

Survivability of starry stonewort bulbils using commonly available decontamination strategies

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ABSTRACT

Starry stonewort, *Nitellopsis obtusa* (Desvaux in Loiseleur) J. Groves, is a benthic green alga native to the Old World. It is considered an aquatic invasive in much of the Great Lakes region. Although numerous strategies exist for limiting the further spread of such aquatic invasives, it is unclear how effective these strategies would be for starry stonewort, which propagates via vegetative propagules termed bulbils. In this study, we test the effects of freezing, desiccation, steam, and bleach on starry stonewort bulbils. A significant negative impact on the survivability of *Nitellopsis obtusa* bulbils was observed after treatments consisting of freezing, desiccation, steam, and bleach. Bulbils subjected to desiccation or freezing were tested by two measures of viability: the germination assay and the tetrazolium assay, and there were significant—apparently complete—reductions in bilbil viability after 24-h treatment durations (100% reduction as measured by the germination assay, and 91% or greater average reduction in viability signal as measured by the tetrazolium assay). The tetrazolium assay in particular, coupled with semiautomated image processing from microscope images, will be useful for similar studies in the future, as it provides a comparatively rapid assessment of bilbil viability. The apparent sensitivity of bulbils to some of these treatments is relevant to both distribution modeling and to the management of this invasive aquatic alga.

Key words: Little Muskego Lake, WI, *Nitellopsis obtusa*, Silver Lake, WI, starry stonewort, tetrazolium assay, Upper Little York Lake, NY.

INTRODUCTION

Nitellopsis obtusa (Desvaux in Loiseleur) J. Groves (commonly known as starry stonewort) is a charophytic green alga native to fresh and brackish waters in Europe and Asia (Soulié-Märsche et al. 2002). Despite rarity in its native range (Kato et al. 2014), it has been labeled an aggressive aquatic invader in the United States (Kipp et al. 2018). Much is yet to be understood about the ecological impacts of *N. obtusa* in North America. It has been observed to be negatively associated with species richness where *N. obtusa* biomass is high (Brainard and Schulz 2016), and could also affect water chemistry and nutrient cycling of a lake in

similar ways to other characean green algae (Kufel and Ozimek 1994, Kufel and Kufel 2002, Larkin et al. 2018). Because of its potential to alter North American water bodies, methods are actively being explored to control and limit its spread.

The earliest confirmed North American record of *N. obtusa* is from a specimen collected in 1974 or earlier in Québec (Karol and Sleith 2017). The species is thought to have been introduced via ballast water along the St. Lawrence river (Geis et al. 1981) and has spread across at least eight states and two Canadian provinces: Indiana, Michigan, Minnesota, New York, Ohio, Ontario, Pennsylvania, Québec, Wisconsin, and Vermont all have confirmed reports (Alix and Scribailo 2010, Eichler 2010, Sleith et al. 2015, VDEC 2015, Wisconsin Department of Natural Resources [DNR] 2015, Midwood et al. 2016, Kipp et al. 2018).

Since its introduction to North America, there has been some speculation as to the manner of dispersal for *N. obtusa*, and whether it mirrors its dispersal in its native range. The available evidence points to vegetative reproduction via bulbils and fragments as the dominant mode of reproduction for *N. obtusa* in its introduced North American range. In its native range, *N. obtusa* propagates both sexually via oospores and asexually via bulbils and fragments (Soulié-Märsche et al. 2002). The sexual propagules (oospores) of some Characean have been observed to survive the digestive tracts of birds, and are likely assisted by birds in dispersal between water bodies (DeVlaming and Proctor 1968, Charalambidou and Santamaría 2002, Brochet et al. 2009). In its introduced range, sexual propagation via oospores has not been documented, as only male plants have ever been observed. *Nitellopsis obtusa* is dioecious, with the female oogonia and male antheridia borne on separate plants (Wood and Imahori 1965). Previous work describes female plants in North America (Pullman and Crawford 2010); however, these images and descriptions of “oocytes” have subsequently been determined to be male antheridia and no vouchered or otherwise confirmed reports of oogonia-bearing plants in North America have been made (Sleith et al. 2015). Dispersal in the introduced range therefore seems to be limited to—or at least heavily reliant on—the vegetative structures of *N. obtusa* and not the sexual structures such as oospores.

