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ENDOD IS LETHAL TO ZEBRA MUSSELS AND INHIBITS THEIR ATTACHMENT

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ABSTRACT The invasion of zebra mussels into the Laurentian Great Lakes has resulted in biofouling of water intakes of public utilities. Both static bioassays and assays using a recirculating-flow system with solutions of the African soap berry *Phytolacca dodecandra,* or Endod. were used to develop the experimental basis for mitigation and control methods. The molluscicides, Lemmatoxins, in Endod were lethal to zebra mussels at concentrations higher than 20mg/L while lower concentrations inhibited attachment of adult mussels. Our results on the lethal effects of Endod. its inhibition of attachment, and its apparent biodegradability show Endod to be a candidate for controlling zebra mussel populations in restricted localities such as water intake pipes of water works.

KEY WORDS: Endod. molluscicides., focal mitigation, *Phytolacca dodecandra, Dreissena polymorpha*

INTRODUCTION

The zebra mussel *Dreissena polymorpha* (Pallas), a native of Eastern Europe, has recently been introduced into the Great Lakes of North America, presumably through the dumping of ballast water from ocean going vessels. Since its discovery in 1988 in Lake St. Clair (Hebert et al. 1989) the mussels have been found in great quantities along lake shores and in water intakes of various water works, especially on Lake Erie. The veligers, larvae, are free swimming resulting in a wide distribution. After one to two weeks, the veligers are able to attach themselves to almost any solid surface and to each other, with their byssal threads, proteinaceous fibers with adhesive at the ends. Their ability to cluster into large aggregates, extensive proliferation and wide distribution, combined with the possible lack of natural predators in the Great Lakes may threaten fish spawning grounds as well as cause problematic fouling of utility pipes, screens etc. Existing chemical and mechanical methods are being used to control the population growth and at the same time the search for new methods has been intensified.

The experimental approach taken in this investigation is in the context of controlling zebra mussels by cost effective methods and simple applications using natural substances. Pilot studies using solutions prepared directly from powdered *Phytolacca dodecandra,* commonly known as Endod, to control populations of the snails *(Minus* and *Biomphakaria)* that are intermediate hosts for the protozoan causing schistosomiasis in some African countries (Mokhubu et al. 1987), indicate that Endod could also be used to control populations of *Dreissena.*

P. dodecandra is an indigenous plant in Africa whose berries have been used as soap for thousands of years. Lemma (1965, 1970) found that the berries possessed molluscicidal property, i.e., they were lethal to various species of snails that were intermediate hosts for the spread of schistosomiasis. Chemical studies of extracts of these berries revealed that the active molluscicidal compounds, called Lemmatoxins, were triterpenoid saponins (Parkhurst et al. 1973a and b, 1974). Using a bacterial system to assay mutagenicity and carcinogenicity, it was found that powder from dry berries was neither mutagenic nor carcinogenic (Lemma and Ames 1975). A solution of Ended powder loses its molluscicidal potency in two to three days presumably due to microbial degradation or modification in streams (Lemma 1970, Lemma and Yau 1974).

Laboratory experiments described in this paper demonstrate that simple preparations from Endod powder (without removing cellular debris and other biological materials) are effective toward zebra mussels. Together with results on the non-toxic nature of Lemmatoxins to mammals (Lambert et al. 1991), Endod appears to be a potential candidate as a mitigation agent in the control of zebra mussel populations.

MATERIALS AND METHODS

Endod powder of approximately 250 µm particle size of *P. dodecandra* (type 44) was obtained from the Institute of Pathobiology, Addis Ababa University, Ethiopia. Stock solutions of 1000 mg/L were prepared by dissolving the powder in aged and aerated tap water (referred as water unless otherwise stated). Insoluble cellular debris was not removed. Working solutions therefore also contained insoluble materials. Endod-S, a reference standard, was obtained from the Department of Pharmaceutical Technology and Biophanneceutics of the State University Groningen, The Netherlands. Endod-S is hot-air dried powder prepared from the soluble fraction of the type-44 Endod berries, therefore containing no insoluble materials. Since both solutions of the raw powder and

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the Endod -S powder were not filtered nor clarified, they both contain cellular components that are essential for the synthesis of the Lemmatoxins (Parkhurst et al. 1973a and b). Freshly made Endod solutions are not active unless they have been incubated at 37°C for one hour or at room temperature for 16 hours. Longer incubation will lead to reduced molluscicidal potency, presumably due to microbial activity (Lemma 1970, Lemma and Yau 1974). Endod stock solutions used in this study were incubated in either manner and stored at 4°C until used. Although no potency tests were done, stock solutions older than one month were not used.

