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Can Hot Water Be Used to Kill Eurasian Watermilfoil?

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ABSTRACT

Hot water has been used as a safe and efficient strategy to control unwanted pest species in row crops and to control zebra mussel infestations of water intake and discharge pipes. The goal of this study was to determine if hot water can be used as an effective prevention strategy for killing an aquatic invasive plant species like Eurasian watermilfoil (*Myriophyllum spicatum* L.) attached to watercraft. In 2006, 20-cm Eurasian watermilfoil fragments with and without an apical meristem were exposed to six different water temperatures ranging from 45 to 80 C for 2-, 5-, and 10-min intervals to see if they could be killed. Fragment viability after treatment was determined in three ways: (a) the presence of living tissue at the end of a 30-day observation period; (b) change in biomass from before treatment to after treatment over the 30-day observation period; and (c) enzyme activity within fragments immediately after heat treatment. The 20-cm fragments were killed at temperatures ≥60 C. All fragments at temperatures ≥60 C lost mass, and enzyme activity was significantly reduced at temperatures ≥60 C for fragments without an apical meristem. Temperature was the most important variable causing death, and time of exposure had little effect at the tested temperatures. These results suggest that using hot water alone to kill fragments of Eurasian watermilfoil attached to watercraft may not be feasible because of the high water temperatures required. More research into additives, such as chlorine or bromine, may reveal ways to reduce the temperature of water necessary to kill plant fragments and make hot water control feasible.

Key words: aquatic invasive plants, enzyme activity, lethal temperature and time exposure, *Myriophyllum spicatum*, watercraft decontamination.

INTRODUCTION

Preventing the transfer and establishment of exotic aquatic species into new environments is a primary concern of managers and researchers. Many different prevention and control methods have been employed, including physical inspection, physical removal, the use of chemicals, and quarantine. Boat cleaning with power washers and treatment with water at 40 C or hotter has been recommended to help prevent introduction of problematic organisms such as zebra mussels (*Dreissena polymorpha*), spiny water flea (*Bythotrephes longimanus*) and, more recently, the viral hemorrhagic septicemia virus (Beall 2005, AveLallemant and Marcquenski 2007). However, the effectiveness of hot water treatments to kill Eurasian watermilfoil (*Myriophyllum spicatum* L.) has not been rigorously evaluated. Such research is needed if hot water is to be recommended to decontaminate boats, trailers, and other gear.

Hot water treatment is one of the most commonly used methods to control mussel fouling in industrial cooling water systems worldwide (Jenner et al. 1998). Rajagopal et al. (2005) showed that 100% mortality could be achieved in all size groups of three separate adult mussel species (*D. polymorpha*, *Mytilus eduli*, and *Mytilopsis leucophaeata*) by raising and maintaining temperatures of 40 C and maintaining for about 30 min. Hicks and McMahon (2002) determined that the acute upper lethal limit for another adult mussel species (*Perna perna*) was 44 C, regardless of acclimation temperature or individual size. In thermal weed control, flame, hot water, or steam is used to kill or at least reduce the competitiveness of unwanted weeds in row crops (Vanhala et al. 2004). The underlying goal of this method is to heat the essential parts of the plant to a lethal temperature. The lethal temperature for most terrestrial plants is around 60 C (Levitt 1980, Sirvydas et al. 2003, Virbickaite et al. 2006).

Little research has been done to determine lethal hot water exposure for any aquatic plant species. One study suggests that the viability of fragments of Eurasian watermilfoil is severely reduced after being subjected to temperatures ranging from 45 to 50 C and that the lethal temperature and time exposure is 5 min at 50 C or 10 min at 45 C. Stanley (1975) used small (4-cm) fragments and based his survival determinations on change in plant mass after a 32-day incubation period.

The purpose of our study was to determine the temperatures and exposure times required to kill larger (20-cm long) Eurasian watermilfoil fragments with and without an apical meristem. We chose to examine fragments with and without an apical meristem because we wanted to determine if fragments with a meristem would be more resistant to heat treatment or more likely to regrow. Three methods were used to determine fragment viability: (a) the presence of observable living plant tissue over 30 days; (b) change in biomass after treatment and a 30-day observation period; and (c) enzyme activity within the fragments immediately following treatment. Enzyme activity was examined to see if it could provide a rapid and definitive assessment of viability.