Vegetative reproduction in *N. obtusa* can occur through fragmentation of the plant thallus or through specialized structures termed bulbils. These bulbils are star shaped—from which the common name “starry stonewort” is derived—and can occur in large numbers at the rhizoidal nodes of the plants. It is unclear what role birds or wildlife

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play in the dispersal of bulbils and *N. obtusa* fragments in the North American range. Human dispersal, though, is thought to play a significant role in its movement across North America (Midwood et al. 2016). A number of decontamination strategies are being explored to limit the impact of human-assisted dispersal, and are similar to strategies used for other aquatic invasive plants including physical removal of plants from boats and gear, drying out contaminated gear for a period of time, steam treating boats and gear, and the use of bleach solutions to kill plants and propagules (Wisconsin DNR 2015). Although Characeae oospores have been observed to remain viable over years of desiccation (Casanova and Brock 1996), and to survive bleach (5% NaOCl for 1 min) or UV (9030 Lux for 24 h) treatments (Wetzel and Mcgregor 1968), the viability of bulbils under different conditions has not been assessed. Understanding physiological limits of bulbils will assist in both the development of management strategies to limit the spread of *N. obtusa*, and in modeling efforts to predict its future occurrences.

The focus of this work was to evaluate the effectiveness of existing decontamination strategies and environmental controls on limiting bulbil survival and germination. We used desiccation, freezing, bleach, and steam treatments in an attempt to render bulbils nonviable compared to control groups.

MATERIALS AND METHODS

Bulbils were collected from sites in the eastern and western portions of the range invaded by *N. obtusa*: Upper Little York Lake, NY (ULYL, 42°42'30"N, 76°9'5"W); Little Muskego Lake, WI (LML, 42°55'23"N, 88°8'31"W); and Silver Lake, WI (SL, 43°23'35"N, 88°12'49"W). Plants were cropped to remove most of the upper vegetative material and transported in 5-gallon buckets or shipped in¹ Whirl-Paks™ to Bronx, NY. Bulbils were separated from the rhizoids using forceps and a dissecting scope. The bulbils were stored at 4 C in Forsberg's Medium II (FMII) liquid media (Anderson 2005). Prior to each treatment, the subset of bulbils to be used were binned into five size classes and equal numbers of each size class were included for each treatment and duration.

Treatment conditions were applied to bulbils in batches of five as outlined below. Bulbils were kept in the designated condition (control, desiccation, freezing, steam, bleach) for a set period of time—the treatment condition—with corresponding “control” bulbils kept at 4 C for the same duration. When possible, we employed two measures of bulbil viability. We tallied germinations (successful germinations interpreted as green *N. obtusa* growth > 5 mm) in the germination assay (outlined below, only for desiccation and freezing experiments compared to controls), or we measured the positive signal (a change from white to red) for cellular respiration as outlined in the tetrazolium assay (also outlined below, used for all treatments). The germination assay served to not only test the efficacy of the freezing and desiccation treatments, but also to provide benchmarks for interpreting the tetrazolium assay. We assumed that the control bulbils for

experiments using the germination assay would have the same viability as those using the tetrazolium assay and a more meaningful interpretation of the tetrazolium assay could be made.

Liquid medium. Forsberg's Medium II (FMII) was used in all experiments following Anderson (2005). The pH was adjusted to 8.0 using HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), to achieve the average measured pH in the native range (Larkin et al. 2018). Two hundred milliliters of FMII was used for the applicable treatments and conditions.

Substrate. Fine-grain sand was rinsed 10 times or until water would clear within 1 to 2 s. The rinsed sand was baked twice for 4 h each time at 220 C with a 20-min soak followed by rinsing in water between baking to decrease the chance of potential external contaminants. Bulbil stock was not sterile, so we made no further attempts to sterilize the substrate. Approximately 30 g of sterilized sand was added as substrate to Magenta™ (GA-7) boxes² for each of the treatment conditions and bulbils were placed on top of the sand substrate. The sand served primarily as anchorage for the bulbils' developing rhizoids.

Controls. For each experiment, control bulbils were placed in a Magenta™ (GA-7) box containing the sand substrate and FMII media. Controls were kept at 4 C for the treatment duration and then subjected to either the germination or tetrazolium assay as outlined below.

Desiccation treatments. Bulbils were placed on substrate within culture boxes kept in desiccators³ maintained at either of the two humidity levels. No liquid FMII media was added until the end of the treatment period, so these are termed “dry” conditions. Two humidity regimes were included as separate treatments. Dry substrate, dry air (dry/dry) conditions were established as 20 to 40% humidity. Dry substrate, humid air (dry/humid) conditions were at 80 to 100% humidity. Humidity was tracked for each desiccator using a digital hygrometer (Fisherbrand™).⁴

Freezing treatments. Culture boxes containing substrate, FMII liquid media, and bulbils were placed into either a –20 or –6 C freezer for the duration of the treatment.

