Use of *Mycoleptodiscus terrestris* as a mycoherbicide for *Myriophyllum spicatum* (Eurasian watermilfoil) management in the open-water system of the Les Cheneaux Islands, Michigan.

R. Smith, M. Clymer, D. Dunn, S. Myers
Les Cheneaux Watershed Council
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Use of *Mycoleptodiscus terrestris* as a mycoherbicide for *Myriophyllum spicatum* (Eurasian watermilfoil) management in the open-water system of the Les Cheneaux Islands, Michigan.

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Abstract: A field trial to evaluate the ability of *Mycoleptodiscus terrestris* (Mt) to control growth of non-native Eurasian watermilfoil (*Myriophyllum spicatum*), or EWM, was conducted in the Les Cheneaux Islands (LCI), Michigan during the summer of 2017. Objectives of the trial were to confirm effective biological control of EWM by Mt observed during a 2014 trial conducted in the Les Cheneaux area; and to learn if Mt efficacy against EWM could be reproduced when the fermentation process was conducted in a different laboratory and at a larger scale. Findings indicate that the Mt strain, NRRL TX-05, did retain efficacy in controlling EWM growth in the Les Cheneaux open-water system during the 2017 field trial. Moreover, it was learned that Mt efficacy in controlling EWM growth continues when water temperatures are lower than optimum. The degree to which Mt infected EWM was quantified by measuring biomass reduction as a function of time. Follow-up observations one year post treatment suggest that EWM vigor might be reduced during the season following Mt treatment. Results from LCI field trials evaluating the mycoherbicidal activity of Mt for EWM management are encouraging. Based upon our studies of 1/6th and 1/8th acre plots as well as previous data, Mt is a strong candidate as a commercial competitor to chemical herbicides for EWM management in lakes, harbors and waterways.

Introduction: Mt has been studied as a biological control agent for use against such aquatic macrophytes as *Myriophyllum spicatum* and *Hydrilla verticillata* since the 1970's (1). Mt has been isolated from over 26 states and is considered ubiquitous in the United States as well as many parts of the world (1,2,4). In the course of Les Cheneaux Watershed Council research, Mt has been recovered from infected EWM plants collected from multiple northern MI lakes and waterways (3). Historically, most experiments have been conducted as greenhouse applications or at microcosm scale, although some studies were conducted as small acreage field trials in ponds or reservoirs. In all known field experiments, the water bodies in which studies were conducted were warmer (25^{o}-30^{o}C) than the Les Cheneaux waters (15^{o}-24^{o}C) in northern Lake Huron. In addition to Les Cheneaux waters being cooler than water bodies previously studied (2), the Les Cheneaux area is an open-water system in which the waters constantly mix with waters of Lake Huron *vs* all other studies having been conducted in confined water bodies. It was previously unknown if Mt would be diluted and remain effective in an open-water application. Sites to which Mt was applied in this study were restricted by the Environmental Protection Agency to semi-enclosed areas. The only open water sites monitored for EWM growth were two untreated control areas.

MATERIALS AND METHODS

Mt fermentation process:

Mycoleptodiscus terrestris (Mt), NRRL strain TX-05, has been used for LCWC field trials in the Les Cheneaux Islands.

Mt was produced by the United States Department of Agriculture (USDA) Northern Regional Research Laboratory (NRRL), Peoria, IL for a 2014 LCWC field trial and was produced by Wisconsin BioProducts, Milwaukee, WI for the 2017 field trial.

The Mt strain culture medium ingredients (5) and fermentation parameters were the same for production of both batches but the fermentation vessel size differed. The NRRL Mt fermentation was produced in two vessels with a 100L working volume vs 400L of Mt produced by Wisconsin BioProducts in a single vessel with a 500L working volume.

Transport and application: Mt whole culture harvest was completed at Wisconsin BioProducts on 26 July, 2017. The culture was chilled to 4^{o}C, transferred to five gallon (20 L) carboys and each carboy was stored in a Styrofoam container inside a protective cardboard shipping box. Sixteen boxes were loaded on a covered pickup truck, layered with dry ice and plastic tarp, then driven from Milwaukee to Les Cheneaux (Plate 1). Boxes were stored on the truck until application on Friday, 28 July. Dry ice remained between shipping boxes and the Mt culture temperature ranged from 39^{o} to 44^{o}F (3.9^{o} to 6.7^{o}C) at the time of application. Material not used during the first application day was stored at 4^{o}C until used.

Application was achieved using gravity feed from a mix tank through a PVC manifold (Plate 2). Mt whole culture was applied at a rate of 45.6 gal/ surface acre (11.4 lb Active Ingredient) but diluted prior to application with lake water with 20 volumes of water per 1 volume of Mt. Whole culture was diluted to improve Mt distribution throughout the plot to which material was applied.

Sites to which Mt was applied on 28 July included: Cedarville launch ramp(37,865 ft^2) Bumpa's waterfront (10,500 ft^2), Cedarville Marine Marina (9500 ft^2), Breezeswept Harbor (9590 ft^2) and Hessel Harbor (7040 ft^2), (Plates 3,4). Dates of observation and sample collection following application were: 23 Aug (25 Days After Testing: DAT), 28 Aug (30 DAT), 12 Sep (45 DAT), and 7 Oct (70 DAT).

Sample collection and quantification:

As stated in the introduction, sites to which Mt was applied in this study were restricted by the Environmental Protection Agency to semi-enclosed areas. The only open water sites monitored were two untreated control areas. Mt was applied on 28 July 2017 and the final observations were conducted 70 days following treatment on 7 October 2017.

Only the Hessel Harbor site was monitored for quantified analysis due to all other areas except the Cedarville launch ramp being compromised by the respective businesses grooming aquatic vegetation using a mechanical harvester. The Cedarville launch ramp was monitored on a qualitative basis because at the time of Mt application the EWM growth was too dense to accurately quantify.

Change in EWM biomass as a function of time was the criterion used to quantify the degree of EWM infectivity by Mt. Biomass change was reported as milligrams (mg) of EWM wet weight per centimeter (cm) of stem length. Decreased biomass recorded post Mt application indicated an adverse effect of Mt on the EWM plant. By comparison, EWM biomass at untreated control sites exhibited constant or increased biomass during the 2017 study.

Sample quantification:

A grapnel hook used for EWM collection is shown in Plate 5. Samples were processed within 24 hours of collection. Specimens were photographed, followed by determination of wet weight and dry weights, and reported as such. Mean water loss upon drying at 80^{o}C (176^{o}F) for 12 Hr averaged 90.1% for ten different sample with the range in water loss of 88% to 92%. Water loss had stabilized after 12 Hr at 80^{o}C. Since water loss was so uniform among samples, only wet weights were determined for samples collected after the first sampling.

The first step in the sample weighing procedure consisted of selecting stems where the distance between leaflets was less than ten cm because that area was most likely to be contacted by EWM. A long stretch of leafless stem would be less likely to be infected by EWM and would, therefore, skew weight measurements. No branched from stems were included in stem length measurements. Representative stems for each sample were selected for a total length of approximately 200-300 inches prior to processing.

After photographing representative stems, the entire set of stems for a specific sample were then dewatered using a centrifugal salad spinner. After dewatering, samples were weighed to the nearest half gram and then air dried to a stable weight for 72-80 Hr protected by a hardware cloth cover. Dry weights were determined by oven drying as mentioned above for 12 hr and weighed to the nearest half gram after cooling to ambient for one hour.

Conversion of wet weight per inch of stem to weight in milligrams (mg)/centimeter (cm)

(a) wet wt in grams / # inches stem weighed = grams wet wt/inch

(b) Conversion of g wet wt/inch to mg wet wt/cm

—-g wet wt from (a) x 1000—mg wet wt/inch

—-(wt/inch) / 2.54 = wet wt in mg/cm

(e.g.) 58 g wet wt/209 inches of stem—> 0.278 g/inch & that quantity /2.54 = 109 mg wet wt/cm stem length

(c) Use same calculations process for dry weight determination

Safety: In addition to having been shown effective in controlling EWM growth since the 1970's, the environmental and human safety profile of Mt is positive (1,6,7). The hose range of Mt is specific without adverse impact to important nontarget aquatic plants. There has been no adverse effect observed to avian, freshwater fish or freshwater invertebrates. Neither have issues occurred during trials of human dermal, ocular or pulmonary tests. Mt does not grow at mammalian temperatures. Finally, no residual post-application of Mt has been found in aquatic studies.