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MATERIALS AND METHODS

Six temperatures (45, 50, 55, 60, 70, and 80 C), three time intervals (2, 5, and 10 min), and two fragment types were used resulting in 36 different treatments. The fragment types were: plant pieces with an apical meristem, comprising the top portion of a stem; and plant pieces without an apical meristem, composed of the lower portion of the stem including at least five leaf nodes. Milfoil was hand-collected in July 2006 from Lake Harriet in Minneapolis, Minnesota, and stored in 379-L outdoor stock tanks filled with dechlorinated water under ambient light and temperature (20-30 C) for 2 to 6 days before use. Eurasian watermilfoil has been present in Lake Harriet since 1991 and chemical control is not used. All the plant fragments used in this study were inspected for herbivores and herbivore damage and trimmed to 20-cm lengths. Fragments that appeared senescent, damaged, or had root or branch growth were not used.

Temperature Treatment

Excess water was removed and each group of fragments was weighed before the time-temperature treatment. A subset of fragments of each type was set aside before each treatment that were used to determine the wet to dry mass conversion and to serve as controls. Groups of two, three, and five similar fragments were selected for each treatment. These fragments were heat-treated by submersing them in a MagniWhirl Constant Temperature Bath filled with 15-L deionized water and divided into three free-flowing sections. Order of temperature treatments was randomized, and for each temperature and fragment type the time interval and placement in the bath were randomized.

Immediately after treatment, each group of two fragments was frozen and used later for enzyme assays. Each group of three fragments was weighed, dried at 60 C for 48 hr, and reweighed to determine dry mass. Each group of five fragments was weighed and placed in 1.1-L Mason jars filled with Smart/Barko medium (Smart and Barko 1985) for direct observation. Smart/Barko medium was used to provide suitable ionic conditions; use of sediments or addition of nutrients was rejected because our aim was to evaluate viability, not growth, and additional nutrients would promote algal growth. Control jars of untreated fragments were also set up for each fragment type, and the remaining untreated fragments were used to establish the wet-to-dry mass conversion for untreated plant pieces. The entire experimental procedure was repeated on five different days, producing five replicates for each time-temperature-fragment type combination.

Survival and Growth

All the Mason jars were placed in an environmental chamber set to 16:8 light:dark (150 to 260 µmoles/m^2/s), 80 to 85% humidity, and constant temperature of 25 C. Plant fragments were observed twice weekly for 30 days. As water evaporated from the jars, it was replaced (250-500 ml/wk). The water remained clear during the experimental period. After 30 days, the fragments were removed, inspected for growth or damage, cleared of epiphytes, and the dry mass was determined. Data from the groups of three fragments that were treated and immediately dried were used to determine if significant loss of mass occurred immediately following treatment. These showed no significant change. To estimate the mass change after the 30-day observation period, estimated dry mass of fragments before treatment was subtracted from the final dry mass after 30 days.

Enzyme Activity

Frozen fragments of heat-treated and control milfoil were ground with a mortar and pestle in buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 1.5% polyvinyl polypyrrolidone, and 0.5% TritonX-100. Washed sand was added to facilitate breaking of plant filaments and cells walls. The resulting slurry was centrifuged at 8000x g for 5 min to remove sand, unbroken cells, and cell debris. The cell-free extract was kept on ice until assayed for glutathione reductase activity (10-30 min).

Assays were done by the procedure of Carlberg and Mannervik (1985). The assay mixture contained 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 3mM oxidized glutathione, and 0.24 mM NADPH in a final volume of 0.5 ml. The reaction was initiated by adding 0.075ml of the milfoil extract. Oxidation of NADPH was determined by monitoring the decrease in absorbance at 340 nm in an HP 8450 A dual beam spectrophotometer. A unit of glutathione reductase activity was defined as the amount of enzyme that catalyzes the oxidation of 1 µmole of NADPH per minute. Protein concentration in the extracts was determined from the absorbance of extracts at 260 and 280 nm (Copeland 1994). Enzyme activity was expressed in units/mg of protein.