Steam treatments. A steam rig was assembled using a handheld steamer⁵ mounted on a stand a fixed distance away from a platform such that the measured temperature of the steam was ~60 C at the platform. Bulbils were introduced five at a time on a Magenta™ (GA-7) box lid for the duration of each of the treatments. Three replicates of five bulbils per replicate were carried out for each control or treatment duration: 1 s, 5 s, 10 s, 30 s, and 1 min.

Bleach treatment. Bulbils were submerged in a 500-ppm NaOCl bleach solution for the duration of the treatment in a 1.5-ml Eppendorf tube. Bleach solution was removed with a pipette and the bulbils were rinsed three successive times using FMII media at the end of each treatment. Three replicates of five bulbils per replicate were carried out for each control or treatment duration: 1 s, 5 s, 10 s, 30 s, 1 min, and 10 min.

Germination assay. Germination assays were carried out for freezing and desiccation experiments only (Table 1). Bulbils were kept in the treatment conditions for the specified treatment duration. At the end of the treatment, 200 ml of

TABLE 1. SAMPLE SIZES, ΔR VALUES, AND PERCENT GERMINATION SUCCESS FOR THE BULBIL TREATMENTS USED IN THIS STUDY. NEW YORK AND WISCONSIN BULBILS WERE USED FOR ALL TREATMENTS AND TIMEPOINTS WHEN AVAILABLE, SUMMARY STATISTICS ARE FOR THE COMBINED SET.

Treatment	Time Point	Tetrazolium Assay		ΔR (mean \pm SE)	Germination Assay		Percent Success
		NYLY Count	WISL Count		NYLY Count	WILM Count	
-20 C	1 h	10	20	49.9 \pm 15.68	10	No data	70.0%
-20 C	3 h	10	20	33.1 \pm 5.22	10	No data	40.0%
-20 C	6 h	10	20	16.3 \pm 3.25	10	No data	10.0%
-20 C	12 h	10	20	5.3 \pm 1.41	10	No data	0.0%
-20 C	24 h	10	20	0.8 \pm 0.2	25	15	0.0%
-20 C	5 d	10	20	2.7 \pm 1.13	20	15	0.0%
-20 C	30 d	No data	No data	No data	15	15	0.0%
-20 C	90 d	No data	No data	No data	10	15	0.0%
-6 C	1 h	10	10	54.3 \pm 6.55	10	No data	0.0%
-6 C	3 h	10	10	57 \pm 8.12	10	No data	30.0%
-6 C	6 h	10	10	47.7 \pm 4.29	10	No data	10.0%
-6 C	12 h	10	10	52.8 \pm 3.85	10	No data	0.0%
-6 C	24 h	10	10	2.4 \pm 1.07	10	No data	0.0%
-6 C	5 d	10	10	4.1 \pm 1.12	No data	No data	No data
Control	1 h	10	20	50.3 \pm 7.03	10	15	#VALUE!
Control	3 h	10	20	39.8 \pm 4.8	10	No data	60.0%
Control	6 h	10	20	53.5 \pm 3.63	10	15	76.0%
Control	12 h	10	20	38.5 \pm 4.42	10	No data	60.0%
Control	24 h	10	20	44.9 \pm 4.03	25	30	70.9%
Control	5 d	10	20	29.4 \pm 4.07	20	15	85.7%
Control	30 d	No data	No data	No data	15	15	66.7%
Control	90 d	No data	No data	No data	10	15	56.0%
Dry/dry	1 h	10	19	44.2 \pm 6.78	10	15	48.0%
Dry/dry	3 h	10	20	26.2 \pm 6.26	10	No data	50.0%
Dry/dry	6 h	10	20	2.3 \pm 0.73	10	15	8.0%
Dry/dry	12 h	10	20	3.3 \pm 0.88	10	No data	40.0%
Dry/dry	24 h	10	20	0.8 \pm 0.27	25	30	0.0%
Dry/dry	5 d	10	19	0.5 \pm 0.15	20	15	0.0%
Dry/dry	30 d	No data	No data	No data	15	15	0.0%
Dry/dry	90 d	No data	No data	No data	10	15	0.0%
Dry/humid	1 h	10	20	55.5 \pm 5.02	10	No data	80.0%
Dry/humid	3 h	10	20	38.7 \pm 6.02	10	No data	50.0%
Dry/humid	6 h	10	20	39.3 \pm 5.62	10	No data	50.0%
Dry/humid	12 h	10	20	2.6 \pm 0.97	10	No data	20.0%
Dry/humid	24 h	10	20	0.9 \pm 0.26	10	No data	0.0%
Dry/humid	5 d	No data	20	0.561075 \pm 0.24	5	No data	0.0%
Steam	Control	20	10	25.6 \pm 3.65	No data	No data	No data
Steam	1 s	20	10	26.2 \pm 3.89	No data	No data	No data
Steam	5 s	20	10	33.4 \pm 4.4	No data	No data	No data
Steam	10 s	20	10	7.4 \pm 2.79	No data	No data	No data
Steam	30 s	20	10	0.1 \pm 0.05	No data	No data	No data
Steam	1 min	20	10	0 \pm 0.02	No data	No data	No data
Bleach	Control	40	No data	47.9 \pm 3.67	No data	No data	No data
Bleach	1 s	20	No data	38.3 \pm 5.08	No data	No data	No data
Bleach	5 s	20	No data	35.3 \pm 4.27	No data	No data	No data
Bleach	10 s	20	No data	41.5 \pm 4.45	No data	No data	No data
Bleach	30 s	20	No data	36.1 \pm 4.54	No data	No data	No data
Bleach	1 min	20	No data	24.8 \pm 4.81	No data	No data	No data
Bleach	10 min	20	No data	32.6 \pm 5.01	No data	No data	No data
Total		570	598		435	270	

