min	10	90	37	0	——	——	50	0	——
MS-222/$MgCl_2$	100 ppm/30 g/L	Bath/drip—90 min	10	90	40	0	——	——	10	0	——
MS-222/$MgCl_2$	100 ppm/30 mg	Bath/inject	10	80	61	10	37	15	0	10	Inject 2 cc of 15 g/L $MgCl_2$ into aperture after 90-min MS-222 bath
MS-222/$MgCl_2$	100 ppm/60 mg	Bath/inject	10	50	43	20	48	19	0	10	Inject 2 cc of 30 g/L $MgCl_2$ into aperture after 90-min MS-222 bath
KCl	10 g/L	Bath	20	0	——	0	——	——	55	0	Trial maxima 2 h
KCl	20 g/L	Bath	20	0	——	0	——	——	45	5	——
KCl	30 g/L	Bath	20	0	——	0	——	——	30	5	——
KCl	40 g/L	Bath	20	0	——	0	——	——	35	20	——
Succinylcholine-Cl	250 ppm	Bath	20	5	60	0	——	——	35	0	Trial maxima 2 h
Succinylcholine-Cl	500 ppm	Bath	20	5	60	0	——	——	55	0	——
Succinylcholine-Cl	750 ppm	Bath	20	15	65	5	25	25	55	0	——
Succinylcholine-Cl	1,000 ppm	Bath	20	15	36	0	——	——	45	5	——
MS-222/Succinylcholine-Cl	100 ppm/0.5 mg	Bath/inject	40	55	44	100	4	23	40	0	Inject 0.05 cc into foot after 60-min MS-222 bath
MS-222/Succinylcholine-Cl	100 ppm/1.0 mg	Bath/inject	40	60	54	100	5	27	15	0	——
MS-222/Succinylcholine-Cl	100 ppm/2.5 mg	Bath/inject	40	75	40	100	4	30	2	0	——
MS-222/Succinylcholine-Cl	100 ppm/5.0 mg	Bath/inject	40	65	56	100	4	24	5	0	——

TABLE 3.

Summary of trials conducted to evaluate 2-phenoxyethanol, dichlorvos, menthol, and clove oil as potential anesthetics for *Elliptio complanata*.