Statistical Analysis

For changes in biomass and enzyme activity, Analysis of Variance (ANOVA) was used to evaluate the significance of responses for temperature, time of treatment, and fragment type combinations. Multiple comparisons, using pairwise t-tests with Bonferroni corrections, were used to test difference among temperatures. Statements of statistical significance without precise indication of probability level refer to a p-value ≤ 0.05.

RESULTS AND DISCUSSION

30-Day Observation

For the purposes of observing living tissue after 30 days, each jar was considered an independent sample. For each of the six temperature treatments, 30 jars (three time intervals × two fragment types **x** five replicates) were observed. Another 36 control jars were observed. After 30 days, all control, 45 and 50 C jars had observable living plant tissue and all 55, 60, 70 and 80 C jars had none. For visual estimates of viability after 30 days, the upper limit of probability for any one independent sample (jar) having observable living tissue, P(T), with a 95% confidence was calculated as $P(T) = 1 - 0.05^{(1/n)}$, where "n" is the number of jars with no living tissue (C. Bing-

ham, School of Statistics, University of Minnesota, Minneapolis, MN, pers. comm.). At 55 C, (n = 30, P(T) = 0.095) there is only a 9.5% chance that observable living material would be found within any one jar. Each jar contained five individual fragments, so the number of independent units experiencing death could be increased 5-fold. Therefore, at 55 C, (n = 150, P(T) = 0.0198) the chance is <2% that observable live material would be found. If all fragments within all jars treated at temperatures ≥55 C were included as independent samples (n = 600, P(T) = 0.00498), the chance is <0.5% that observable live material would be found. However, given that each individual fragment was not in its own jar and therefore not independent, the possibility of death from some source other than temperature cannot be ruled out. Our tests indicate conservatively that at 55 C, the chance of any fragments surviving is <10%, and the probability is likely <2%. To more accurately determine probability of a complete kill, much larger independent sample sizes should be tested.

Change in Dry Biomass

A three-way ANOVA (time, temperature, and fragment type) of change in biomass over the 30-day observation period indicated that fragment type ($p < 0.001$) and temperature ($p < 0.001$) were both significant factors, but time was not ($p = 0.721$). For this reason, each fragment type was analyzed separately for time, temperature, and temperature by time interaction.

For fragments with an apical meristem, a significant effect of temperature on change in biomass ($p < 0.001$) was observed, but no effect of time ($p = 0.474$) or a temperature by time interaction ($p = 0.328$) occurred. Fragments with an apical meristem gained biomass over the 30 days in the control, 45 and 50 C treatments, and lost biomass in the warmer treatments (Figure 1). No significant difference was observed between the 45 and 50 C treatments. These treatments gained significantly more biomass than the warmer treatments. No significant difference was observed between treatments ≥55 C (Figure 1).

For fragments without an apical meristem, a significant effect of temperature on change in biomass ($p < 0.001$) was observed. Time had no effect ($p = 0.355$), but a significant temperature by time interaction ($p = 0.018$) was observed. A time effect appeared to have occurred at 50 C, but not at other temperatures. Comparisons of the 50 C treatments showed less gain in biomass for fragments without an apical meristem treated for 10 min when compared to the 2- and 5-min treatments (Bonferroni t-test; $p = 0.044$), but it is not clear that longer treatment times resulted in greater treatment effects at other temperatures.

Fragments without an apical meristem gained biomass over the 30 days in the control, 45, 50, and 55 C treatments (Figure 1). They lost biomass in the warmer treatments. No significant difference occurred between the 45, 50. and 55 C treatments. The warmer treatments were different from the 45, 50, and 55 C treatments, but not different from each other (Figure 1).