FMII media was added and the bulbils and media were placed in the growth conditions (indirect lighting at 20 C). Frozen bulbils were thawed by placing the sealed Magenta™ box under cold tap water. Once thawed, they were moved to the growth conditions. Germinations were tallied over the course of 90 d, with any green growth longer than 5 mm considered a successful germination. Care was taken to not manipulate the bulbils during this phase, lest the manipulation damage or otherwise alter a bulbil's germination success. Growth to 5 mm was considered adequate to distinguish *N. obtusa* from other green algae that may be attached to the bulbil. Germinations were considered

unsuccessful if after 90 d no 5-mm or longer growth was observed for a given bulbil.

Tetrazolium assay. 2,3,5 triphenyl tetrazolium chloride⁶ (TTC) has been used as an indicator of seed viability (Moore 1976), and we have extended its use to *N. obtusa* bulbils. At the conclusion of all treatments and prior to imaging, bulbils were incubated at 21 C overnight in 1% TTC. The TTC solution was prepared after Verma and Majee (2013), using autoclaved distilled water as the solvent. TTC causes formazan precipitate to develop in the presence of active cellular dehydrogenases. This red precipitate accumulates in viable cells causing them to turn red, and nonrespiring

cells should not change color (in the case of bulbils, they should remain white; Verma and Majee 2013). Bulbils were imaged immediately after incubation in TTC (imaging steps below). No preconditioning steps were performed (e.g., scarification).

Image analysis. Images were taken using a Nikon SMZ1500 microscope⁷ with a Nikon DS-Ri1 camera.⁸ A color card⁹ and white balancing was used to establish consistent lighting and color conditions, and all images were captured at the same magnification. Individual bulbils in each image were cropped, roughly first to remove extraneous debris, then using a custom plugin in the software package ImageJ v. 1.50i (<http://imagej.nih.gov>) to edge detect the extents of each bilbil (Schindelin et al. 2015). The ImageJ plugin was built using the “record” function and specifying “mean dark” as the threshold cutoff for edge detection using “setAutoThreshold.” This setting removed reflections of the bilbil that would often be found on the acrylic dish used to image them, and manual inspection deemed this a suitable, reproducible representation of the bilbil extents whether the bilbil was treated with TTC or not. This edge detection does not eliminate rhizoids or other solid debris that may be attached to the bilbil, so care was taken to eliminate such debris prior to imaging. Area measurements and measurements of the red values for each bilbil were carried out using the custom plugin (available upon request). The shift to red, here termed ΔR , was normalized to the total light intensity by subtracting green and blue color bands and then averaging these values. The formula used to do this was:

$$\Delta R = \frac{(R - G) + (R - B)}{2}$$

where B, G, and R correspond to the pixel values (between 0 and 255) for blue, green, and red respectively. Higher ΔR values are inferred to represent viable bulbils, whereas low ΔR values represent nonviable bulbils. A biologically meaningful level of signal from the tetrazolium assay is somewhat elusive. Early attempts to germinate bulbils that had been incubated in TTC were unsuccessful, so viability thresholds were inferred from the percent germinations of the control bulbils from the germination assays. Three germination rate estimates were used to assist in the interpretation of ΔR values. A conservative, average, and liberal germination rate value was set as the minimum (56%), average (72%), and maximum (85.7%) germination success from the germination experiments (Table 1). These thresholds were projected onto untreated (control) bulbils treated with TTC, by sorting the measured ΔR values for all TTC-treated controls from highest to lowest. The threshold ΔR values (7, 20, and 35, respectively) were the values for which 85.7, 72, and 56% of the tetrazolium assay control bulbils were above. This was the target range to interpret bulbils as nonviable in the tetrazolium assay.