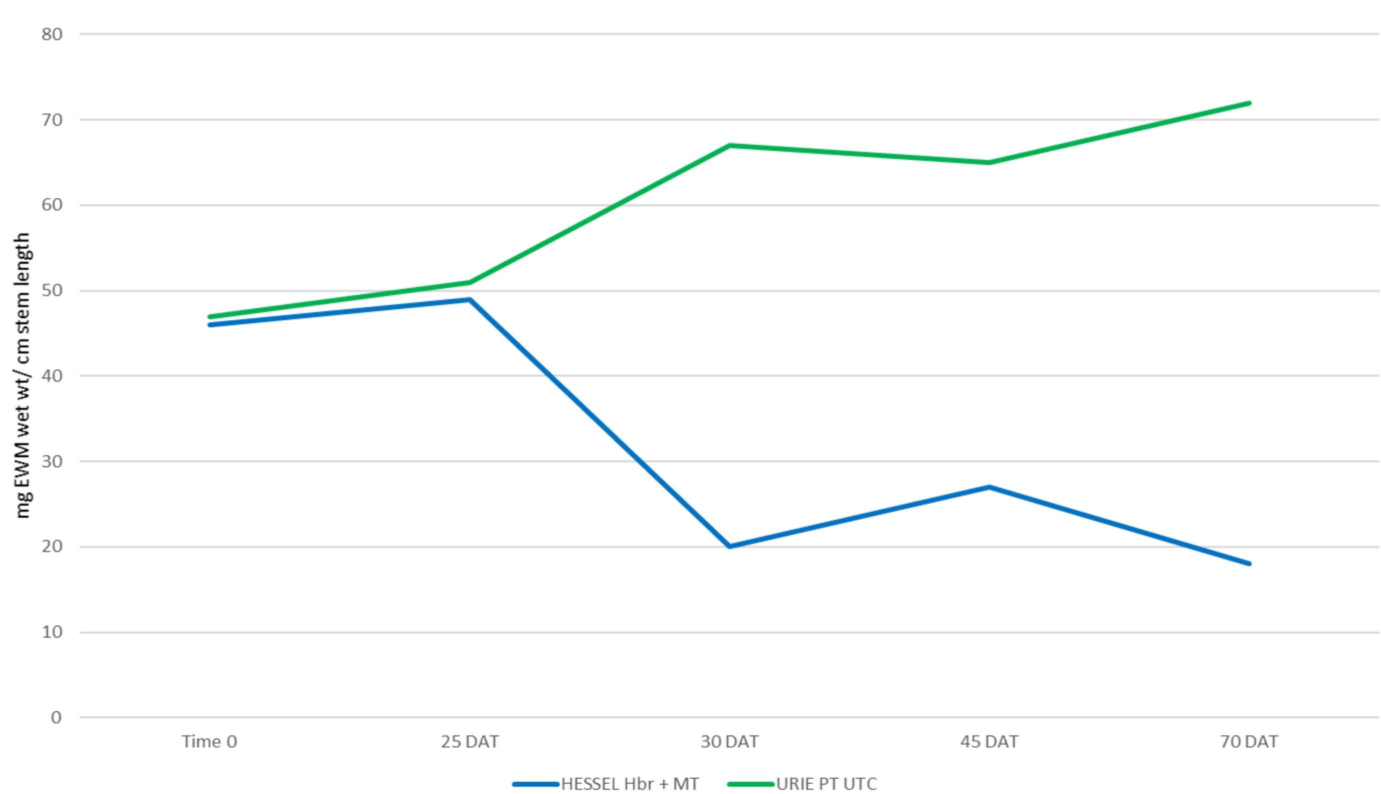
RESULTS AND DISCUSSION

Infectivity and EWM biomass loss was demonstrated during Les Cheneaux field trials in 2014 using Mt produced by NRRL . In 2017 Mt was produced by Wisconsin Bioproducts. Microscopic observation and agar plate counts of greater than 1×10^4 viable microsclerotia mL^{-1} indicated that the physical and viability characteristics of Mt resulting from fermentation at both laboratories were comparable (Plate 6). Reproducibility of Mt culture viability and EWM infectivity between two laboratories indicates the Mt strain stability as well as the reproducibility of the fermentation process and recovery process between laboratories. These parameters are important as the process is scaled up to commercial volumes.

Changes in EWM biomass were measurable between 25 Days After Treatment (DAT) and 30 DAT (Fig. 1). Visual differences were apparent at 25 DAT as indicated by the chlorotic appearance of EWM in the treated area (Plate 7). EWM biomass diminished between 30 DAT and 70 DAT at the treated Hessel Harbor site compared to EWM biomass at the untreated control (UTC) plants at the Urie Point and Howell sites at which EWM remained robust throughout the test period (Plates 8,9). The trial observation period ended at 70 DAT, 7 Oct 2017, because EWM typically enters senescence in LCI shortly after this date. After 70 days EWM plants infected by Mt had no evidence of chlorophyll and leaflets were necrotic, brittle or missing (Plate 10). Observation of continued EWM control throughout the tourist season and EWM growing season indicates that a single Mt treatment can be expected to manage EWM in LCI waters.

Mt application timing was dictated by water temperature and the stage of EWM growth. Water temperature should be in the 20°-25°C range for optimum Mt growth (2,5). Water temperatures in the higher EWM growth areas within LCI typically attain this range by early July. EWM plants are stressed due to low carbohydrate reserves as they approach inflorescence which, in LCI, is late July through mid-August (4). Mt application was scheduled for late July to take advantage of the naturally stressed EWM which would be more susceptible to pathogen incursion.

Fig. 1. Biomass shift as a function of time expressed as mg EWM wet wt /cm EWM stem length <MT FIELD TRIAL DAT 2017> SHT2 DP83....PLUS SHT 5 T40



Two untreated control sites were monitored to reduce bias from a single control site comparison with the test site, due to either higher or lower untreated EWM growth densities (Fig. 2). When EWM biomass at the treated site, Hessel Harbor, was compared to two different untreated control sites, Urie and Howell, about 60% EWM biomass loss was evident by 45 DAT and greater than 70% biomass loss was measured by 70 DAT.

Fig. 2 indicates that the relation of treated site biomass to the untreated sites was virtually the same as reflected by a correlation coefficient of r= 0.99, thereby accurately reflecting the loss in biomass at the Hessel Harbor site when compared to EWM growth at both untreated control sites.

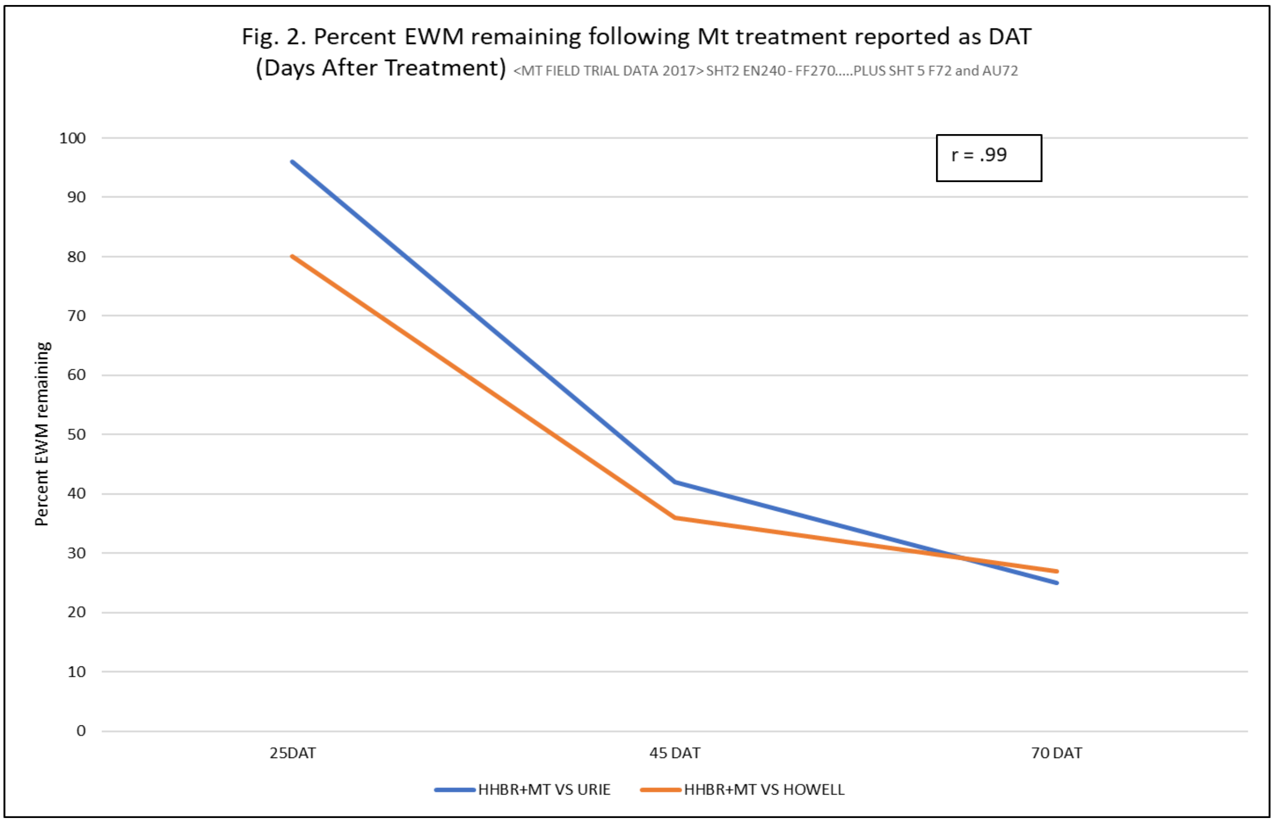


Fig. 2. Percent EWM remaining following Mt treatment reported as DAT (Days After Treatment) <MT FIELD TRIAL DATA 2017> SHT2 EN240 - FF270.....PLUS SHT 5 F72 and AU72

EWM biomass decreased ca 85% in the 2014 LCI trial and ca 75% in the 2017 trial (Fig. 3). Although the 30 DAT was less in 2017, the final biomass decrease was comparable to 2014 results. To achieve efficacy this similar for field trials conducted three years apart is excellent. Having the Mt culture produced by two different laboratories and achieving the observed degree of EWM control is also encouraging. Although Mt culture characteristics were within expectations and the final EWM biomass reduction was similar for both trials, the lower EWM biomass observed during the 2017 trial raised questions. An f-test comparison of the data sets gave a relationship of only 0.2.