To test biodegradability or modification of the molluscicidal activity of Endod, solutions of 25 mg/L were filter-sterilized through 0.45 mm millipore filters and were stored in sterilized bottles for three days at room temperature before being used. Controls were not filtered.

Adult zebra mussels were collected between June and September 1990 at the shore of Lake Erie near the Toledo and Oregon pump stations, where there is no public access or industrial activity. The animals were scraped off the rocks and brought to the laboratory. The animals were then rinsed with water and placed in half-gallon fish bowls which were immersed into an 80-gallon recirculating aquarium (Living Stream, Frigid Unit, Inc. Toledo, Ohio) at 10°C. They were fed every other day with the unicellular alga *Clamydomonas.* Dead animals were removed daily. It was estimated that at least 70% have survived.

Prior to using the mussels in experiments, the fish bowls containing the animals were placed at room temperature with aeration overnight. For all bioassays, the animals were separated from their clusters. Unless stated otherwise, the mussels were placed in the appropriate vessels and allowed to acclimate for at least 24 hours before the addition of Endod. Animals of approximately 1.0-l.5 cm length were used. All experiments were done in water and some in lake water as indicated. Unless otherwise indicated, Endod containing water was replaced with clean water at 24 hours after treatment. Animals were not fed during exposures to Endod, but were fed afterwards.

To assay the effect of Endod in a static system, animals were placed in beakers, either 250 or 400 ml, depending on the number of animals used. The solutions were aerated.

A diagram of the recirculating-flow system is presented in Fig. 1. The pump was a Minipus-II (Gibson, Inc.) with a flow rate of 1.3 L/hr which was the sum of all four lines. 150 ml or 100 ml of Endod solution at 1000 mg/L was delivered via one of these four lines. Detailed mathematical analyses of the flow dynamics, the precise Endod concentrations as a function of time and their relationship to the biological effects of Endod will be presented elsewhere.

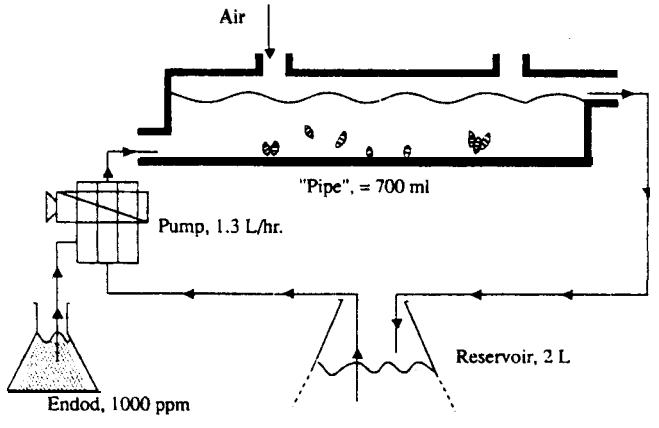

Figure 1. A recirculating system in which mussels were treated with Endod. Results of experiments using this system are shown in Tables 4 and 5.

Animals were considered dead when they failed to close their shells upon mechanical stimulation.

A probit analysis was performed to determine the LC_{50} and LC_{90} values and their 95% confidence limits for the experiment in which zebra mussels were exposed to Endod. The analysis was done with the SAS/STAT PROBIT computer program using SAS Version 5.18 (SAS Institute, Cary, NC) on ^{10}log-transformed Ended concentrations.

RESULTS AND DISCUSSIONS

In one static bioassay done at room temperatures, the animals were not acclimated prior to the addition of Endod, i.e., Endod was added at the same time that the individual animals were placed in the beakers. The results of this experiment are shown in Table 1. Even after only 7 hours of treatment. a clear dose-effect relationship is evident (Fig. 2). The 24-h LC_{90} and LC_{50} values (with 95% confidence limit) were 19 (14.5-33.9 mg/L) and 8.8 (6.4-11.0 mg/L), respectively. Even though the animals were transferred to clean water after 24 hours of treatment with Endod, mortality continued to occur. For example, mortalities at 54 hours after treatment with 6.25 and 12.5 mg/L Endod reached respectively 85 and 100%. Therefore, it appeared that there was an effective exposure time during which no death occurred but which resulted in subsequent mortality in the absence of Endod.

