EFFICACY OF CANDIDATE CHEMICALS FOR PREVENTING ATTACHMENT OF ZEBRA MUSSELS (*DREISSENA POLYMORPHA*)

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Abstract—Forty-seven chemicals having potential for preventing the attachment of zebra mussels *Dreissena polymorpha* were identified and tested. For each chemical, 15 zebra mussels (5–8-mm shell length) in each of two replicates and six treatments were exposed for 48 h followed by a 48-h postexposure period in untreated water. Eleven of the chemicals inhibited the reattachment of zebra mussels after the 48-h exposure; eight had EC50 values ranging from 0.4 to 5.4 mg/L, and three had EC50 values ranging from 19.4 to 29.0 mg/L. Based on an analysis of chemical cost, solubility in water, anticipated treatment concentrations, and potential hazards to humans or the environment, three of the most promising chemicals, all antioxidants, (butylated hydroxyanisole [BHA], *tert*-butylhydroquinone, and tannic acid) were tested on nontarget fish (bluegill, *Lepomis macrochirus;* channel catfish, *Ictalurus punctatus;* and rainbow trout, *Oncorhynchus mykiss*). These chemicals were not selectively toxic to zebra mussels; only the tests with bluegill and BHA and with channel catfish and tannic acid had 48-h LC50 values greater than the concentrations effective for preventing the reattachment of zebra mussels. Although the attachment of zebra mussels can be prevented with selected antioxidants, an alternative formulation should be investigated to minimize effects on nontarget organisms, such as fish.

Keywords—Zebra mussel　　*Dreissena polymorpha*　　Antioxidant　　Toxicity

INTRODUCTION

Zebra mussels, *Dreissena polymorpha*, attach to almost any solid substrate with a byssus or tuft of byssal threads [1,2], which allows the mussels to resist detachment by wave action and currents. The chemical composition and pathways for development of byssal threads in zebra mussels have not been fully determined. However, recent ultrastructural analyses of the byssal threads of zebra mussels and their marine counterpart, the blue mussel *Mytilus edulis,* have revealed similarities in structure, mechanisms of attachment, secretion behavior, and frequency of byssal thread formation [3,4]. Based on these studies, similarities may also exist in byssal thread composition and production pathways.

In contrast to the situation for zebra mussels, the chemical composition and pathways for development of byssal threads in *M. edulis* have been extensively characterized [5–8]. Five glands or groups of cells (phenol gland, enzyme gland, collagen gland, byssus gland, and mucous cells) in the foot of the mussel contribute to the composition and formation of the byssus [5–7]. Each of these glands or groups of cells secrete products of enzymatically catalyzed reactions. Many of these enzymatic reactions are catalyzed by the phenolase group of enzymes, which carry out oxidation reactions in the production of a byssal thread protein [5–7]. Likewise, a final set of reactions that lead to the formation of the attachment plaque and the finished byssus are also enzymatically catalyzed, oxidation-type reactions [7–9].

The similarities between biochemical pathways for byssus development in zebra mussels and their marine counterparts are unknown; however, reactions in the pathway of zebra mussels are likely similar to *M. edulis* and may be targeted for inhibition as a potential control method. Our objectives were to identify candidate chemicals that may inhibit either byssal-

thread development or attachment of zebra mussels, to test the efficacy of the candidate chemicals on zebra mussels, and to assess their toxicity to nontarget fish.

MATERIALS AND METHODS

Identification of test chemicals

A total of 47 chemicals having the potential to inhibit the attachment of zebra mussels were chosen for testing, based on their antioxidant properties or properties that inhibit critical catalytic enzymes involved in byssus development. Generally, we selected chemicals with existing regulatory approval status (e.g., fishery therapeutants, toxicants, or antioxidants used as preservatives in human and livestock foods approved by the U.S. Environmental Protection Agency or the U.S. Food and Drug Administration). Groups of chemicals tested included the carotenoids, tocopherols, flavonoids, urates, gallates, synthetic phenolics, and other related compounds (Appendix). The solubility of all candidate chemicals was assessed before testing on zebra mussels. Water was the preferred solvent for all test chemicals; however, if water was unsuitable, ethanol or acetone (listed in order of preference) was used as the diluent for preparing stock solutions.