Water temperature was fairly constant during the 2014 field trial which contributed to rapid infection and decrease in EWM biomass, but with little increased biomass loss after the first 30 days (Fig. 4A, 4B). Water temperature varied to a greater extent during 2017 which was very likely reflected as a slower Mt infection rate during the first 30 days. That the Mt infection continued during the remainder of the trial is significant for two reasons: First, that biomass loss continued even with the seven degree temperature drop, and two: the final biomass loss was similar to that observed during the 2014 trial.

Using biomass as an indicator of Mt effect of continued impact on EWM, it appears that EWM growth at the Mt application plots was less vigorous than prior to Mt application in 2017 (Appendix B). One year after EWM treatment with Mt, the EWM biomass in the previously treated area was one-half the biomass recorded for an untreated area. This limited evaluation suggests that EWM vigor might be reduced during the season following Mt treatment, based upon EWM biomass in previously treated and untreated areas. If annual reduction in EWM vigor were to occur during successive annual Mt treatments, it is possible that multiple Mt applications could reduce EWM growth to a "minimum nuisance macrophyte" relative to aquatic ecology and recreational activities. Moreover, there appears to be no obvious impact on non-target macrophytes.

Observations from these two open water field trials indicate that Mt can reproducibly and significantly reduce EWM biomass in LCI waters, even when the water temperature is less than optimum.

Summary: The two objectives of this field trial have been achieved: First, mycoherbicidal activity of Mt strain NRRL TX-05 against the target macrophyte, EWM, was retained when the organism was produced by a different laboratory and at a larger scale. Second, the degree of EWM biomass decrease was comparable to findings from a 2014 Les Cheneaux field trial. Finally, it was learned that Mt efficacy in controlling EWM growth continues when water temperatures are lower than optimum. Results from LCI field trials evaluating the mycoherbicidal activity of Mt as well as residual activity for EWM management are encouraging. Based on our studies and previous data, Mt is a strong candidate as a commercial competitor to chemical herbicides for EWM management in lakes, harbors and waterways.

Fig. 3. Percent EWM decrease following Mt application in 2014 and 2017 studies

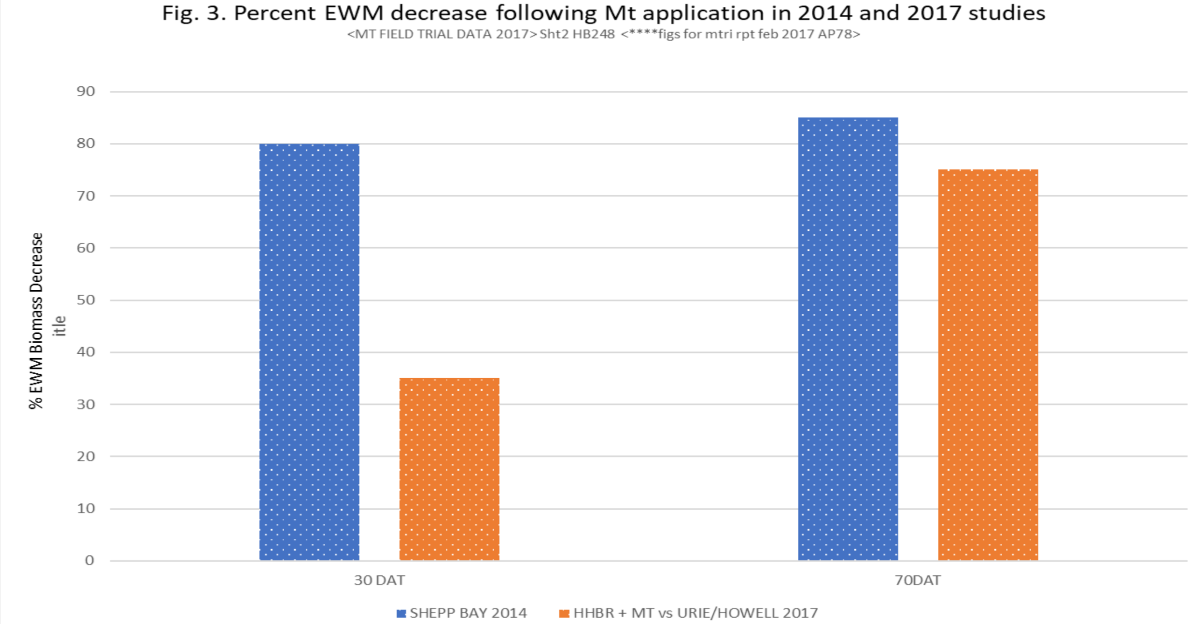


Fig. 4A. Percent EWM biomass decrease in Sheppard Bay 2014 field trial with temperature profile (degrees C).<WATER TEMP COMPARISON BC 2014 vs 2017 201217> AL62 SHT2<figs for mtri rpt feb 2017>

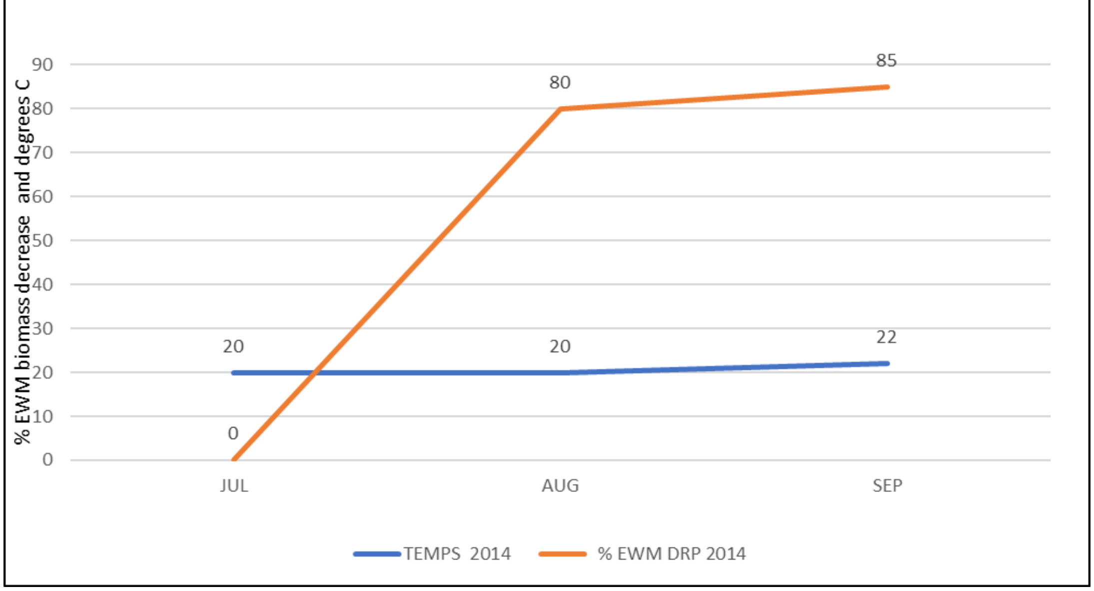
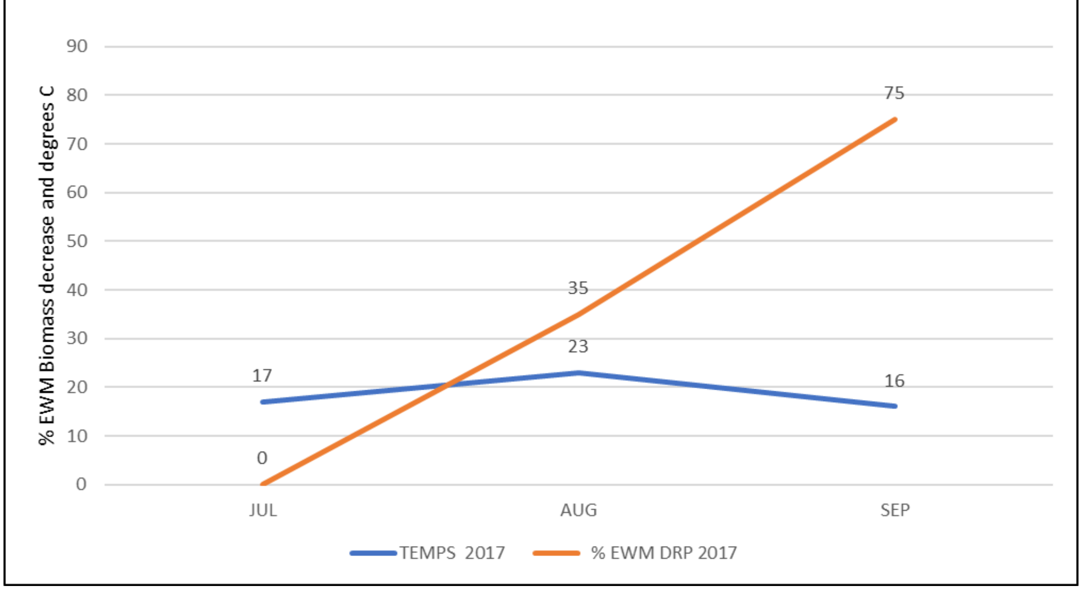


Fig. 4B. Percent EWM biomass decrease in Hessel Harbor 2017 field trial with temperature profile (degrees C). <WATER TEMP COMPARISON BC 2014 vs 2017 201217> AL62 SHT2<figs for mtri rpt feb 2017>



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(3) Shearer, J.F. 2012, 2015; Pers. Comm.