All treatments ≥60 C lost biomass regardless of fragment type or time. At 55 C, fragments with an apical meristem lost biomass, but fragments without an apical meristem gained biomass. Observations over 30-days of the fragments without an apical meristem treated at 55 C showed no evidence of new growth. The cause of the increase in biomass for frag-

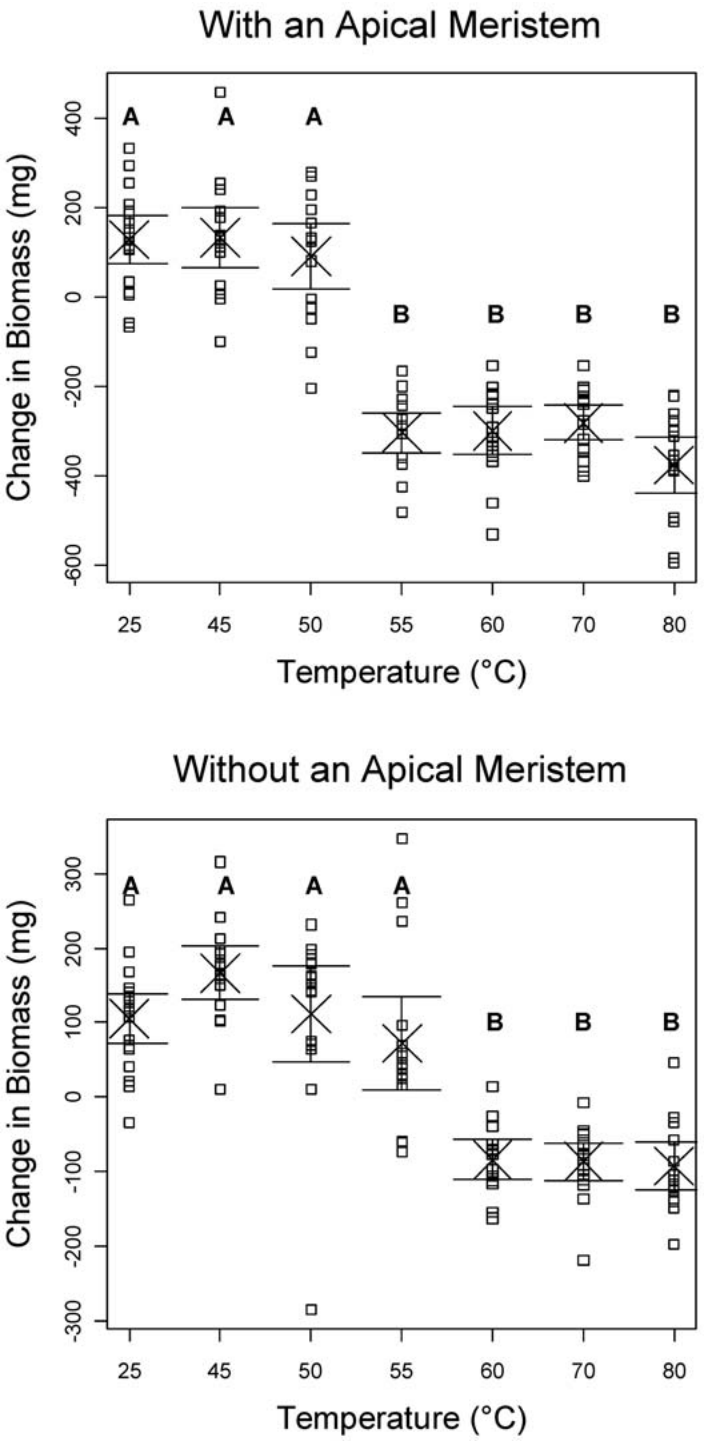

Figure 1. Change in dry mass before treatment and after 30-day observation period vs. temperature treated for all fragments with an apical meristem (top) and without an apical meristem (bottom). The control is indicated at 25 C. Mean (X), and two standard errors (horizontal lines) are included for each temperature (n = 15). Common letters represent no significant difference between temperature treatments (paired t-tests, Bonferroni correction for $\alpha = 0.05$).

ments without an apical meristem treated at 55 C is unknown because no new growth was noted, and epiphyton was removed.

Stanley (1975) considered treated fragments that did not gain biomass after a 32-day incubation period to be dead. In our study, all of the original control and treated 20-cm fragments in every replicate, regardless of time, temperature, or fragment type, were in a state of decay at the end of the 30-day observation period. In many cases the original fragments were almost completely decayed with only one or several small buds of new growth or a few adventitious roots visible. Therefore, treated fragments could lose significant biomass but remain alive. The degree of new growth observed after treatment likely depends on the length of time considered and growing conditions. Stanley (1975) grew fragments in sediment, possibly resulting in greater growth. We grew our fragments in Barko/Smart solution and did not provide nutrients because our aim was to determine if the plants were dead or alive and to avoid algal growth in the jars. The lack of nutrients likely reduced the amount of growth we found but did not appear to affect survival. Our plants survived higher temperature treatments than did the plants used by Stanley (1975), and new growth was supported by existing plant tissue. A combination of change in biomass along with an assessment of new growth is recommended for viability assessment.