Test organisms

Zebra mussels. Zebra mussels used in tests were collected by divers from relatively nonindustrialized areas of Lake Michigan (near Racine, WI, USA) or Lake Erie (near Put-in-Bay, OH, USA). During collection, zebra mussels were severed from their substrate, placed in ice chests with overlying water from the collection site, and transported within 12 h to the laboratory.

Once at the laboratory, zebra mussels were placed in flow-through tanks (3.0 × 0.81 × 0.43 m; flow rate 1.0 L/min), supplied with well water (pH 7.9, alkalinity 107 mg/L as $CaCO_3$, hardness 134 mg/L as $CaCO_3$ and conductivity 281 μS/cm), and acclimated to 12°C. The length of the acclimation

period depended on the water temperature during collection, but did not exceed 5°C in a 24-h period. The mussels were allowed to attach to polyvinyl chloride (PVC) plates and held horizontally in PVC racks in the flow-through tanks.

The diet of the stock culture of zebra mussels consisted of a mixture of both live and dried algae. The zebra mussels in the stock culture tanks were daily fed 10 L of live *Ankistrodesmus,* dried *Chlorella,* and a mixture of pond-cultured algae. The maximum length of time zebra mussels from a given collection were held in the laboratory and used for testing was 60 d.

Fishes. Three species of fishes were tested to assess the potential toxicity of candidate chemicals to nontarget organisms. Juvenile bluegill, *Lepomis macrochirus,* channel catfish, *Ictalurus punctatus,* and rainbow trout, *Oncorhynchus mykiss,* were obtained from the fish culturist at the Upper Mississippi Science Center in La Crosse, Wisconsin, USA, and held in isolation tanks supplied with well water (pH 8.0, alkalinity 106 mg/L as $CaCO_3$, hardness 141 mg/L as $CaCO_3$, conductivity 291 μS/cm and temperature 12°C). Fish were graded by size before being placed in the isolation tank to ensure that all fish were of similar size before the tests. The mean length and wet weight of fishes used in tests were 39 mm (range 37–42) and 1.03 g (range 0.97–1.09) for bluegill, 46 mm (range 39–52) and 0.93 g (range 0.76–1.22) for channel catfish, and 43 mm (range 35–61) and 0.87 g (range 0.48–1.64) for rainbow trout. All tests were initiated within 14 d after these measurements were taken. Silver Cup® #1 trout feed (Sterling Nelson and Sons, Murray, UT, USA) was fed to the fishes ad libitum daily.

Test conditions

Well water was used as test water for both zebra mussels and fishes. The temperature, dissolved oxygen, and pH of the test water in each exposure chamber were measured daily, whereas alkalinity, hardness, and conductivity were measured on a sample of the water used to fill the exposure chambers at the beginning of each test. Water quality characteristics were determined with standard methods [10]. The temperature and dissolved oxygen were determined with a Yellow Springs Instrument Model 58 oxygen meter (YSI, Yellow Springs, OH, USA), pH with a Beckman Model Φ 11 meter (Beckman Instruments, Fullerton, CA, USA), and conductivity with a Hanna Instruments Model HI-8733 meter (Hanna Instruments, Woonsocket, RI, USA). The mean characteristics of the test water were similar in tests with zebra mussels and fishes (temperature 17.0°C, range 16.3–17.4; dissolved oxygen 8.3 mg/L, range 6.0–11.2; pH 8.0, range 7.3–8.7; alkalinity 109 mg/L as $CaCO_3$, range 98–120; hardness 146 mg/L as $CaCO_3$, range 136–156; and conductivity 260 μS/cm, range 212–298).

All tests (zebra mussel and fish) were conducted at 17°C, which was maintained by a Remcor model CFF-501 thermostatically controlled, liquid-circulation pump (Remcor Products Company, Franklin Park, IL, USA) connected to a water bath. A 16-h light : 8-h dark photoperiod was used in all tests (zebra mussel and fish).