(4) Smith, C.S. and J.W. Barko. 1990. Ecology of Eurasian watermilfoil. J. Aquat. Plant Manage. 28:55-64

(5) U.S. Patent 6,569,807 B1. Shearer, J.F. and M.A. Jackson. 27 May 2003. Mycoherbicidal compositions and methods of preparing and using the same.

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Acknowledgements

We thank Mark Jackson for providing experimental Mt culture for our 2014 field trial and for consulting in the Mt fermentation process. Thanks to Judy Shearer for Mt isolation studies from EWM samples collected from Michigan waterways. This work was supported by US EPA grant GL-00E01928-0 “Les Cheneaux Islands Eurasian watermilfoil Control”.

APPENDIX

Appendix A Contents:

LOCATION OF LES CHENEAUX ISLANDS ON THE NORTH SHORE OF LAKE HURON AND EAST OF THE STRAITS OF MACKINAC

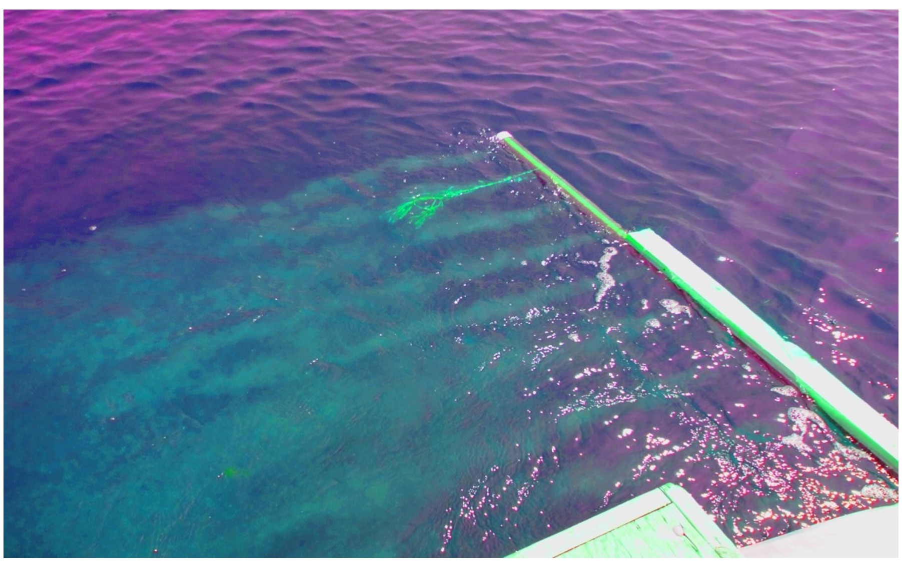
Plate 1

LCWC Mt application apparatus

Mt aqueous suspension was applied through a manifold constructed from PVC pipe.

Manifold was kept on deck en route to applications sites. (Left)

Manifold was lowered into the water during application for transfer efficiency and to prevent aerosol.



Mt application:

Lower photo was enhanced to demonstrate application plume uniformity. No dyes were used.

Plate 2

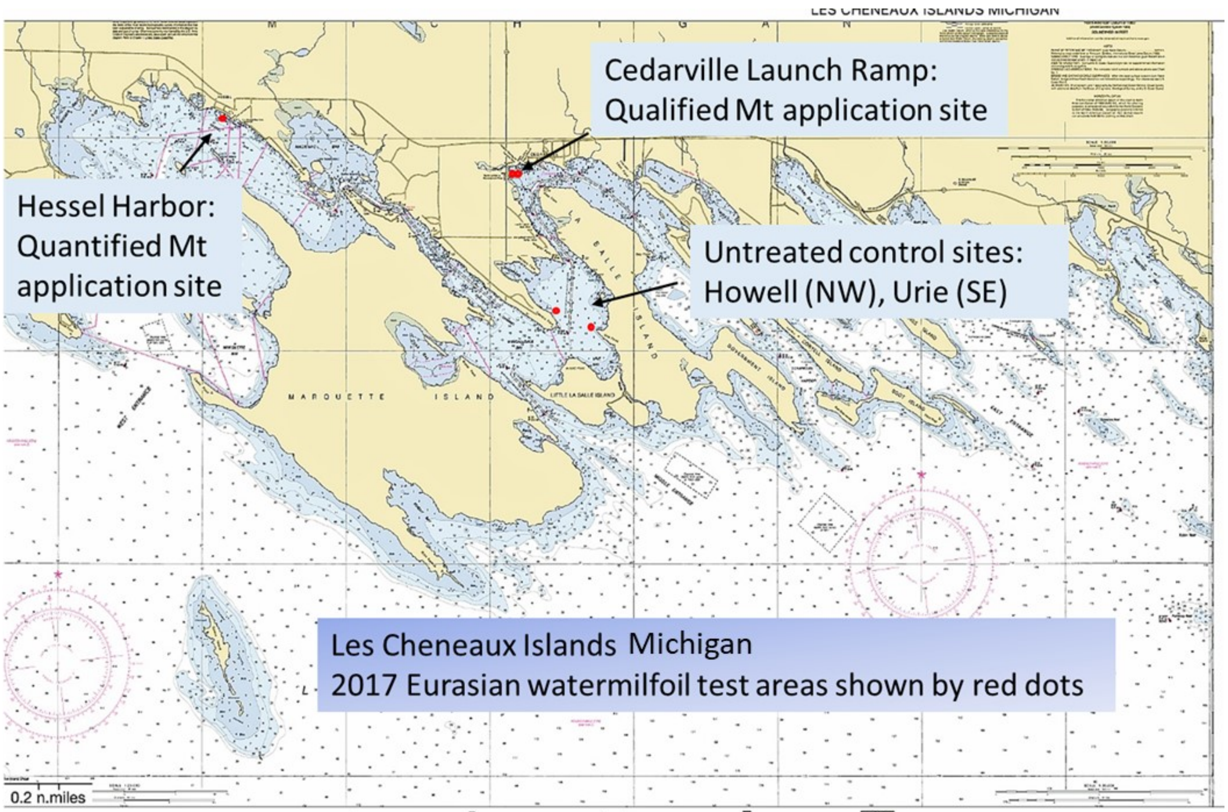

LES CHENEAUX ISLANDS MICHIGAN
Cedarville Launch Ramp:
Qualified Mt application site
Hessel Harbor:
Quantified Mt
application site
Untreated control sites:
Howell (NW), Urie (SE)
MARQUETTE ISLAND
Les Cheneaux Islands Michigan
2017 Eurasian watermilfoil test areas shown by red dots
0.2 n.miles