This hypothesis of an effective exposure time was tested with a time course experiment. The Endod solutions were replaced with clean water at intervals after the beginning of treatment. The results are shown in Table 2. It appears that treatment for 4-8 hours was needed to yield a significant effect. The existence of the effective exposure time indicates a latent physiological response of the zebra mussels to Endod. This response was not investigated in

TABLE 1.

Mortality in *Dreissena polymorpha* exposed to various concentrations of Endod solution. Endod solutions replaced with freshwater after 24 hours. Ten mussels per replicate.

Exposure Concentr. (mg/L)	Replicate	Mortality (%) @7h.	@24h.	@30h.	@54h.
0	A*	0 (l0)**	0 (l0)	0 (l0)	0(10)
	B*	0 (8)	0 (10)	0 (10)	0 (10)
6.25	A	0 (0)	30 (2)	60 (0)	90 (0)
	B	0 (0)	40 (2)	70 (0)	80 (0)
12.5	A	20 (0)	50 (0)	80(0)	100
	B	0 (0)	70 (0)	90(0)	100
25	A	70 (0)	100		
	B	40 (0)	100		
50	A	60 (0)	100		
	B	70 (0)	100		
100	A	90 (0)	100		
	B	80 (0)	100		

* Mussels clustered together.
** Values in parentheses are number of mussels attached to the surface o the beaker.

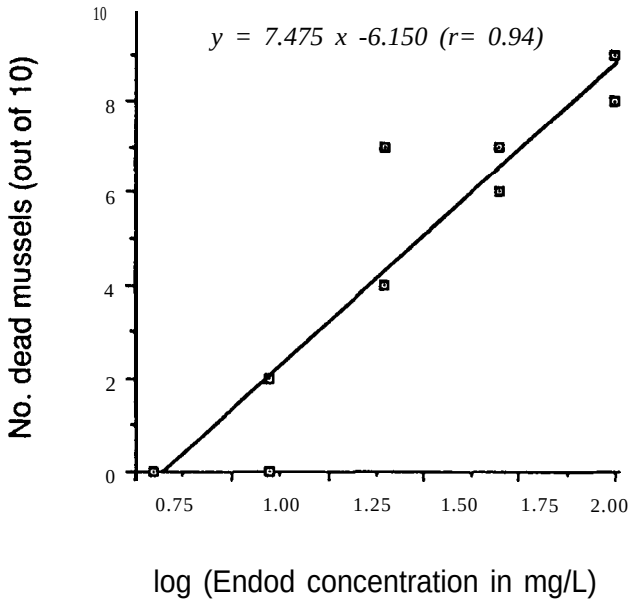

Figure 2. A semi-log plot of the mortalities in zebra mussels, 7 hours after being treated with Endod for 24 hours (data from Table 1).

the studies on the African snails. This result has significant implications in that prolonged and continuous applications may not be necessary to control mollusc populations.

In contrast to the exposure to the solution prepared directly from the powdered berries, in which 7 of the 20 animals were dead after 24 hours exposure to 6.25 mg/L (Table 1), a 22 hour exposure to Endod-S solution at the same concentration resulted in a mortality of 19 of 20 mussels (Table 3). Endod-S is more potent than the solution prepared from dry powder in this laboratory. Parkhurst et al. (1974) demonstrated that Lemmatoxins were present in the soluble fraction (the cytosol). Since Endod-S contains no insoluble materials, the concentrations of Lemmatoxins in the Endod-S are therefore expected to be proportionally higher than in the powder of the whole berries. Methods of isolation and purification of Lemmatoxins have been achieved (Parkhurst et al. 1973a, 1974) and the procedures have been patented (U.S.A. #3,81 3,383 and 3,886,272). The 24h-LC_{50} for the toxicity of a butanol extract of Endod to the snail *Biomphalaria glabrata* is less than 3 mg/L (Stobaeus et al. 1990). However, the cost of producing Endod-S or purifying Lemmatoxins for large scale applications may be prohibitively high. In spite of the lower effectiveness of the powder of dry berries, their use may be cost-effective when a large quantity is required for controlling zebra mussels in intake pipes.

TABLE 2.

Mortality in *Dreissena polymorpha* at 24 hours after start of exposures of different durations, to 25 or 50 mg/L Endod solution.