Zebra mussels. Static toxicity tests were conducted according to standard methods for testing macroinvertebrates [11], which were modified for testing the reattachment of zebra mussels. Zebra mussels were acclimated to the test temperature (17°C) over a 4-d period before the test and were not fed during this time to allow clearance of the digestive tract. The experimental design for each chemical tested was completely ran-

domized with two replicates in each of five or six treatments (four exposure concentrations, one solvent control [used only for tests requiring ethanol or acetone], and one well water control). The experimental unit for each test was a 3.8-L glass jar, which contained 2.5 L of well water. In each replicate, 15 zebra mussels (ranging from 5–8 mm in shell length) were exposed to the chemical for 48 h followed by a 48-h postexposure period in untreated water to assess latent effects.

At the time of testing, 15 zebra mussels were removed from the PVC substrates held in the stock tank by carefully severing the byssus with a scalpel. All zebra mussels were confirmed as *D. polymorpha,* according to the shell characteristics described by May and Marsden [12], and then placed in a glass petri dish (100 mm in diameter). Petri dishes were preconditioned in an aquarium containing well water for 5 d before the test to allow the development of a biofilm, which seems to facilitate the attachment of zebra mussels [13]. The petri dish with zebra mussels was then placed onto the bottom of a randomly selected exposure chamber, and the zebra mussels were turned on their side (left valve) with a blunt probe. Zebra mussels were not fed during the test.

The ability of zebra mussels to right themselves (righting response) and reattach was assessed at the end of the 48-h exposure period by gently touching the shell with a blunt probe. Any mussels that were not upright and attached were transferred to a separate 3.8-L glass jar containing 2.5 L of untreated well water and placed back on their side on the bottom of the jar for a 48-h postexposure period. Reattachment and mortality of zebra mussels were assessed at the end of the 48-h postexposure period.

Fishes. Static toxicity tests were conducted according to standard methods for fish [11]. Fishes were acclimated to the test temperature (17°C) over a 4-d period before the test and were not fed during this time to allow clearance of the intestinal tract. The experimental design for each chemical tested was completely randomized with two replicates in each of five or six treatments (four exposure concentrations, one solvent control [used only for tests requiring ethanol or acetone], and one well water control). The experimental unit for each test was a 20-L glass jar, which contained 15 L of well water. In each replicate, 10 fish were exposed to the chemical for 48 h. The mortality of test fish was measured at 1, 3, 6, 24, and 48 h. Fishes were not fed during the test.

Statistical analyses

The results of tests assessing the ability of zebra mussels to reattach to a substrate were expressed as an effective concentration (EC) value. The EC50 and EC90 values were defined as the concentrations of chemical required to inhibit the reattachment of 50 and 90%, respectively, of the test organisms after the 48-h exposure to the test chemical and were calculated by Probit analysis [14]. The primary criterion used in our initial evaluation of chemical efficacy was an EC50 value of ≤10 mg/L, but we considered all chemicals with EC50 values below 50 mg/L effective for preventing the attachment of zebra mussels. We chose 10 mg/L as the evaluating criterion because this concentration would likely be realistic and achievable during a control treatment in an industrial setting [15]. The LC50 (lethal concentrations resulting in 50% mortality) values for tests with fish were also calculated by Probit analysis [14]. The LC50 values (and EC50 and EC90 values) were considered significantly different when the 95% confidence intervals did not overlap.

Table 1. Efficacy of candidate chemicals for inhibiting the reattachment of zebra mussels, listed in order of decreasing effectiveness based on the 48-h effective concentration (EC50) (95% confidence interval in parentheses)