Statistical analyses. Statistics were carried out using Python 2.7 as part of the Jupyter Notebook 5.7.8 (<https://jupyter.org>) with the numpy 1.15.1, matplotlib 2.2.3, pandas 0.23.4, scikit-posthocs 0.5.2, scipy 1.1.0, scikit-learn 0.20.3, and statsmodels 0.9.0 packages enabled. Germination data were treated as discrete (binary) variables. Logistic regression was

carried out on germination data using a logit link function in the statsmodels api. Measured ΔR values were found to not be normally distributed (data not shown, Shapiro-Wilk $P < 0.05$), so nonparametric statistics (Kruskal Wallis and the post hoc Dunn test with Bonferroni correction for multiple comparisons) were used to interpret these data. Statistical significance was set at $P < 0.01$ for all of the treatment comparisons measured with the tetrazolium assay.

RESULTS AND DISCUSSION

Limiting the spread of aquatic organisms through effective decontamination strategies is a major concern for agencies in the Great Lakes Region and northeastern United States (Johnstone et al. 2014, VDEC 2015, Wisconsin DNR 2015, Larkin et al. 2018). Both physical and chemical strategies exist to decontaminate boats and gear of other aquatic plants, with a preference for physical decontamination (removal) over chemical decontamination because of the negative equipment and environmental effects of the latter (Johnstone et al. 2014). The methods described in this study are intended to extend the tools and priorities already in place of other aquatic hitchhikers, and assist with management of *N. obtusa* by effectively suppressing the viability of its primary propagule in its invaded range: the bilbil.

In this study, both physical (desiccation, freezing, and steam) and chemical (bleach) treatments were tested, and all treatments were found to be effective at reducing bilbil viability to varying degrees. For two of these treatment methods (freezing and desiccation), two measures of bilbil viability were obtained: germination success and the red color shift associated with interactions with reducing agents (an indication of living cells) and the chemical indicator TTC in the tetrazolium assay. There was a 100% reduction in bilbil germination success at 24-h treatment durations and longer for both freezing (at -6 and -20 C) and desiccation (in dry/dry and dry/humid conditions) treatments (Table 1, Fig. 1). A logistic regression on treatment durations 5 d or less (due to missing data at longer treatment durations) indicated that there was roughly a 10-fold difference in the negative effect of a given treatment on bilbil survivability (Table 2). The largest effect, irrespective of treatment duration, was the -6 C, with a 10.2-fold more lethal effect than the least effective dry/humid treatment (Table 2). However, the -6 C treatments also have the highest error respective to their regression coefficient visible as a lower z value compared to both the -20 C and dry/dry treatments, and it seems likely that these other two treatment methods could have a similarly negative effect. Additionally, much of the negative effect of the -6 C treatment seems attributable to the fact that no germinations were observed for the 1-h treatment duration, accounting for about 22% of the total nonviable observations for the -6 C treatment (Fig. 1). All of the treatment durations had negative coefficients (predicting bilbil survival rather than bilbil death) ranging from slight and not statistically significant (24-h and 5-d durations), to comparably high and statistically significant (1 h, 3 h, 6 h;

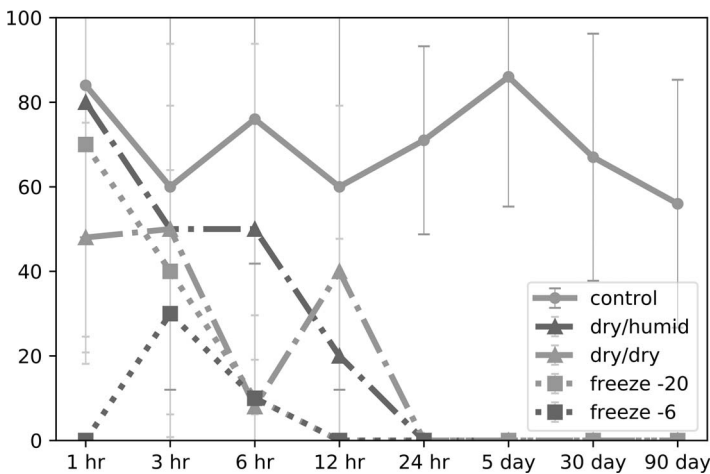


Figure 1. Bulbil germinations after freezing or desiccation. Line graph of germination success for six treatment durations (five for the -6°C treatment). Complete icing of the growth media occurred by 6 h (and longer) for the -20°C treatment, and by 12 h (and longer) for the -6°C treatment. Though 30- and 90-d treatments are shown; these represent no data for the -6°C , and dry/humid treatment conditions. Bars represent the 95% confidence interval.