Treatment	Level	Application Method	Mussels #	Mussels Relaxed (%)	Time to Relax (min)	Mussels Anesthetized (%)	Time to Anesthesia (min)	Time to Recover (min)	Active at 24 h (%)	Active at 7 days (%)	Mortality at 7 days (%)	Notes
2-Phenoxyethanol	0.25%	Bath	20	10	50	5	88	57	70	——	0	Trial maxima 5 h
2-Phenoxyethanol	0.50%	Bath	20	10	98	25	62	60	50	——	0	——
2-Phenoxyethanol	0.75%	Bath	20	0	——	20	151	28	95	——	0	——
2-Phenoxyethanol	1.00%	Bath	20	5	30	25	104	34	65	——	0	——
2-Phenoxyethanol	1.50%	Bath	20	0	——	60	47	36	40	——	0	——
2-Phenoxyethanol	2.00%	Bath	20	0	——	70	17	64	60	——	5	——
2-Phenoxyethanol	2.50%	Bath	20	0	——	70	32	49	25	——	5	——
2-Phenoxyethanol	3.00%	Bath	20	0	——	75	36	53	20	——	5	——
MS-222/2-Phenoxyethanol	100 ppm/0.25%	Bath/bath	10	80	51	80	43	31	40	100	0	Trial maxima 3 h
MS-222/2-Phenoxyethanol	100 ppm/0.50%	Bath/bath	10	80	47	100	23	48	0	30	0	Add phenoxyethanol after 60-min MS-222 bath
MS-222/2-Phenoxyethanol	100 ppm/0.75%	Bath/bath	10	60	40	100	14	34	10	40	0	——
MS-222/2-Phenoxyethanol	100 ppm/1.00%	Bath/bath	10	90	42	100	18	36	0	30	20	——
MS-222/2-Phenoxyethanol	100 ppm/0.25%	Bath/drip—35 min	20	70	24	35	74	28	35	——	0	Trial maxima 4 h
MS-222/2-Phenoxyethanol	100 ppm/0.50%	Bath/drip—35 min	20	75	28	70	48	22	30	——	0	Drip phenoxyethanol after 60-min MS-222 bath
MS-222/2-Phenoxyethanol	100 ppm/0.75%	Bath/drip—35 min	20	80	28	75	50	28	25	——	0	——
MS-222/2-Phenoxyethanol	100 ppm/1.00%	Bath/drip—35 min	20	60	32	85	40	30	15	——	10	——
Clove oil	0.25 mL/L	Bath	20	10	58	65	79	46	15	60	0	Trial maxima 2 h
Clove oil	0.50 mL/L	Bath	20	15	42	70	68	76	65	75	0	——
Clove oil	0.75 mL/L	Bath	20	0	——	70	56	65	20	95	0	——
Clove oil	1.00 mL/L	Bath	20	25	26	95	57	100	40	80	0	——
MS-222/Clove oil	100 ppm/0.125 mL/L	Bath/bath	20	55	64	55	90	37	15	80	0	Trial maxima 3 h
MS-222/Clove oil	100 ppm/0.250 mL/L	Bath/bath	20	70	62	70	74	46	15	80	0	Add clove oil after 60-min MS-222 bath
MS-222/Clove oil	100 ppm/0.375 mL/L	Bath/bath	20	50	42	85	51	56	15	80	0	——
MS-222/Clove oil	100 ppm/0.500 mL/L	Bath/bath	20	70	56	90	38	59	30	75	0	——
Menthol crystal	125 mg/L	Bath	20	0	——	0	——	——	45	75	0	Trial maxima 3 h
Menthol crystal	250 mg/L	Bath	20	0	——	0	——	——	35	85	0	——
Menthol crystal	375 mg/L	Bath	20	0	——	0	——	——	45	90	0	——
Menthol crystal	500 mg/L	Bath	20	0	——	0	——	——	35	80	0	——
Dichlorvos	0.1 ppm	Bath	20	10	34	0	——	——	65	80	0	Trial maxima 6 h
Dichlorvos	1 ppm	Bath	20	0	——	0	——	——	55	85	0	——
Dichlorvos	5 ppm	Bath	20	10	180	0	——	——	45	55	0	——
Dichlorvos	10 ppm	Bath	20	35	170	0	——	——	40	55	0	——
Dichlorvos	25 ppm	Bath	20	25	184	80	267	24–36 h	0	15	0	——
Dichlorvos	50 ppm	Bath	20	35	108	100	196	24–36 h	0	35	0	——

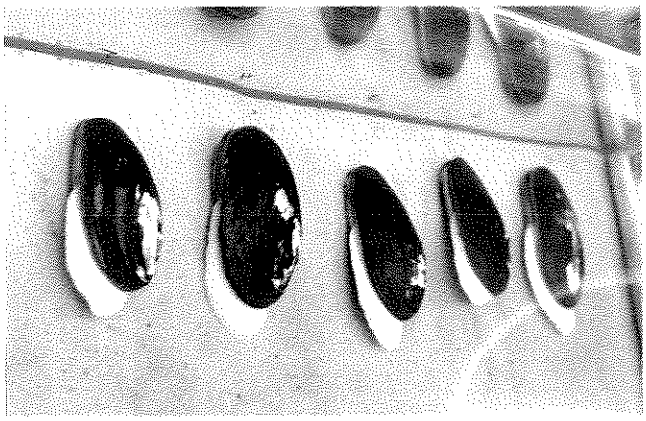

Figure 1. ***Elliptio complanata*** **relaxed and anesthetized with 750 ppm buffered MS-222.**

anesthetized increased ($P = 0.003$) in a nonlinear fashion (cubic response $P = 0.005$) with increasing dosage to an apparent maximum of about 70% anesthesia at 2.0% 2-phenoxyethanol. Mussels exposed to 2-phenoxyethanol solutions of 1.0% or less continued siphoning throughout the 5-h trial if they did not reach anesthesia; whereas, mussels exposed to concentrations greater than 1.0% closed tightly and ceased siphoning if not anesthetized. Time to anesthesia decreased ($P = 0.005$) with increasing dosage in a nonlinear fashion (cubic response $P = 0.001$) to an apparent minimum at approximately 1.50% 2-phenoxyethanol. Dosage level did not affect time to recovery ($P = 0.30$) nor 24-h activity ($P = 0.16$). Mortality (5% of mussels tested) occurred at 2-phenoxyethanol doses greater than 1.50%. Mussels exposed to 3.0% 2-phenoxyethanol produced copious mucus discharge after being returned to fresh water.