Chemical	48-h EC50[a] (mg/L)	48-h EC90[a] (mg/L)
L-3,4-Dihydroxyphenylalanine (L-3,4-DOPA)	0.4 (0.01–0.9)	2.6 (1.6–3.6)
tert-Butylhydroquinone (TBHQ)	1.0 (0.8–1.2)	1.9 (1.5–2.3)
Butylated hydroxytoluene (BHT)	1.3 (0.8–1.8)	2.4 (1.3–3.5)
Nordihydroguaiaretic acid (NDGA)	2.0 (1.5–2.7)	4.5 (3.2–5.9)
Ethoxyquin	2.9 (1.9–4.0)	6.0 (3.6–8.5)
Butylated hydroxyanisole (BHA)	3.4 (2.8–4.0)	5.8 (4.6–6.9)
Capsaicin	4.9 (3.6–6.1)	7.9 (5.8–9.9)
Gum guaiac	5.4 (3.7–7.1)	13.0 (9.1–16.9)
(+)-δ-Tocopherol	19.4 (13.7–25.1)	42.3 (30.7–53.8)
n-Propyl gallate	17.8 (11.7–23.8)	35.5 (23.2–47.8)
Tannic acid	29.0 (23.1–34.9)	47.9 (38.6–57.2)

[a] EC50 and EC90 = effective concentration of chemical required to inhibit reattachment of 50 and 90%, respectively, of zebra mussels after the 48-h exposure to the test chemical.

RESULTS

Eleven of the 47 chemicals tested inhibited the reattachment of zebra mussels. Eight of these had EC50 values ranging from 0.4 to 5.4 mg/L, and three had EC50 values ranging from 19.4 to 29.0 mg/L (Table 1). None of the other 36 chemicals tested prevented the attachment of zebra mussels at the range of concentrations tested (0–50 mg/L). The corresponding EC90 values for the eight most effective chemicals had values ranging from 1.9 to 13.0 mg/L, whereas the remaining three had EC90 values ranging from 42.3 to 47.9 mg/L (Table 1).

A strong concentration–response pattern was observed for all chemicals that inhibited the reattachment of zebra mussels. For example, the test with butylated hydroxyanisole (BHA) showed that exposure concentration was highly correlated ($r = -0.92$, $p < 0.01$) with the reattachment of zebra mussels. In that test, all zebra mussels in the control treatment reattached, whereas none reattached in the 10 mg/L treatment after the 48-h exposure to BHA (Fig. 1). However, when the unattached zebra mussels were transferred to untreated water for the 48-h post-exposure period, they rapidly reattached (Fig. 1). Thus, reattachment was inhibited at exposure concentrations that were not lethal to the zebra mussels. Similar concentration–response patterns were observed for the other 10 chemicals that were effective at preventing the reattachment of zebra mussels.

Based on an analysis of chemical cost, solubility in water, anticipated treatment concentrations [15], and potential hazards to humans or the environment, three of the 11 candidate chemicals, all antioxidants, showed promise for future investigation; these were BHA, *tert*-butylhydroquinone (TBHQ), and tannic acid.

The toxicity of the three most promising chemicals (BHA, TBHQ, and tannic acid) to three nontarget organisms (bluegill, channel catfish, and rainbow trout) showed that these chemicals were not selectively toxic to zebra mussels. The 48-h LC50 values for the fish species tested were generally less than the 48-h EC50 values for zebra mussels (Table 2). Only the tests with bluegills and BHA and with channel catfish and tannic acid had 48-h LC50 values exceeding the concentrations effective for preventing the reattachment of zebra mussels. Moreover, the toxicity (based on a comparison of LC50 values) of all three candidate chemicals tested was substantially greater (3.4- to 787-fold) to fishes than to zebra mussels (Table 2).

DISCUSSION

Preventing the reattachment of zebra mussels was feasible with selected chemicals in laboratory tests. The range of chemical concentrations that inhibited reattachment was not lethal to the exposed zebra mussels, which reattached after transfer to untreated water (e.g., Fig. 1). Although the mode of action of these chemicals for preventing the reattachment of zebra mussels is unknown, the reattachment upon transfer to untreated water indicates that the pathways for byssus production and development were affected during exposure.