Plate 3

Hessel Harbor Mt application area: 7040 ft^2

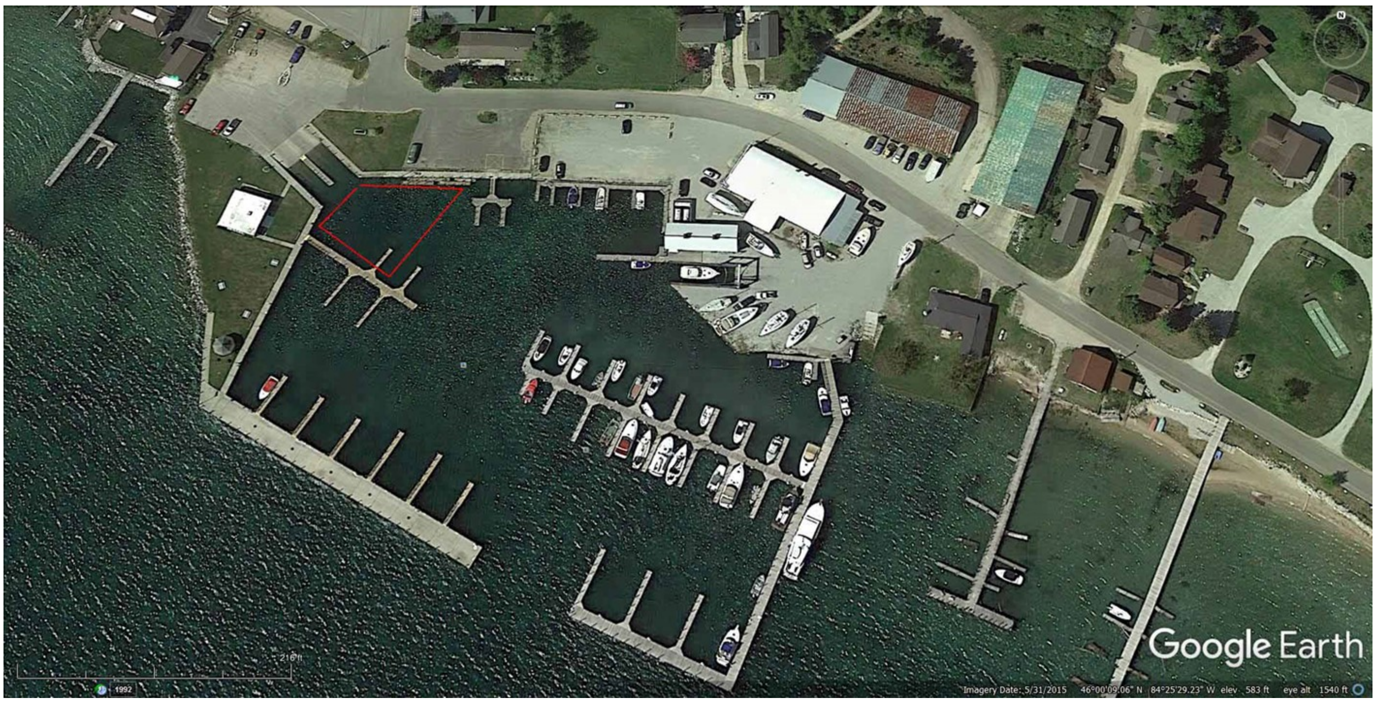
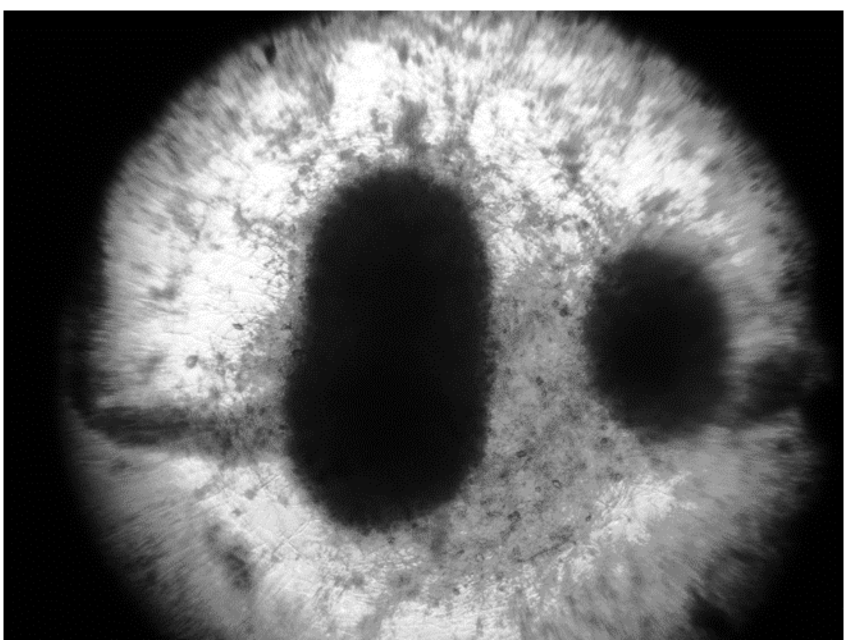


Plate 4

Grapnel hook used for EWM sampling.

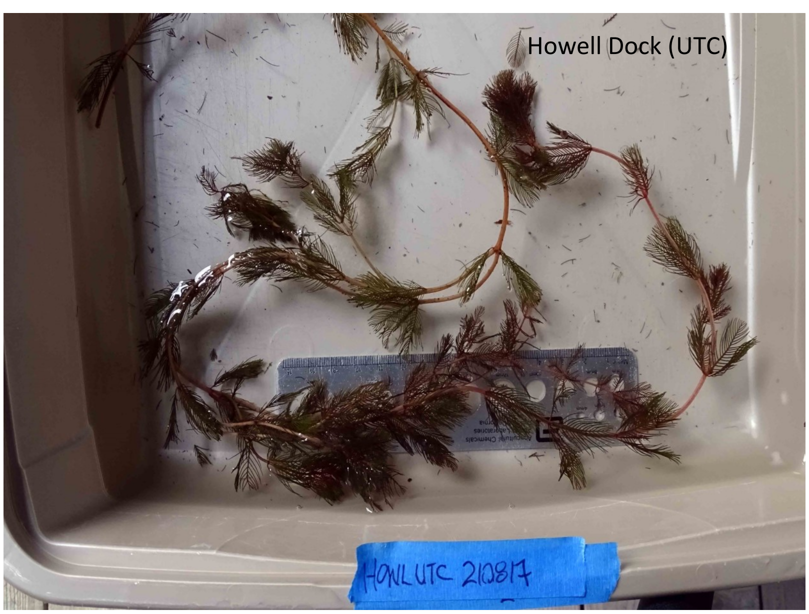
Plate 5

Mt vegetative mycelial growth phase midway through fermentation process shown at 400 magnifications



Mt microsclerotia at end of fermentation process shown at 40 magnifications. Microsclerotia are considered the structures which infect *M. spicatum.*

Plate 6



Although biomass among treated (TX) and untreated control (UTC) sites was indistinguishable at 25 DAT two differences are worth noting.

First the leaflets from untreated sites appear healthier vs the seemingly chlorotic treated Hessel Harbor sample.

Second, stems from the untreated sites were the more typical burgundy color.

These observations may indicate stress of the treated EWM.

TX: Hessel Harbor

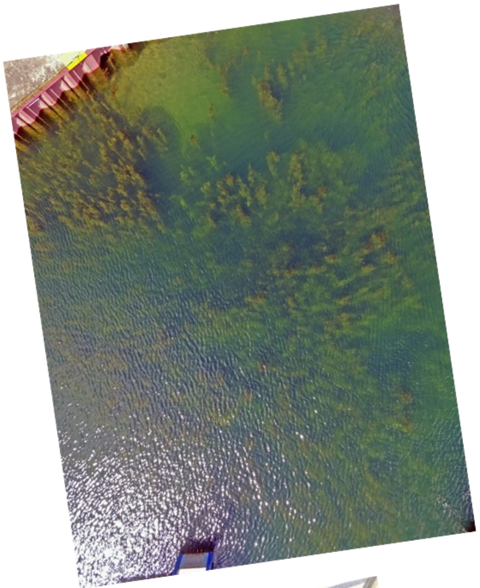
UTC: Urie Point and Howell Dock

Plate 7

Mycoherbicidal effect of Mt on EWM in Hessel Harbor during 70 day trial.

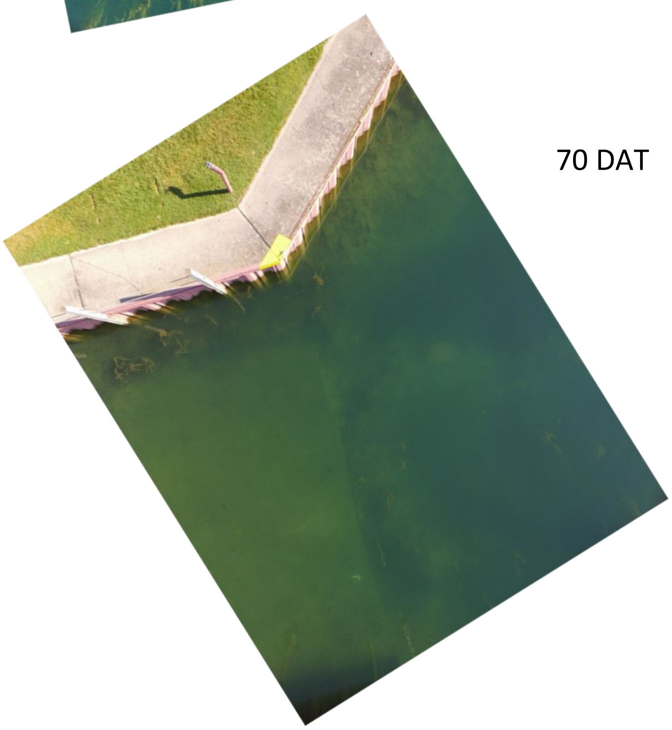
Results indicate a single Mt application is capable of controlling EWM growth for one season.