Enzyme Activity

Heat treatment is expected to denature cellular proteins and lead to a decline in or complete loss of enzyme activity. This may provide a rapid assessment of plant viability. Glutathione reductase was chosen for analysis because the enzyme is produced constitutively, and relatively large amounts are present in living cells. Reduced glutathione is ubiquitous in plant cells, where it maintains an intracellular reducing environment, and is also required for enzymes involved in cell division and xenobiotic detoxification (Maughan and Foyer 2006). There are also simple and sensitive assay procedures for the reductase that yield rapid results (Smith et al. 1989, Kalt-Torres et al. 1984).

A subset of the treated fragments was assayed for enzyme activity, and the results of 17 enzyme assays for each fragment type were used for further statistical analysis based on consistent activity in replicate samples. Because a limited number of assays were used, data from fragments with and without an apical meristem were first analyzed together by a three-way ANOVA. This analysis showed that time and fragment type were not significant ($p = 0.19$ and $p = 0.52$, respectively), but temperature was highly significant ($p < 0.001$), as expected. Separate analyses for each fragment type revealed that enzyme activity in fragments with an apical meristem was marginally affected by temperature ($p = 0.076$), and enzyme activity in fragments without an apical was significantly affected by temperature ($p = 0.012$). Time was not significant for either type of fragment, and no temperature-time interaction was noted.

After heat treatment at 45 and 50 C, fragments with an apical meristem had levels of enzyme activity that overlapped with values found for untreated control plants (0.410 ± 0.153 U mg^{-1}). Activity after 55 to 60 C treatments was somewhat lower, but still within the range of controls (Figure 2). Based on enzyme activity, most of these plants would be viable. Activity in fragments after 70 and 80 C treatments was only