Exposure of mussels to a 60-min bath of 100 ppm MS-222 before 2-phenoxyethanol treatment increased ($P < 0.0001$) anesthetic rate among mussels subjected to low concentrations of 2-phenoxyethanol (Table 3). Time to anesthesia ($P = 0.02$) and time to recovery ($P = 0.04$) were also decreased among these treatment groups by preconditioning with MS-222. However, MS-222 also decreased the dosage level of 2-phenoxyethanol at which mortality occurred to about 1.0%. Bath application of 2-phenoxyethanol was more effective than drip application in inducing anesthesia. Although anesthetized mussels generally constricted their feet while actively exposed to 2-phenoxyethanol, feet became relaxed and extended within a few minutes of return to fresh water.

Bath solutions of 0.25–1.00 mL/L clove oil induced anesthesia in 65–95% of *Elliptio complanata* tested without significant foot relaxation (Table 3). Although anesthetic rate was similar among treatment levels ($P = 0.20$), time to anesthesia decreased (quadratic response $P = 0.006$), and time to recovery increased (linear response $P = 0.01$) with increasing dosage level. Similar to 2-phenoxyethanol, mussels became anesthetized in a constricted position, but relaxed and extended feet upon transfer to fresh water. Exposure of mussels to 100 ppm MS-222 for 60 min before clove oil addition did not increase anesthetic rate at 0.25 mL/L or 0.50 mL/L dosage levels ($P = 0.29$), but did reduce time to anesthesia ($P = 0.03$). There were no mortalities among the 160 mussels exposed to clove oil during these trials.

Powdered menthol crystal had no anesthetic or relaxing effects on *Elliptio complanata* at 125–500 mg/L dosage levels (Table 3). Mussels ceased siphoning upon initial exposure to menthol crystal and remained tightly closed for the duration of the 3-h trial. There were no mortalities associated with exposure to menthol in this trial.

Bath solutions of 0.1–10 ppm dichlorvos had no affect on *Elliptio complanata* within a 6-h exposure period (Table 3). Concentrations of 25–50 ppm produced anesthesia in 80–100% of the mussels tested in an average of 196–267 min. Feet were retracted during anesthesia. Recovery time was prolonged as compared to other treatments, requiring 24–36 h to regain full responsiveness to touch. Mussels anesthetized by dichlorvos were less active ($P = 0.01$) at 7 days post-treatment than mussels that had been exposed to dichlorvos but not anesthetized. Most anesthetized mussels required in excess of 2 weeks to upright themselves and resume burrowing activity. No mortalities occurred among the 120 mussels exposed to dichlorvos during this study.

DISCUSSION

The goal of this study was to identify a nondestructive method to anesthetize unionid bivalves in a relaxed position to allow collection of biological samples and examination of internal anatomy. Relaxation was defined as extension of the foot at least 2 cm beyond the valves and anesthesia as the gaping of valves with unresponsiveness to touch. These two events are separate and unconnected, because a relaxed mussel may be fully responsive to touch; whereas, an anesthetized mussel may be in a constricted position. Because foot extension also occurs with burrowing activity, some control groups were recorded as having relaxation even though no chemical treatment was applied (e.g., MS-222 0 ppm, Table 1). However, foot extension associated with burrowing can often be distinguished from chemical relaxation, because burrowing was a more active process and typically took longer to initiate, usually in excess of 100 min. An exception occurred within the buffered MS-222 control group in which a single animal extended its foot after 19 min. The term "anesthesia" often implies the loss of consciousness or sensitivity, but gaping and lack of response can also be attributable to muscular paralysis. No distinction was made between these two causes of gaping in this study.

Using these definitions, *Elliptio complanata* were relaxed by MS-222 and anesthetized by MS-222, succinylcholine chloride, 2-phenoxyethanol, clove oil, and dichlorvos. MgC_2, KCl, and menthol crystal had no appreciable effect on the mussels. Pretreatment with MS-222 before addition of other primary chemical agents often decreased time to anesthesia and/or dosage requirements for the primary anesthetic and placed the mussels in a relaxed position during anesthesia.