The chemical composition and pathways for development of byssal threads in zebra mussels remain uncertain [16,17]. However, given the similarities in byssal thread structure and formation between zebra mussels and *M. edulis* [3,4], many of the oxidation reactions (enzymatically catalyzed by the phenolase group of enzymes) known to occur in the production of byssal thread proteins, attachment plaque, and finished byssus of *M. edulis* [5–7] may have been similarly affected by the test chemicals in the exposed zebra mussels. Interestingly, all of the chemicals (except the L-3,4-dihydroxyphenylalanine [L-3,4-DOPA]) that prevented the reattachment of zebra mussels were phenolic (synthetic or natural) antioxidants used in the preservation of human and livestock foods [18–20]. Phenolic antioxidants inhibit oxidation reactions by chelating catalytic metals and terminating free radicals [20] and may have interfered with the oxidation reactions in the pathways for byssal thread production in zebra mussels by these mechanisms.
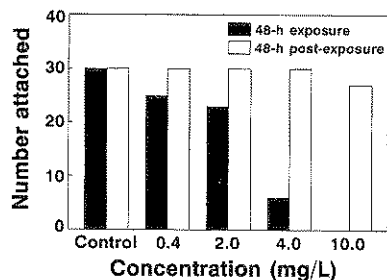

Fig. 1. Reattachment of zebra mussels after exposure to butylated hydroxyanisole (BHA) for 48 h (solid bars) followed by a 48-h postexposure period in untreated water (open bars).

Table 2. Relative sensitivity of fishes and zebra mussels to candidate chemicals for inhibiting the reattachment of zebra mussels (95% confidence interval in parentheses)

Chemical	48-h LC50[a] (mg/L)				48-h EC50[a] (mg/L)
	Rainbow trout	Bluegill	Channel catfish	Zebra mussel	Zebra mussel
TBHQ[a]	0.37	0.15	0.37	118	1.0
	(0.34–0.39)	(0.14–0.16)	(0.34–0.39)	(104–133)	(0.8–1.2)
BHA[a]	1.0	4.8	1.5	65	3.4
	(0.87–1.2)	(4.5–5.0)	(1.2–1.9)	(48–88)	(2.8–4.0)
Tannic acid	20	24	46	156	29
	(19–21)	(21–26)	(43–50)	(140–177)	(23–35)

[a] LC50 = lethal concentration, EC50 = effective concentration, TBHQ = *tert*-butylhydroquinone, and BHA = butylated hydroxyanisole.

The L-3,4-DOPA, which was the most effective chemical (EC50 = 0.4 mg/L) at preventing the reattachment of zebra mussels, is a protein that has been isolated from the byssal adhesive complex of both zebra mussels [17] and *M. edulis* [21–23]. L-3,4-DOPA is an intermediate catecholamine transmitter synthesized from the amino acid tyrosine and is used in the treatment of Parkinson's disease in humans [24]. Proteins isolated from the byssus of zebra mussels [17] and *M. edulis* [6] are rich in tyrosine and DOPA and are believed to be involved in oxidation reactions leading to the completed attachment plaque and finished byssus [21,23].

The type of observed response for the effective chemicals follows enzyme theory for a reversible inhibitor [24], because the reaction and pathway apparently resumed function and the zebra mussels reattached after transfer to untreated water. Alternatively, the chemicals may have affected the neuromuscular responses of the foot (especially the L-3,4-DOPA, a known catecholamine transmitter), which are necessary in byssal thread production [6]. When a chemical was effective, the mussels were generally not able to right themselves.

Although the attachment of zebra mussels may be prevented with selected antioxidants, our tests of nontarget fishes (rainbow trout, bluegill, and channel catfish) showed that the three most promising chemicals (BHA, TBHQ, and tannic acid) were not selectively toxic to zebra mussels. The 48-h LC50 values for the fish species tested were generally less than the 48-h EC50 values for preventing the reattachment of zebra mussels (Table 2). Moreover, the toxicity (based on a comparison of LC50 values) of all three candidate chemicals was substantially greater (3.4- to 787-fold) for fishes than for zebra mussels (Table 2). Similarly, Waller et al. [25], who evaluated the toxicity of 18 candidate molluscicides to zebra mussels, two nontarget fishes (rainbow trout and channel catfish), and a unionid mussel (*Obliquaria reflexa*), found that the toxicity of the candidate chemicals was generally greater for fishes than for zebra mussels or for the unionid mussel. The toxicity (based on a comparison of 48-h postexposure LC50 values) of the candidate chemicals tested by Waller et al. [25] showed that zebra mussels (5–8 mm in length—the same length range used in the present study) were generally more sensitive than the unionid mussel. We did not test unionid mussels in evaluating potential effects on nontarget organisms, but adult unionids that lack a byssus [26] would likely not be adversely affected by nonlethal concentrations of chemicals targeting the pathway for byssus development in zebra mussels.