Altitude: 10 M



Pretreatment

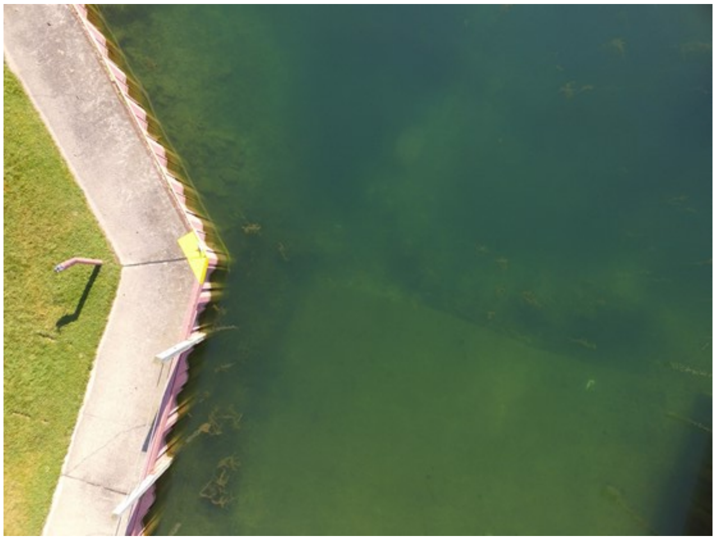
30 DAT



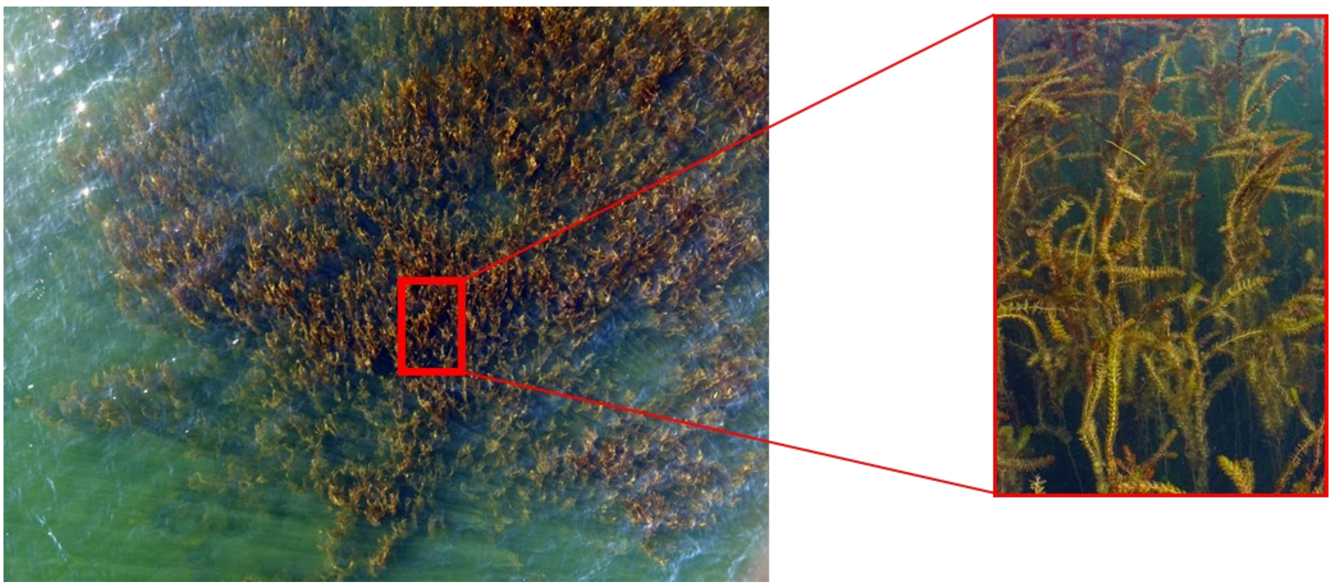
70 DAT

Plate 8

Comparison of Mt-treated vs untreated control EWM beds 70 DAT



RIGHT: Mt-treated EWM bed virtually void of growth 70 DAT. Altitude 10M



ABOVE: EWM untreated control bed at Urie Pt 70 DAT from 10M. Inset view from research vessel deck.

BELOW: EWM untreated control bed at Howell site 70 DAT. Inset view from 10M.

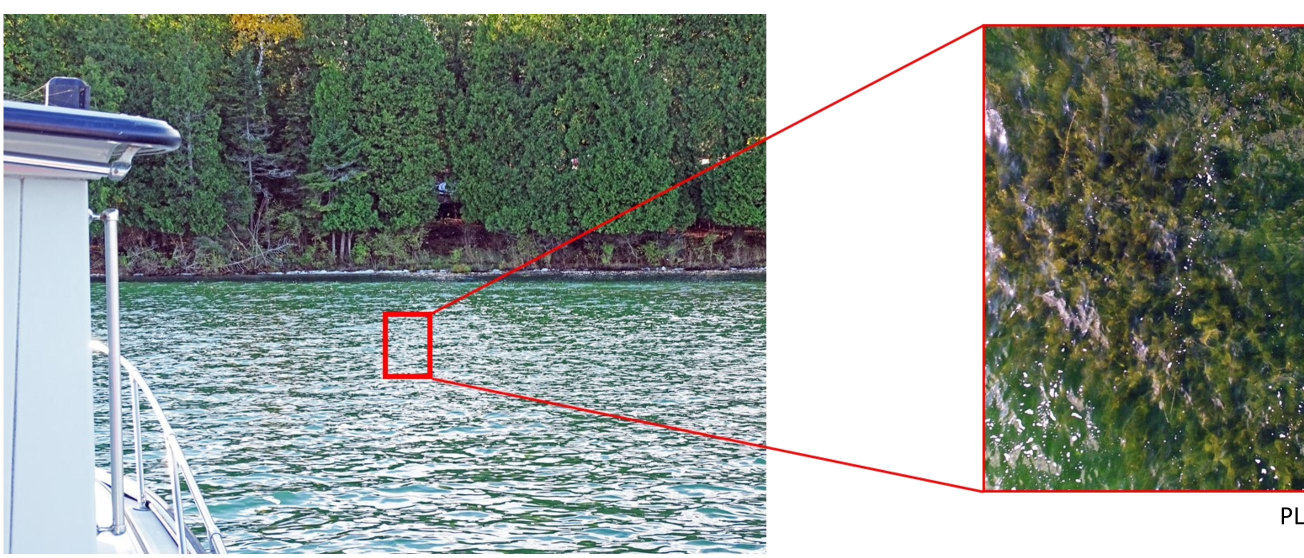


PLATE 9

Mt mycoherbicidal effect on EWM at Hessel Harbor field trial site 70 days post treatment.

EWM pretreatment

EWM 70 days post Mt treatment

Plate 10

APPENDIX B

Seasonal residual effect on *Myriophyllum spicatum* (Eurasian watermilfoil) vigor following *Mycoleptodiscus terrestris* (Mt) treatment.

APPENDIX B

Seasonal residual effect on EWM vigor following Mt application.

Summary: *Mycoleptodiscus terrestris* (Mt) was found to effectively control *Myriophyllum spicatum* (Eurasian watermilfoil, or EWM) growth during multiple 1/8th acre field tests conducted in the Les Cheneaux Islands in 2014 and 2017.

This is a report of observations conducted in areas treated with Mt one year after application in 2017 to assess residual impacts from the treatment. Using biomass as an indicator of Mt effect of continued impact on EWM, it appears that EWM growth at the Mt application plots was less vigorous than prior to Mt application in 2017. One year after EWM treatment with Mt, the EWM biomass in the previously treated area was one-half the biomass recorded for the untreated area. Post treatment EWM biomass was also less in a plot assessed qualitatively. This limited study suggests that the EWM vigor might be reduced during the season following Mt treatment, based upon EWM biomass in previously treated and untreated areas. If annual reduction in EWM vigor occurs during successive annual Mt treatments, it is possible that multiple Mt applications could reduce EWM growth to a "minimum nuisance macrophyte" relative to aquatic ecology and recreational activities. Moreover, there appears to be no observed impact on non-target macrophytes. Additional field trials are recommended to confirm these observations by organizations intending to develop Mt on a commercial scale.

Method:

A custom grapnel hook 18 cm diameter with 8 tines 19 cm in length was secured to a 15.2 M nylon line 6 mm in diameter for use in sampling macrophyte growth. The hook was tossed the length of the line and allowed to settle to the bottom before beginning a slow, steady retrieval. Macrophytes were removed from the hook and placed in a labeled bag for later identification and analysis. Samples were rinsed with fresh water until the water ran clear before photographing at 2.7 X. The entire biomass for each macrophyte type was weighed and recorded. Wet weights were determined to the nearest 0.5 g after spinning off excess water using an Oxo household centrifugal salad spinner. Macrophytes were identified according to Skawinski, 2011: ISBN-13: 978-1-4507-9247-9.

Results and Discussion:

Hessel Harbor was the site where Mt activity against EWM was quantified in 2017.

EWM biomass at Hessel Harbor in 2018 was minimal among other macrophytes when compared to a virtual EWM monoculture at the untreated Urie Pt control site (Fig. 1). Overall macrophyte density at Hessel Harbor appeared similar to growth observed 30 days following Mt application in 2017 (Fig.2).