MS-222 (tricaine methanesulfonate) is the most commonly used anesthetic for finfish in North America (Summerfelt and Smith 1990). Mechanism of action is presumed to be through stabilization of cellular membranes in nervous and cardiac tissue, preventing transient increases in sodium permeability and thus decreasing excitability (Letcher 1992). Dosage of 60–250 ppm produces anesthesia in Atlantic halibut, red drum, and goldfish in 3–6 min with recovery in less than 10 min (Malmstrøm et al. 1993, Massee et al. 1995). MS-222 has been used less frequently to anesthetize bivalves. Coney (1993) used 75–100 ppm for 12–36 h, and Araujo et al. (1995) used 500–2,000 ppm for 24 h to relax unionids before lethal fixation. Norton et al. (1996) relaxed the pearl oyster *Pinctada albina* with 1,000 ppm MS-222, but recommended buffering to pH 8 to prevent excess mucus production and

reduce recovery time. Heasman et al. (1995) could not anesthetize the scallop *Pecten fumatus* with 1,000 ppm MS-222 within 60-min exposure.

In this study, buffered MS-222 at concentrations greater than 100 ppm produced foot relaxation in 85% of *Elliptio complanata* within 30–60 min, and concentrations above 500 ppm produced anesthesia within 2–3 h. Mussels further relaxed after transfer to fresh water, but recovered within 30–60 min. Unbuffered MS-222 produced foot relaxation but not anesthesia, presumably because of low pH of the higher dosages. The inability of unbuffered MS-222 to produce anesthesia may not be completely attributed to cessation of siphoning activity, inasmuch as direct injection of 1,000 ppm unbuffered MS-222 into the incurrent aperture also failed to anesthetize the animals (Table 1). Although this may simply indicate that the volume of injected MS-222 was insufficient, it may also indicate that low pH causes physiologic changes in *Elliptio complanata* or chemical changes in MS-222 that affect absorption or metabolism of the compound.

Magnesium chloride solutions of 30–50 g/L have been used to induce valve gaping in such marine bivalves as the Pacific oyster *Crassostrea gigas* (Whyte and Carswell 1983), the European flat oyster *Ostrea edulis* (Culloty and Mulcahy 1992), and the scallop *Pecten fumatus* (Heasman et al. 1995), but not the pearl oyster *Pinctada albina* (Norton et al. 1996). Mechanism of action was considered to be inhibition of muscular contraction attributable to displacement of calcium ions from tissue by magnesium (Whyte and Carswell 1983). In this study, the freshwater mussel *Elliptio complanata* showed some signs of foot relaxation at $MgCl_2$ concentrations exceeding 30 g/L, but remained responsive throughout the 2–4-h trials.

Beeman (1968) reported that injections of 0.05 mg/g of the myoneural blocking agent succinylcholine chloride (ester dimethochloride) produced rapid relaxation of the sea hare *Aplysia californica* with recovery within 45–90 min. The drug was dissolved in seawater and pH of the solution adjusted to 6.4–7.0 with HCl to prevent alkaline hydrolysis of the ester linkage. Chung (1985) found that injections of 0.012 mg/g succinylcholine chloride combined with 2.4 mg/g $MgCl_2$ produced quick and pronounced anesthesia in the land snail *Helix aspersa* with no mortality. In the present study, succinylcholine chloride injections of 0.5–5.0 mg/mussel produced rapid anesthesia (< 5 min) of *Elliptio complanata* with recovery within 30 min. Inasmuch as average live animal mass was 100 g, with about 20% of that being soft tissue, injected dosage was approximately 0.005–0.05 mg/g whole body and 0.025–0.25 mg/g soft tissue weight. All succinylcholine chloride treatments had equal affect on the mussels, except that 24-h activity was depressed at the higher levels. Therefore, smaller dosages than those used in this study would probably be effective in anesthetizing *Elliptio complanata*. MS-222 pretreatment was used to allow easier access to the injection site, and its affects on anesthetic rate are believed to have been minimal.

Propylene phenoxetol and a related compound, 2-phenoxyethanol, have been used to anesthetize a variety of gastropods and bivalves, such as the giant clam *Tridacna maxima* (Rosewater 1963), the abalone *Haliotis midae* (White et al. 1996), and the pearl oyster *Pinctada albina* (Norton et al. 1996). Effective dose of 2-phenoxyethanol was 0.3–0.4% for these species. Heasman et al. (1995) could not relax the scallop *Pecten fumatus* with 0.06% 2-phenoxyethanol and Araujo et al. (1995) could not relax unionid mussels with 1.0% 2-phenoxyethanol solutions. In the present study, *Elliptio complanata* were anesthetized in less than 30 min using 2.0% 2-phenoxyethanol with recovery in about 1 h. This dosage could be reduced to 0.5% or less when combined with an MS-222 pretreatment. Although the sticky, adhesive consistency and noxious fumes of 2-phenoxyethanol made the substance difficult to handle, it produced quicker and deeper anesthesia in *Elliptio complanata* than MS-222 treatment alone. However, exposure to humans may cause irritation to sensitive tissue and damage to kidney and liver, and Summerfelt and Smith (1990) recommend discontinued use as a fish anesthetic because of inherent toxic effects.