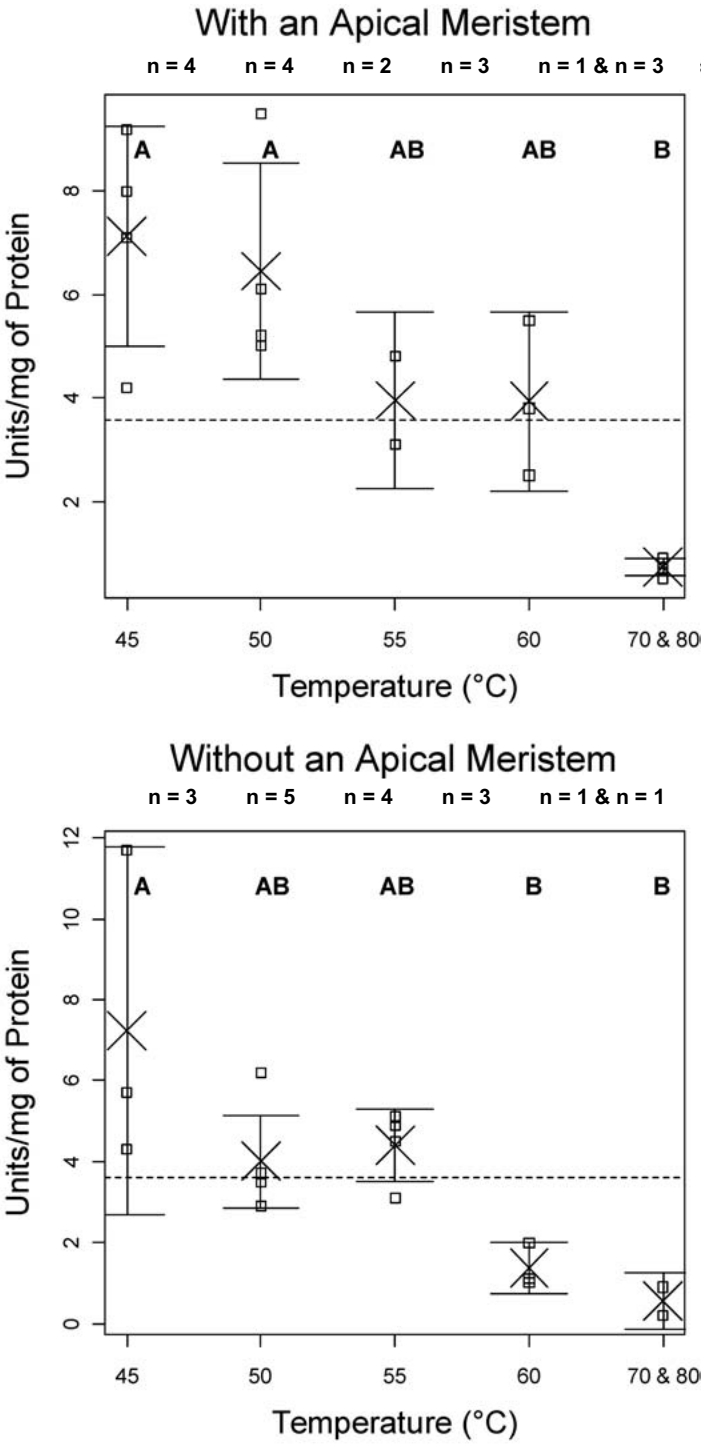

Figure 2. Post-treatment enzyme activity in fragments with (top) and without (bottom) an apical meristem vs. temperature. Mean (X), two standard errors (horizontal lines), and number of samples (n) are included for each temperature. The 70 and 80 C treatment results were combined to allow for pairwise comparisons. Common letters represent no significant difference between treatment temperatures (paired t-tests, Bonferroni correction for $\alpha = 0.05$). The dashed line represents a level of enzyme activity that is 50% of the mean activity of 45 C treatments. Treatments with a mean enzyme activity above this line may still be viable.

slightly above the background oxidation (0.081 ± 0.01 U mg^{-1}) determined on boiled milfoil extract; this treatment is lethal. Fragments without an apical meristem also showed activity in the normal range after treatment at 45 C. Enzyme activity in these fragments declined more rapidly at higher temperatures than in the fragments with an apical meristem (Figure 2). After treatment at 60 C, the activity in the stem fragments with an apical meristem was on the margin of the background oxidation. The difference in the heat stability of enzyme activity in the two types of fragments may be due to differences in size. Ascard (1995) found that plants with less biomass and thinner or fewer leaves were more heat sensitive. In this study, fragments without an apical meristem had less biomass (mean = 1.51 g dm) and fewer leaves than fragments with an apical meristem (mean = 2.22 g dm). The enzyme activity data clearly show that treatment of either type of fragment with temperatures at or below 55 C is not sufficient to kill all the plants. Treatment at 60 C can be lethal but may leave viable tissue, particularly in plants with apical meristems.

Synthesis

Results from all treatments and all methods of determining the viability of fragments (Table 1) show that for the range of temperatures and exposure times we examined, the temperature at which fragments were exposed was more important to viability than the time of exposure. Our results also suggest that fragments with and without an apical meristem may react differently under conditions in the 55 to 60 C range. Although exposure time was generally not a significant factor affecting viability, longer time periods at marginal temperatures in the 50 to 55 C range had greater effect on fragments without an apical meristem than did shorter time periods. Exposure times of 2 min or more likely heated fragment tissue thoroughly for most treatments, but shorter exposure times (e.g., 30 sec or 1 min) may not be sufficient to ensure death.

Our study shows that for any 20-cm fragment, time and temperature exposures much greater than those proposed by Stanley (1975) are necessary to kill fragments. Why did this occur? Perhaps because Stanley (1975) used smaller (4-cm) fragments and assessed the growth of his fragments when placed in sediment. Smaller fragments have less tissue mass to be thoroughly heated to lethal temperatures, so time of exposure may be important. The general lack of temperature-time interactions in our study suggests that our fragments were thoroughly heated under all conditions. Smaller fragments may generally be less viable and more susceptible to stress. Fragments incubated in sediment may be susceptible to increased infection and decay. Finally, lack of growth, as assessed by Stanley (1975), may not be a suitable response by itself to determine fragment viability. Our results, based on three different methods of response, clearly show that treatments at 45 to 50 C for 10 min are not adequate to kill larger fragments.