Table 2). The model predicts a slight chance of bulbil survival for 12-h treatment durations (the 95% confidence interval includes only negative numbers, whereas longer durations have 95% confidence intervals that include zero; Table 2).

The tetrazolium assay largely corroborated the germination observations, but with some important differences. Significant reductions in measured redness values (ΔR) were detected at 6-h treatment durations and longer (Kruskal-Wallis, $P < 0.01$; Fig. 2). The post hoc Dunn test was conducted to compare each treatment to one another and the -20°C treatment and dry/dry treatment were found to be significantly different from the control group for all treatment durations of 6 h or longer (Dunn's test, $P < 0.01$). Findings in similar experiments have established 4 h as a suitable time frame to desiccate bulbils (Glisson et al. 2019, this issue). The post hoc Dunn's test similarly found significant differences between the measured ΔR values for controls and dry/humid conditions at 12-h treatment

TABLE 2. SUMMARY OF LOGISTIC REGRESSION CARRIED OUT ON GERMINATION DATA CODED AS 1 = NOT GERMINATED, 0 = GERMINATED (POSITIVE REGRESSION COEFFICIENTS ASSOCIATED WITH HIGHER GERMINATION).

Variable	Regression Coefficient	SE	z	$P > z $	Odds Ratio	95% Confidence Interval
Control	-0.2779	0.254	-1.0941	0.2739	0.76	-0.78 0.22
Freeze -20°C	3.8012	0.4333	8.7718	0	44.76	2.95 4.65
Freeze -6°C	4.7335	0.6574	7.2004	0	113.69	3.45 6.02
Dry/dry	3.5731	0.3915	9.1268	0	35.63	2.81 4.34
Dry/humid	2.4111	0.4663	5.1706	0	11.15	1.50 3.33
1 h	-3.3071	0.4432	-7.4611	0	0.04	-4.18 -2.44
3 h	-2.8459	0.4947	-5.7533	0	0.06	-3.82 -1.88
6 h	-1.4617	0.4457	-3.2798	0.001	0.23	-2.34 -0.59
12 h	-1.1465	0.5458	-2.1007	0.0357	0.32	-2.22 -0.08
24 h	-0.2638	0.3497	-0.7545	0.4506	0.77	-0.95 0.42
5 d	-0.739	0.3975	-1.8588	0.0631	0.48	-1.52 0.04

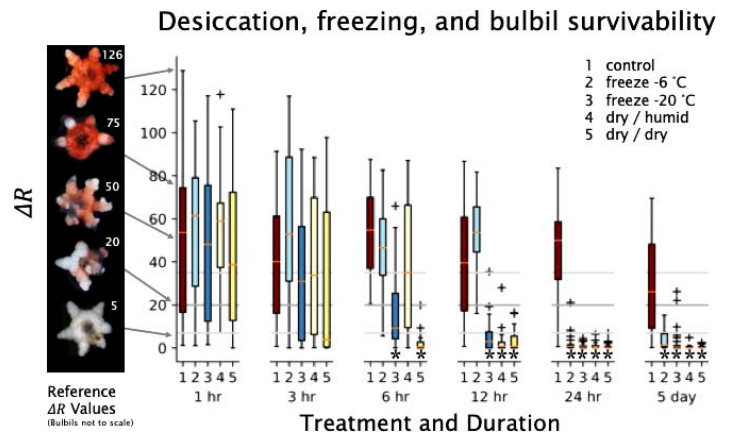


Figure 2. Desiccation, freezing, and bulbil survivability. Bulbils representing reference ΔR values (actual values as white numbers) from low (lowest bulbil pictured, $\Delta R = 5$) to high (highest bulbil pictured, $\Delta R = 126$) along the left. Box and whisker plots of measured ΔR values resulting from tetrazolium assays of controls (magenta), desiccation (yellows), and freezing (blues) treatments. The bulbils pictured are intended to show the ΔR color shift only and are not to scale, but range in size from about 1.5 to about 5 mm across. The box of each box plot ranges from the lower to upper quartile values. The whiskers extend up to the last data point that is less than 1.5 times the interquartile range. Plus signs are outliers that are beyond 1.5 times the interquartile range. The median value is portrayed as an orange line. Statistically significant treatments indicated by an asterisk.