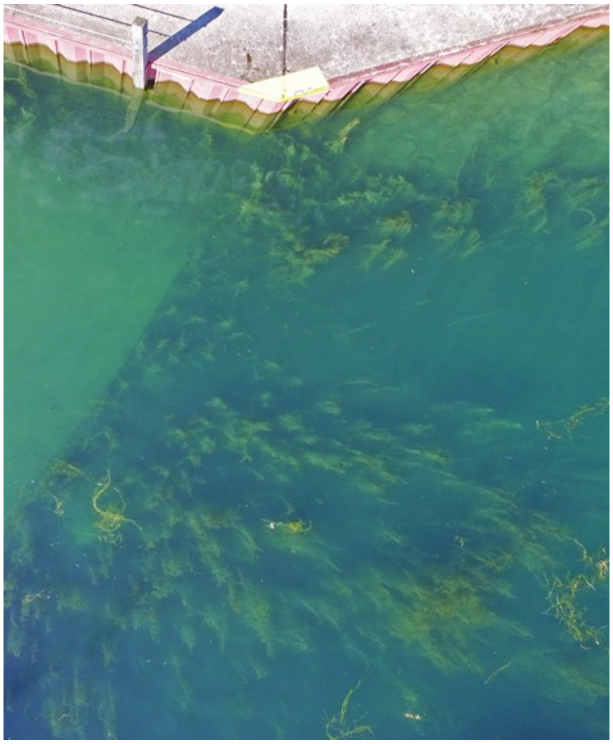
As in 2017, multiple grapnel hook tosses were required at Hessel Harbor to obtain sufficient EWM for evaluation. Macrophytes competing with EWM during this survey and average wet weights included: *Elodea canadensis* (12g); *Potamogeton zosteriformis*; Flatstem pondweed (49g); *P. crispus*, Curly-leaf pondweed (81g); *P friesii* , Fries' pondweed (196g) and *Ceratophyllum demersum*, Coontail (<10 g); which when combined accounted for 86% (338g) of the Hessel Harbor biomass vs. 14% (57 g) of EWM (Fig. 3, 4). See Plates 1-6.

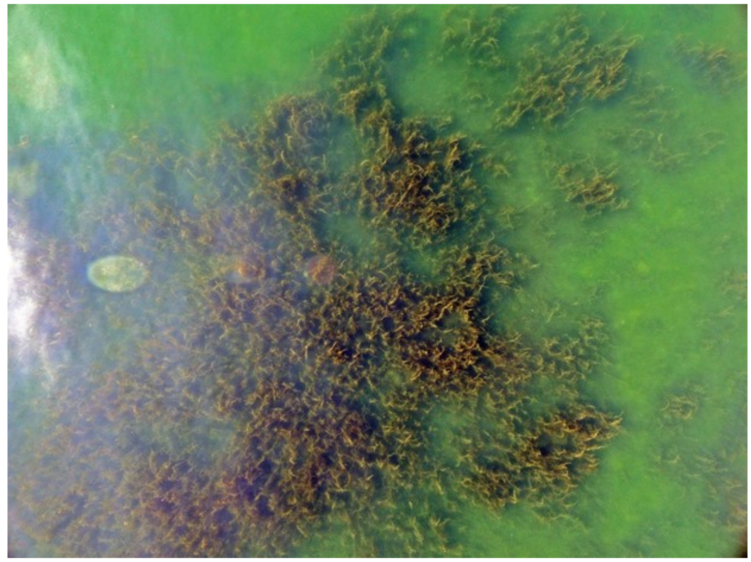
Cedarville Launch Ramp (CRAMP)

Efficacy of Mt at the Cedarville launch ramp was reported as qualitative data in 2017 due to dense macrophyte growth and due to part of the site being compromised during the season by mechanical harvesting of EWM.

Aerial images showed 2018 macrophyte density at CRAMP less than in 2017 (Fig. 5). No EWM was recovered from three grapnel tosses although the total macrophyte biomass per hook toss was *ca*. 1 Kg (Fig.7). Macrophytes present included: *Elodea canadensis, Ceratophyllum demersum* (Coontail) and *Potamogeton friesii* (Fries' pondweed). See plates 7-9.

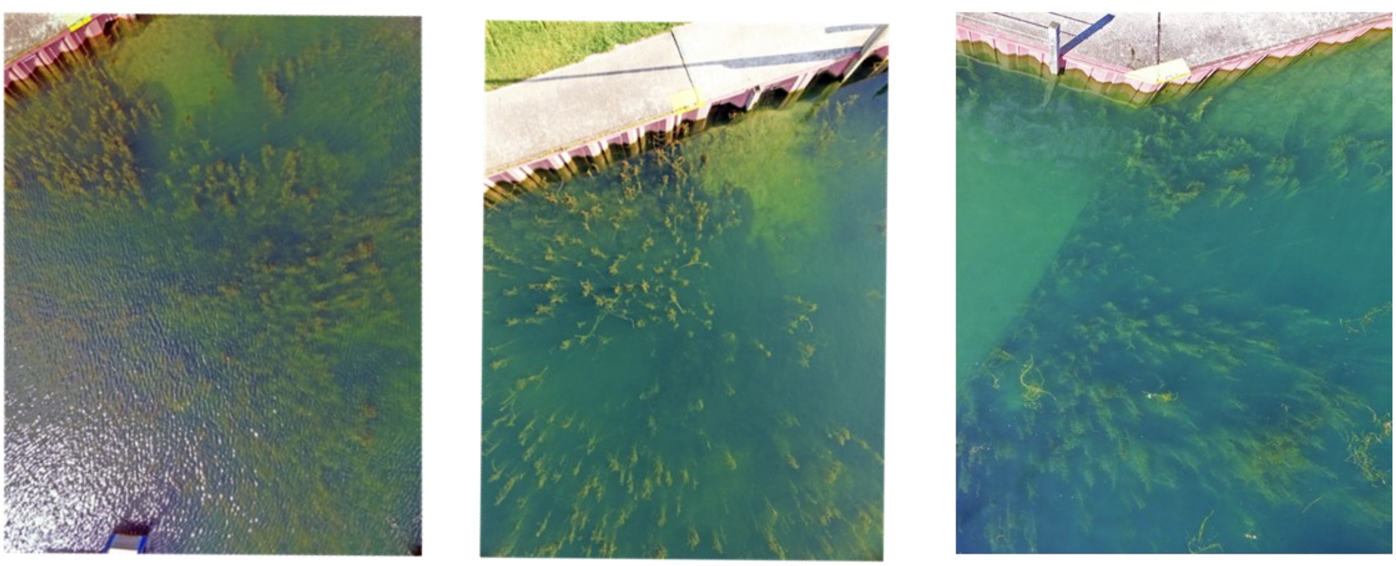
FIG. 1. COMPARISON OF MACROPHYTE DENSITY AT HESSEL HARBOR (treated) vs URIE POINT





Hessel Harbor, left. Urie Point, above. Difference in macrophyte appearance is due to minimal EWM growth at the Hessel site. Urie Pt was a monoculture of EWM. Bare area at upper left of Harbor image is the end of a concrete ramp.

Fig. 2. Hessel Harbor Eurasian watermilfoil growth one year after Mt treatment.



Pretreatment 2017 | 30 days post treatment 2017 | One year following treatment

Non-EWM macrophyte biomass was 6X greater than EWM one year post treatment.

Fig. 3. Eurasian watermilfoil shown as percent of total biomass one year after Hessel Harbor (HHBR) treatment.