Exposure Duration (hours)	Mortality (%) 25 mg/L	50 mg/L	Sample Size
10	0	0	10
20	0	0	10
4	0	0	10
8	70	40	10
24	65	100	20

TABLE 3.

Mortality in Dreissena polymorpha exposed to various concentrations of Endod-S. Encod-S solutions replaced with freshwater after 22 hours. Ten *mussels per* replicate.

Exposure Concentr. (mg/L)	Replicate	Mortality (%) @22h.	@72h.	@96h.
0	A	0	0	0
	B	0	0	0
3.125	A	0	0	0
	B	0	0	0
6.25	A	90	90	100
	B	100	100	
12.5	A	100		
	B	100		
25	A	100		
	B	100		
50	A	100		
	B	100		

In the static experiment described above, individual animals were placed in beakers at the start of the experiments. In the controls, the animals were able to attach onto the pyrex glass surface and to cluster together within 12 hours. Some of the animals treated at 6.25 and 12.5 mg/L of Endod were not dead at 24 hours post-treatment, but most failed to attach (Table 1). The ability of Endod to inhibit the attachment and aggregation may or may not be related to the molluscicidal component. This observation has important implication for the control of zebra mussels in water intakes by the prevention of attachment of adults, and possibly post-veligers.

Prior to their exposure to Endod in the static bioassay, adult mussels were separated from the clusters and placed in beakers. This traumatic manipulation may have increased their sensitivity to Endod. Periodic tests using animals already attached to the beakers did not show significant differences from those reported here.

To study response of zebra mussels to Endod in a continuous flowing condition that water intake pipes operate, a recirculating-flow system was designed (Fig. 1). In this system the concentra-

TABLE 4.

Effects of Endod (150 MI) on zebra mussels in a recirculating system.

	Hours of Endod Treatment 2	3	4	6
Percent Mortality	34	50	53	82
Total # Animals	59	50	65	60
Attach	firm	weak	weak	none
Cluster	loose	very loose	very loose	dispersed

Endod at 1000 mg/L was delivered via one of the four tubes (Fig. 1). Water in the reservoir was changed at the times indicated (after start of Endod). Mortality was recorded at 24 hours.

TABLE 5.

Effects of Endod (100 ml) on zebra mussels in a recirculating system.

Hours Post Treatment	Percent Mortality				
	Hours of Endod Treatment				
	0.5	1	2	4	8
24 Hrs.	0	0	0	2	14
48 Hrs.	0	0	0	2	46
72 Hrs.	0	0	0	6	52
% Hrs.	0	2	0	14	54
120 Hrs.	o	2	0	18	62

Endod at 100 mg/L was delivered via one of the four tubes (Fig. 1). Water in the reservoir was changed at the times indicated (after start of Endod). Fifty animals were used in each treatment. Mortality was recorded at 24 hour intervals after initiation of Endod treatment. Attachment and clustering were markedly reduced (none to weak, 0 to <35%) in the eight hour treatment.

tion of Endod in these experiments was calculated to be approximately 140 mg/L after 30 minutes when a total of 150 ml was delivered, assuming that at this point a negligible amount of Endod had entered the reservoir. It took 30 minutes to deliver 150 ml and approximately 60 minutes to reach equilibrium at about 50 mg/L in the system (pipe and reservoir). The water in the reservoir was changed to clean water directly after the exposures (lasting 2 to 6 hours), resulting in an equilibrium concentration of about 15 mg/L. After another change of the water 4 hours later, the remaining Endod concentration was about 4 mg/L. Mortality increased as the duration of exposure to Endod increased (Table 4). Since the animals had established their attachment prior to the treatment with Endod, the data of this experiment show that Endod treatment resulted in a loss of firm adhesion, and in some cases (last column, Table 4) dispersion from the clusters.

While the experiment described above used 150 ml of Endod stock, the second experiment used 100 ml of 1000 mg/L Endod in the recirculating-flow system. In this experiment the Endod concentrations were approximately two-thirds of the concentrations mentioned above. In this experiment, the animals were transferred to beakers with clean water at 24 hours after the beginning of the treatment. Mortality was determined daily. Although mortality was lower than in the first experiment in the same recirculating system, a latent effect was observed similar to the static bioassays (Table 5). Virtually no mortality was observed when the animals were exposed to Endod for 0.5, 1, or 2 hours. The 4 and 8 hour treatment however clearly show the latent physiological response of zebra mussels to Endod; mortalities in Endod-free water increased 4 to 6 fold between 24 and 120 hours after the treatment.