durations and longer ($P < 0.01$), and at 24 h and longer for the -6°C treatments ($P < 0.01$; Fig. 2). The robust effect seen by the -6°C treatment (particularly for the 1-h treatment duration) in the germination assay was not replicated here in the tetrazolium assay. Rather, the -6°C treatment was not statistically different from the other treatments (or from the controls) for the 1-h time point (87 Dunn's test, $0.23 < P < 0$), suggesting either experimental error or an external, unmeasured effect (e.g., organisms overgrowing the bulbils).

At the 12-h treatment duration, the tetrazolium assay predicts a high reduction of bulbil viability for the dry/dry treated bulbils with the mean ΔR value falling below all three germination thresholds (Fig. 2), whereas the germination assay measures 40% germination success, an increase over the 6-h time point (Fig 1). It is unclear where this discrepancy comes from, but could be attributed to the variability in the treatment conditions for the germination trial. Glisson (2019, this issue) observed longer desiccation times for clumped material. We made no effort to distribute bulbils once they were placed in MagentaTM boxes, and so we might expect this to influence some of the drying times.

The combined evidence of the germination and tetrazolium assays suggest that treatment durations of 24 h and longer would be sufficient to eliminate bulbil viability provided the minimum treatment conditions can be replicated in the field. Guidelines that are already in place in some states urge a drying time of five consecutive days (Wisconsin DNR 2015) or 5 to 12 d depending on temperature and humidity (Johnstone et al. 2014). These guidelines should serve to eliminate bulbil viability in light of this study. Freezing at -10°C for at least 4 h has been recommended to decontaminate equipment (Johnstone et al. 2014). Freezing at this temperature appears to be

Bulbil survival after bleach and steam treatments

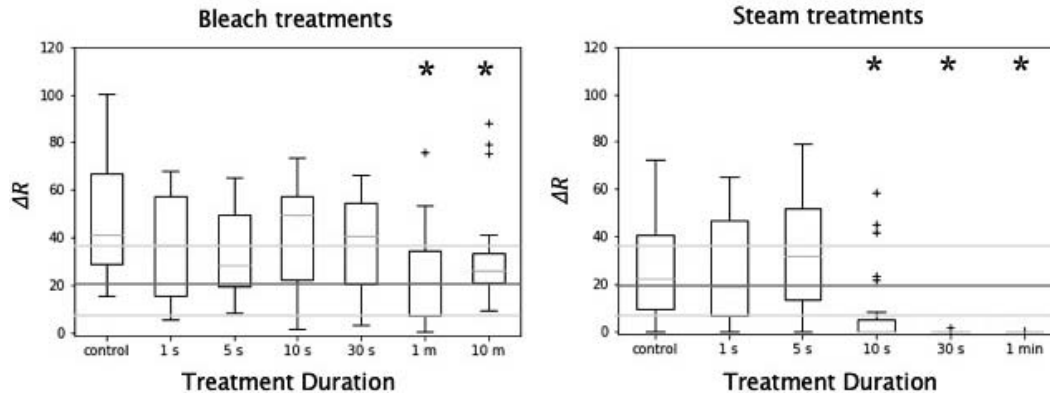


Figure 3. Bulbil survival after bleach (left) and steam (right) treatments as measured by the tetrazolium assay. Box and whisker plots show the measured ΔR values resulting from tetrazolium assays of bleach and steam treatments. The box of each box plot ranges from the lower to upper quartile values. The whiskers extend up to the last data point that is less than 1.5 times the interquartile range. Plus signs are outliers that are beyond 1.5 times the interquartile range. The median value is portrayed as an orange line. Statistically significant treatments indicated by an asterisk.

effective against *N. obtusa* bulbils given the data presented here, but the efficacy of this treatment is potentially dependent on ice formation or a combination of ice formation and temperature, and undrained gear may not freeze completely in a 4-h time frame.

Currently, *N. obtusa* is distributed across much of the Great Lakes region in the United States and Canada, these results suggest that further sustained colonization northward could be limited to lakes that do not freeze completely. Given that *N. obtusa* is readily found in shallow waters from 0.5 to 7 m in its introduced range (Sleith 2015), its susceptibility to freezing could be exploited through the use of lake drawdowns timed with freezing temperatures.