Clove oil has been used as a fish anesthetic in Southeast Asia, because it is inexpensive, readily available, and simple to apply. Soto and Burhanuddin (1995) reported that rabbitfish *Siganus lineatus* lost consciousness within 3 min of exposure to 0.1 mL/L clove oil and recovered within 3 min of transfer to fresh water. Araujo et al. (1995) found clove oil to be an effective anesthetic for the freshwater mussel *Unio sp.* and the clam *Pisidium amnicum*, but not for the Asian clam *Corbicula fluminea*. Norton et al. (1996) relaxed the pearl oyster *Pinctada albina* with 1.5 mL/L clove oil. In the present study, *Elliptio complanata* reached anesthesia within 90 min of exposure to 0.125–1.0 mL/L. Lack of dose response in percentage mussels anesthetized may indicate that lower doses of clove oil can be used for relaxing *Elliptio complanata* than were applied in this study. However, clove oil fumes were found to be particularly irritating to the eyes and respiratory tract, and experimentation was discontinued. Use of this substance may be limited to outdoors or to indoors within ventilated hoods.

Menthol has been used as a general anesthetic for invertebrates, because it is readily available, inexpensive, easily handled, and gives acceptable results over a wide range of species (Araujo et al. 1995). Smith (1996) successfully narcotized freshwater mussels by subjecting them to powdered menthol solutions for 24 h. Response, however, is often unpredictable, and neither Coney (1993) nor Araujo et al. (1995) had success using menthol to anesthetize freshwater unionids. Norton et al. (1996) used 250 mg/L menthol crystal to relax the pearl oyster *Pinctada albania*, but in the present experiment *Elliptio complanata* were unaffected by 125–500 mg/L during 3-h trials. Success with menthol may be related to water temperature, considering that Runham et al. (1965) reported improved results by transferring animals to hot water.

Dichlorvos is an organophosphate that affects the nervous system of animals by inhibiting function of the enzyme acetylcholinesterase (Murison et al. 1997). This results in elevated levels of the neurotransmitter acetylcholine, leading to exhaustion and possibly death by continuous neuromuscular stimulation. Bath treatments of 1.0 ppm are used in commercial salmon farms to kill such ectoparasitic crustaceans as sea lice (MacKinnon 1997). Le Bris et al. (1995) found that dichlorvos concentrations of 0.1–1.0 ppm caused adductor muscle relaxation in Manila clams (*Ruditapes philippinarum*) and Japanese oysters (*Crassostrea gigas*) within 2 h of exposure. Recovery occurred within 12 h after removal from treatment with no latent mortalities. In the present experiment, *Elliptio complanata* required much greater dichlorvos concentrations (25–50 ppm) to initiate gaping within the 6-h allotted exposure time. Recovery required more than 24 h, and activity levels remained depressed in excess of 7 days. Thus, dichlorvos would not be appropriate for field use or when immediate recovery and burrowing are required, but may be useful in laboratory situations where extended anesthesia is necessary.

In summary, this study identified several compounds useful in relaxing and/or anesthetizing the freshwater mussel *Elliptio com-*

planata, each with differing induction times, recovery rates, ease of use, and danger to the operator. Our present protocol is to de-water the mussels for 30–60 min before immersion in 500 ppm buffered MS-222. Mussels are taken to near, but not full anesthesia, then held open with either a finger or reversing plier during sample collection and internal examination. Several hundred *Elliptio complanata* were sexed using this technique and held in captivity for over 1 year with no mortality or apparent affect on behavior. MS-222 has also been used to anesthetize *Alasmidonta undulata, A. varicosa, Lasmigona subviridis*, and *Strophitus undulatus*, although reaction time is quicker for these species than for *Elliptio complanata*. *Pyganodon cataracta* did not respond to 500 ppm MS-222 and may require a different chemical agent and/or technique for anesthesia. Thus, anesthetic protocols will likely need to be developed independently for each new species under investigation.

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