Although the flow rate in our recirculating system is not the same as in water intakes, results did indicate that Endod is effective in a flowing system. The effective dose in this system appeared similar to that obtained in the static bioassay. A detailed analysis of the flow dynamics and its relationship to biological effects, which may be fundamental in designing a delivery system for Endod, will be presented elsewhere.

Earlier observations in field studies in Ethiopia suggested that Endod might be biodegradable (Lemma and Yau 1974). It was attributed to microbes present in the water and on the Endod berries. This preliminary observation was confirmed in the present study (Table 6). When microbes were filtered out from the 25 mg/L Endod solutions, Endod's molluscicidal potency was retained even after a 72 hour storage at room temperature. Storage of the non-filtered preparations at room temperature caused a reduced effect on zebra mussels, especially for sample #1. However, there appeared to be differences among preparations. Freshly prepared Endod was effective in all three preparations, with a weakest activity in the sample that was prepared in lake water (sample #3). The difference among the preparations may be attributable to differences in chemical compositions of the water samples. Future studies should include water chemistry and its effects on Endod's efficacy. The molluscicidal effect could therefore be modified biologically or chemically. Nevertheless, our results indicate that the reduction of the effectiveness of Endod when kept at room temperature is (at least partly) due to biodegradation, although no tests were done to compare microbial growth among the different preparations.

It is evident that the solutions of powder of dried Endod berries contains molluscicidal components which may be useful in the control of zebra mussel populations in certain restricted environments, such as water intake pipes. Recent safety evaluations of Endod-S were conducted in North American and European laboratories in accordance with the Minimal Data Requirements (Tier I) and with the Guidelines for Pre-Market Chemicals of the Organization for Economic Cooperation in Development (Lambert et al. 199 1). In Acute Mammalian Toxicity Tests, with the exception of the eye irritation toxicity test which indicated severe

TABLE 6.

Effects of sterilization on the molluscicidal activity of Endod.

Hours Post Treatment	Percent Mortality											
	Controls						Treatments					
	Water			Endod			Non-Filtered			Filtered		
	1	2	3	1	2	3	1	2	3	1	2	3
24 Hours	0	0	0	10	10	0	0	0	0	10	10	0
48 Hours	0	0	0	95	35	10	0	10	0	85	35	5
72 Hours	0	0	0	95	70	20	0	45	0	85	75	5
96 Hours	15	0	0	100	80	35	5	75	0	85	75	25

Endod solutions at 25 mg/L in aged, aerated tap water (1,2) and lake water (3) were divided m half and processed as outlined in Materials and Methods. A freshly prepared Endod control (25 mg/L) and a water control were included in each set of assays. Twenty zebra mussels were used in each assay.

irritation. results were classified as either non-toxic or only slightly toxic. Ecotoxicity tests indicated that Endod is not any more toxic than synthetic molluscicides such as niclosamide (Monkiedje 1990). Because of these positively encouraging studies, Endod has been recomended for field trials in streams in Ethiopia and other African countries (Lambert et al. 1991). In addition to its molluscicidal activity, Endod also exhibits larvicidal (mosquito), hirudinicidal, trematodicidal, spermicidal (human), and fungicidal properties (Lemma 1971). The molluscicidal component, the saponins, may or may not be responsible for the multiple actions of Endod. In addition, the biodegradability of Endod clearly indicates its potential for control of schistomiasis as well as for focal control of *Dreissena,* This notion is further strengthened by the comprehensive study of Monkiedje (1990). This study indicated that Endod could be used in the environment because of results from US-EPA required tests including isotherm absorption to carbon and soil in accordance with Freundlich parameters. New analytical procedures (thin layer chromatography and hemolytical assays with red blood cells) have been worked out to determine concentrations of Endod (Monkiedje 1990, Monkiedje et al. 1990). Monocultivation of Endod type-44 has been successful in Ethiopia and other African nations. The berries of iype-44 have the highest molluscicidal saponin content among about 600 varieties assayed, approximately 25% by weight (Monkiedje, 1990). That large scale preparations of Endod powder can be available on demand (Mokhubu et al. 1987) suggests that the use of Endod may be a cost-effective means for zebra mussel mitigation.

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