Bleach and steam treatments were also tested in this study. Although steam appeared to reduce positive viability signal quickly, as measured by the tetrazolium assay, bleach treatments did not (Fig. 3). Steam treatments significantly reduced bulbil viability by 10-s treatment durations ($P < 0.01$), and virtually eliminated positive viability signal altogether between 10 and 30 s (Fig. 3). Because these ΔR values for the 30-s and 1-min steam treatment durations fall completely below the thresholds inferred to be the biologically relevant range for germination success (gray lines, Fig. 3), it seems likely such treatments would be effective in decontamination efforts.

Bleach treatments had a statistically significant impact on the measured ΔR values at the 1- and 10-min time points (Dunn's test, $P < 0.01$). Even so, there appears to be positive ΔR signal associated with viable bulbils as many samples are at or above the viability thresholds established by germination experiments (Fig. 3). It is unclear whether the positive signal in this case would truly result in viable, germinating bulbils after being treated with bleach. Bleach treatment with 5% NaOCl is commonly used for sterilizing *Chara* zygotes before attempting to grow them axenically, for instance (Wetzel and Mcgregor 1968) and could similarly yield viable bulbils here as well. Alternatively, the bulbils could be experiencing the negative impacts of the bleach, and the tetrazolium assay is capturing a latent respiring (or

otherwise TTC reducing) state too soon after the treatment. Additional experimentation might be useful to address these questions.

Although both the tetrazolium and germination assays were largely in agreement, there are important limitations to each approach. The tetrazolium assay has likely overestimated the viability of bulbils in some cases. There are three outlier values in the 5-d -20 -C treatment that could be interpreted as having positive, viable ΔR values as they exceed the most liberal threshold, and two of them exceed the average threshold of germination success (Fig. 2). On inspection of these three bulbils, one appears damaged (a before picture, not shown, confirms this) and the positive signal occurs surrounding the damage (Supplemental Figure 1). The other two bulbils have a tetrazolium signal that is diffuse and weak across the whole bulbil, giving them a pink appearance, and in no part of the bulbils is there a carmine red that we would expect from a viable bulbil (Supplemental Figure 1). This oversensitivity of the tetrazolium assay has also been observed in assessing freeze damage to corn kernels (Bennett and Loomis 1949). We suspect the false-positive signal comes from a couple of possible sources: organisms living on or in the bulbils (e.g., bacteria and/or fungi), latent reducing ability after the bulbil has died (is dying and will not germinate), or introduced reducing agents either through breakage or diffusion. The germination approach conversely appears to have the opposite problem. Germination could sometimes take weeks, during this time organisms could overgrow and limit growth of the bulbils. The growth of these organisms—sometimes algae or sometimes fungi—would affect the entire box and in some cases lead to no viable germinations where it seems reasonable to expect that there should be, for instance, the 1-h time point for the -6 C treatment (Fig. 1).

Although some of the decontamination strategies in this study worked more definitively than others, each seems to have utility for limiting the spread of *N. obtusa* by reducing bulbil viability. Steam treatments followed by desiccation seem to be the most effective (highest reduction in the

shortest amount of time), but freezing was also effective after 12 to 24 h, the time frames required for complete icing of the media. Bleach treatments of longer duration, with different time frames for incubation in TTC, or coupled with germination experiments, should be considered. Bulbils do not appear to be as resilient to these treatments as other propagules, such as *Chara* zygotes or the seeds of aquatic embryophytes, and the decontamination methods described here can fit into existing management practices set in place for other aquatic invasive plants.

Sources of Materials

¹Whirl Pak. Nasco-Wisconsin, 901 Janesville Avenue, P.O. Box 901, Fort Atkinson, WI 53538-0901.

²Magenta polycarbonate vessel (GA-7). Magenta LLC, 15160 New Avenue, Lockport, IL 60441.

³Thermo Scientific™ Nalgene™ Acrylic Desiccator Cabinets. Fisher Scientific, www.fishersci.com.

⁴Fisherbrand™ Hygro-Thermometer with Jumbo Display. Fisher Scientific, www.fishersci.com.

⁵My Little Steamer Go Mini. Joy Mangano, joymangano.com.

⁶2,3,5-triphenyltetrazolium chloride. Sigma-Aldrich, sigmaaldrich.com.

⁷Nikon SMZ1500 microscope. www.nikoninstruments.com.

⁸Nikon DS-R1i camera. www.nikoninstruments.com.

⁹ColorGauge Micro Target, matte with coated glass. Image Science Associates, LLC, 6898 Townline Road, Williamson, NY 14589-9707.

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