Millions of dollars are being spent annually by industries and municipalities in the United States and Canada in attempts to control the attachment and biofouling of pipes and other solid structures by zebra mussels [27]. Toxicants such as chlorine and commercially available molluscicides are the primary methods being used to control zebra mussels [15]. These methods are often costly and may adversely affect industrial components, nontarget organisms, and the environment [15,25].

Nonlethal methods for preventing the attachment of zebra mussels, such as those identified in our study, may be an alternative approach to toxicant-based control. For example, Giamberini et al. [28], who studied the effects of an organic molluscicide (Mexel 432) on byssal thread development in zebra mussels, found that the exposed (2–10 mg/L for 10 d) zebra mussels produced fewer byssal threads than the controls. In addition, McMahon et al. [29] found that exposure of zebra mussels to the organic molluscicide PQ 2 (5–9 mg/L for 14 d) caused detachment and inhibited reattachment of the exposed zebra mussels.

The biofouling potential of zebra mussels would be negated if they were unable to attach to a substrate, thereby reducing the cost of removal of dead organisms from a toxicant treatment. Moreover, these molluscistatic techniques would be extremely beneficial to municipal water suppliers and other water users where the mass mortality of zebra mussels from a toxicant treatment in the intake pipe would result in undesirable taste and odor problems. In addition, these techniques would presumably be less harmful to industrial components and the environment than toxicants such as chlorine [15].

We found that the reattachment of zebra mussels may be prevented with selected antioxidants. However, the chemicals (BHA, TBHQ, and tannic acid) selected for further evaluation were lethal in waterborne exposures to most fish species tested at the concentrations effective for preventing the reattachment of the zebra mussels. Therefore, an alternative formulation should be investigated to minimize the effects on nontarget organisms, such as fish. Because antioxidants are readily incorporated into foods and other products as preservatives and stabilizers [20], an alternative formulation of antioxidants into paints and coatings may be a feasible alternative. Moreover, antioxidants formulated into an antifouling paint for pipes and other solid structures may provide an environmentally acceptable application of these compounds because exposure would be directed toward the target organism at the substrate surface.

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APPENDIX

Alphabetized list of 47 candidate chemicals identified and tested for preventing the reattachment of zebra mussels

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L-Ascorbic acid
Benzoic acid
Borax
Boric acid
Butylated hydroxyanisole (BHA)
Butylated hydroxytoluene (BHT)
Caffeic acid
Capsaicin
β-Carotene
Citric acid (monohydrate)
Citric acid (trisodium salt)
p-Coumaric acid
D-3,4-Dihydroxyphenylalanine (DOPA)
L-3,4-Dihydroxyphenylalanine (DOPA)
L-3,4-Dihydroxyphenylalanine methyl ester
Dimethylsulfoxide
Ellagic acid
Ethoxyquin
Ferulic acid
Gallic acid (monohydrate)
Gallic acid (methyl ester)
Gluconic acid lactone
Gum guaiac
Hydroxylamine hydrochloride
Kaempferol
Methimazole
Morin
Myricetin
Nordihydroguaiaretic acid (NDGA)
n-Propyl gallate
Protocatechuic acid
Rutin
Tannic acid
tert-Butylhydroquinone (TBHQ)
Thiopropionic acid
Thiopropionic acid lauryl sulfate
Thiourea
Tiron®
(+)-α-Tocopherol acetate
(±)-α-Tocopherol acetate
(+)-α-Tocopherol acid succinate
(+)-δ-Tocopherol
Trolox®
Uric acid (potassium salt)
Uric acid (sodium salt)
Vanillic acid
Vanillin