Temperatures ≤60 C may not be enough to be lethal when considering multiple indicators of death. Visual inspection suggested that 55 C was adequate to kill fragments, but it is unknown if the apparently dead plants would have shown growth after a longer period of observation. In many of the treatments, growth did not become apparent until the last week of observation. Loss of biomass and enzyme activity suggests that temperatures of 60 C or higher are necessary to ensure complete kill. These results agree with the results of thermal weed control done on terrestrial plants, which suggest that essential parts of a plant need to be heated to at least 60 C to be lethal (Levitt 1980, Sirvydas et al. 2003). Note that enzyme activity measured immediately after treatment is an indication of an immediate, acute affect. Plants may be able to recover from some loss of enzyme activity, but structural or other damage may also be important to long term viability. We did not assess enzyme activity in fragments after the initial treatment because our purpose was to get an immediate measure of thermal effect. Furthermore, only a small portion of the plant needs to remain viable to regrow. Determining which part of a fragment remains undamaged or viable is difficult with whole-fragment assays. Further assessment of enzyme assays and their ability to predict viability is warranted.

Based on our results, the use of hot water to kill Eurasian watermilfoil on boats and trailers at boat landings is not a feasible alternative to watercraft inspection. Current recommendations to wash watercraft with 40 C water (Beall 2005) may help to wash invaders off watercraft but will do little to kill moderate size fragments of Eurasian watermilfoil. Temperatures much greater than 40 C are necessary if fragments are to be killed, but higher temperatures could create substantial liability issues. For example, exposure to 60 C water can cause serious burns in adults in less than 5 sec (Grant 2004).

It may be possible to place a chemical additive in wash water to kill fragments at lower temperatures. Adding a chemical oxidant such as chlorine to water is a commonly used and economical way to increase the effectiveness of mussel control in North America. Harrington et al. (1997) found that compared to heat alone, the combined use of heat and oxidants decreased time to 95% mortality in zebra mussels by

TABLE 1. DEAD OR ALIVE RESULTS OF ALL THREE METHODS FOR DETERMINING THE VIABILITY OF FRAGMENTS AFTER TEMPERATURE TREATMENT. AM FRAGMENTS = FRAGMENTS WITH AN APICAL MERISTEM. BS FRAGMENTS = FRAGMENTS WITHOUT AN APICAL MERISTEM.

	45 C		50 C		55 C		60 C		70 C		80 C	
Fragment Type	AM	BS	AM	BS	AM	BS	AM	BS	AM	BS	AM	BS
30-day Obs.	Alive	Alive	Alive	Alive	Dead	Dead	Dead	Dead	Dead	Dead	Dead	Dead
Change in Mass	Alive	Alive	Alive	Alive	Dead	Alive	Dead	Dead	Dead	Dead	Dead	Dead
Enzyme Activity	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Dead	Dead	Dead	Dead	Dead

more than 95% at 30 C. Watkins and Hammerschlag (1984) reported that low levels of chlorine reduce Eurasian watermilfoil growth and vigor. They did not, however, determine lethal concentrations.

A preliminary assessment of two different oxidants, chlorine and bromine, applied at concentrations approved for hot tubs and swimming pools for 10 min at 50 C was promising. In our original study of hot water alone, all jars treated for 10 min at 50 C had observable growth after 30 days. With the addition of chlorine or bromine, plants in only one of the 10 jars treated had observable growth after 30 days. Within that one jar, only one leaf node on one fragment showed growth. Loss of biomass at this treatment level was much greater than with hot water alone. Enzyme activity in the fragments was, however, similar to results with hot water alone. Further assessment of the temperature plus additive effects is warranted and may lead to a practical treatment to kill aquatic plants at temperatures that are safe for routine use in washing watercraft. In addition, assessment of the response of other aquatic plant species to hot water is also needed, and additional tests of Eurasian watermilfoil with smaller fragments, shorter time intervals, and a narrower temperature range will expand our understanding of the utility of hot water to reduce invasive plant spread.

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