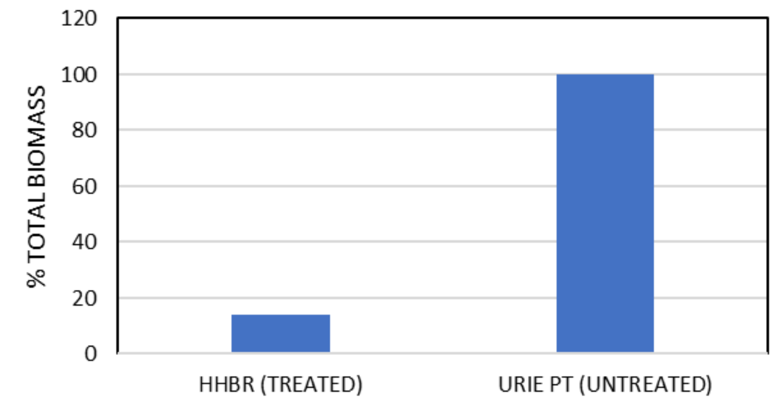


Fig. 4. EWM biomass one year following Hessel Harbor (HHBR) Mt treatment

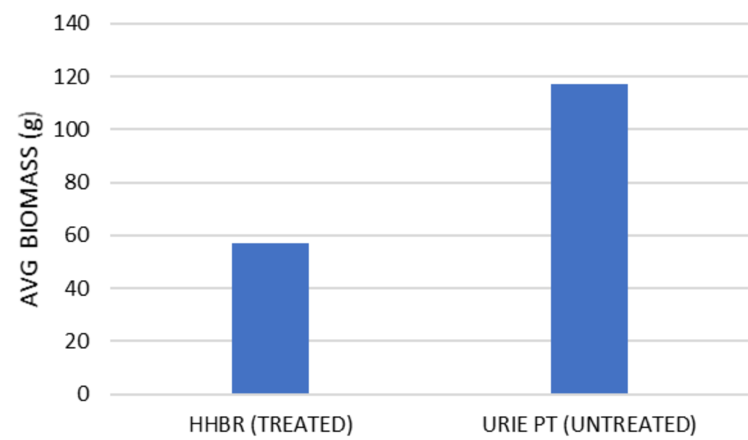
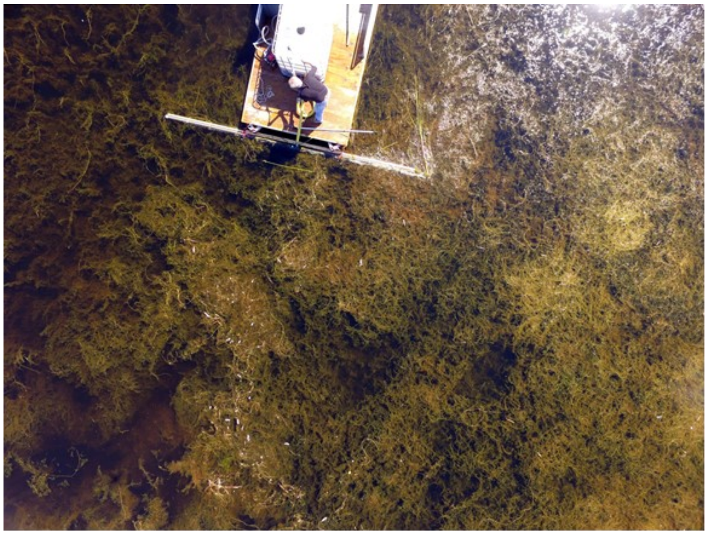


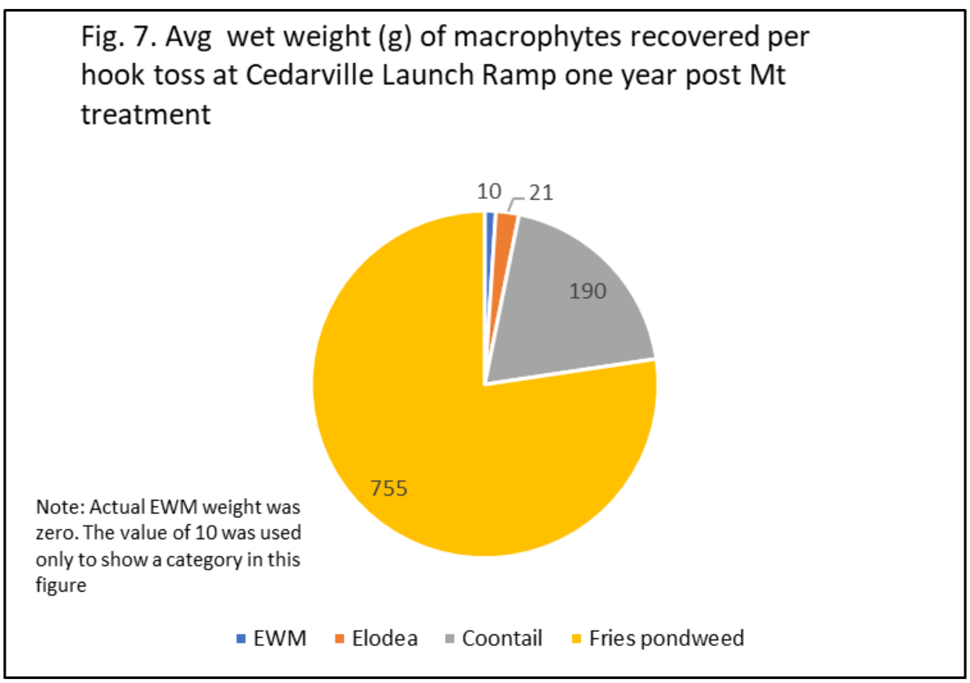
Fig. 5. Cedarville launch ramp one year following Mt treatment.



Biomass during 2017 treatment

Biomass one year post Mt treatment

Three non-EWM macrophytes dominated Cedarville launch ramp growth one year post treatment.



Hessel Harbor Macrophytes Recovered One Year Following Mt Treatment

Plate 1:

Myriophyllum spicatum: Eurasian watermilfoil

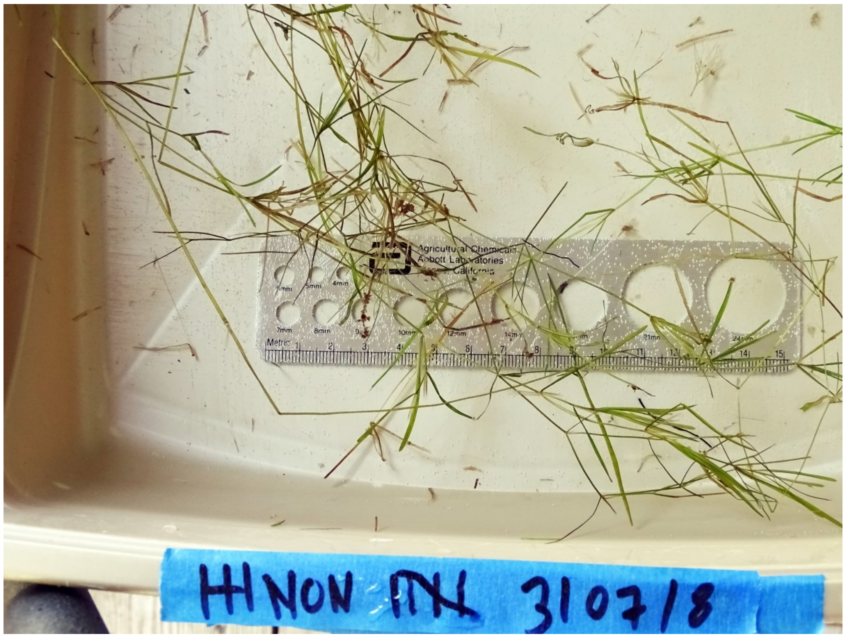
Plate 2. *Elodea canadensis*:

Plate 3. *Potamogeton zosteriformis*: Flatstem Pondweed

Plate 4. *Ceratophyllum demersum*: Coontail

Plate 5. *Potamogeton crispus*: Curly-leaf Pondweed

Hessel Harbor Macrophytes Recovered One Year Following Mt Treatment, cont'd



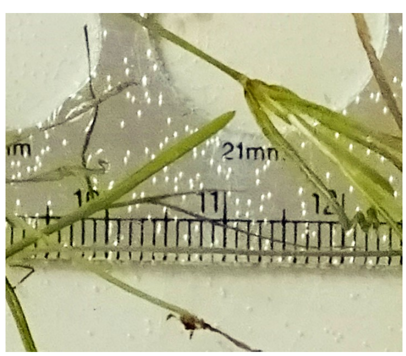


Plate 6.

Potamogeton friesii, or

Fries Pondweed

All three images are of the same sample. The lower two images exhibit diagnostic features of *P. friesii* with the bottom image showing a distinguishing leaf tip.

CEDARVILLE LAUNCH RAMP MACROPHYTES ONE YEAR AFTER Mt TREATMENT

<CRAMP MACROPHYTES ONE YEAR POST 050818>

Plate 7

Ceratophyllum demersum: Coontail

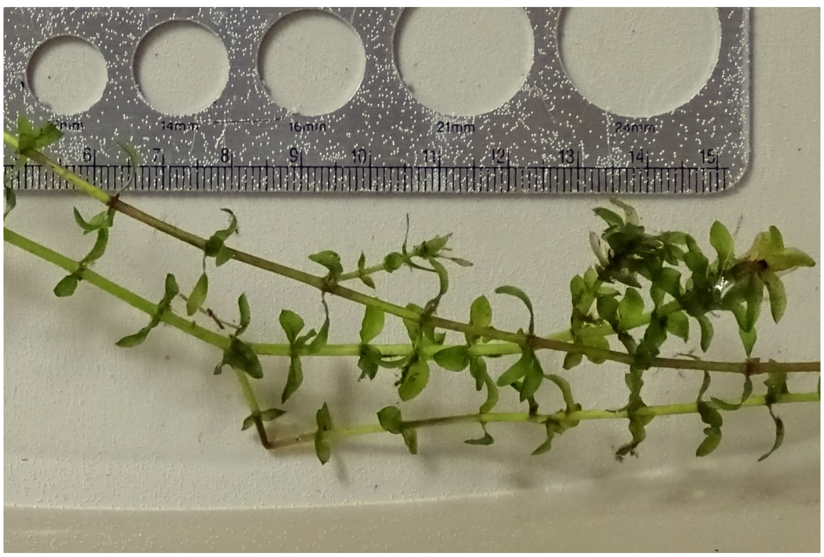


Plate 8

Elodea canadensis: Elodea

Plate 9

Potamogeton friesii: Fries Pondweed