Laboratory evaluation of copper-based algaecides for control of the invasive macroalga starry stonewort (*Nitellopsis obtusa*)

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Abstract

*Nitellopsis obtusa* ([Desvaux] J. Groves [1919]; Characeae), known as starry stonewort, is an invasive macroalga in Laurentian Great Lakes states and provinces in North America. Because of its potential negative impacts on native ecosystems and recreation, *N. obtusa* has become a high-priority target for management. However, there is a critical lack of foundational information on the efficacy of different algaecides, and concentrations thereof, for *N. obtusa* control. Additionally, control of *N. obtusa* bulbils—asexual reproductive structures that are the main pathway for the establishment of new plants—has proven difficult. We tested the efficacy of six commonly used copper-based algaecides, at a series of copper concentrations up to the maximum labeled rate, on *N. obtusa* thalli (photosynthetic aboveground tissues) and bulbils in controlled laboratory experiments. Bulbils were placed above and below sediment in separate experiments to evaluate whether sediment acted as a barrier to treatment. At 14 days after treatment (DAT), there were significant reductions in thalli biomass (34% and 40%) for two algaecides at the highest concentration evaluated (1.0 mg Cu L⁻¹) and significant thalli discoloration at 0.75 and 1.0 mg Cu L⁻¹ for four algaecides. There were no significant negative effects on *N. obtusa* thalli biomass or discoloration at lower concentrations of any product. For below-sediment bulbils, none of the algaecides reduced *N. obtusa* viability compared to untreated controls by 56 DAT, and viability was significantly greater than in controls for three different algaecides at 0.25 mg Cu L⁻¹. For above-sediment bulbils, there was low sprouting across all algaecide treatments and untreated controls, indicating inhospitable growing conditions. These findings provide a baseline for improvement of chemical treatment options for *N. obtusa*, provide guidance for future research on *N. obtusa* control, and underscore the challenges in achieving sustained *N. obtusa* control.

Key words: bulbil, charophyte, herbicide, invasive species, treatment, viability

Introduction

*Nitellopsis obtusa* ([Desvaux] J. Groves, 1919; Characeae), commonly known as starry stonewort, is a freshwater green macroalga native to Europe and Asia, where it is of wide conservation concern. It has been Red List classified as near-threatened in Baltic Sea countries and Switzerland (Auderset Joye and Schwarzer 2012; HELCOM 2013), vulnerable in the
Nitellopsis obtusa can grow in dense stands and has been associated with decreases in native aquatic plant abundance, biomass, and species richness (Brainard and Schulz 2017; Ginn et al. 2021; Wagner 2021). It has also been associated with broader ecological changes, including increases in cyanobacterial blooms and invasive zebra mussels (*Dreissena polymorpha* Pallas; Harrow-Lyle and Kirkwood 2020), decreases in diversity of phytoplankton and macroinvertebrates (Harrow-Lyle and Kirkwood 2021c), and alterations to biogeochemical processes (Harrow-Lyle and Kirkwood 2021b). *Nitellopsis obtusa* can grow over a broad range of depths (Larkin et al. 2018), increasing its potential for ecological impacts and making its detection more difficult. The ability of *N. obtusa* to form dense beds can also interfere with recreation, such as boating, fishing, and swimming (Pullman and Crawford 2010).

Due to its ecological and recreational impacts and ongoing spread, *N. obtusa* has become a high-priority target for management (Pullman and Crawford 2010; Hackett et al. 2017; Pokrzywinski et al. 2020a). In the United States, algae (including cyanobacteria, green planktonic and filamentous algae, and characean macroalgae) are routinely managed with copper-based contact algaecides (Gettys et al. 2014), and these products have been adopted for control of *N. obtusa*. In general, copper suppresses algal growth and causes degradation of algal cells; more specifically, copper’s toxicity to algae occurs via several mechanisms, including inciting free radical production, and inhibiting photosynthesis, ATP production, and enzyme function (Florence and Stauber 1986; Stauber and Florence 1987; Cid et al. 1995). Because direct contact with algal cells is required for copper algaecides to work, higher copper concentrations are typically recommended on product labels for treatment of macroalgae like *N. obtusa* (SePRO Corporation 2014). However, there has been limited evaluation of the effectiveness of *N. obtusa* treatment with copper-based algaecides,
Despite the fact that such management has been ongoing for decades (Pullman and Crawford 2010; Larkin et al. 2018). To our knowledge, only a single study has reported on the effectiveness of N. obtusa management in situ: Glisson et al. (2018) observed > 95% reduction in N. obtusa biomass following a treatment with the copper-based algaecide Cutrine®-Plus at 0.9 mg L\(^{-1}\) elemental copper. Controlled experimental studies are similarly scarce. A single study was conducted outdoors in small containers, and three copper-based algaecides at 1.0 mg Cu L\(^{-1}\) reduced biomass 23–44% compared to untreated controls (Pokrzywinski et al. 2021). We know of no published studies that have evaluated the efficacy of copper-based algaecides on N. obtusa through controlled laboratory experiments—a critical foundational step in determining best management strategies. Hence, there is a lack of basic information on the efficacy of different algaecides and their effective concentrations for N. obtusa control. Without sound guidance from applied research, management efforts are likely to be less effective, risking poor treatment outcomes, misapplication of limited resources, and potentially further spread of N. obtusa. Such research is also crucial to help lake managers balance control of N. obtusa with documented non-target, negative effects of copper-based algaecides on fish, amphibians, and invertebrates (Mastin and Rodgers 2000; Bishop et al. 2014; Christenson et al. 2014; Closson and Paul 2014). Applied research is urgently needed to advance sound management of N. obtusa.

The ability of N. obtusa to spread and reproduce via bulbils further complicates control efforts. Bulbils are asexual reproductive structures that grow from nodes, primarily along underground rhizoids, and are a main pathway for the establishment of new plants (Bharathan 1987). Bulbils of N. obtusa and confamilial species are normally produced beneath the sediment (Bharathan 1987), but may be dislodged or occur within the alga above the sediment. While there has been little work on control of N. obtusa bulbils, Glisson et al. (2018) found greater bulbil density and no reduction of bulbil viability in an area of a lake treated with a copper-based algaecide (86% viability compared to 84% in an untreated reference area). These results are concerning for ongoing N. obtusa management efforts, but may be due to natural processes (e.g., phenology; Glisson et al. 2022) or other factors not directly related to algaecide application (Glisson et al. 2018); hence, further investigation under more controlled conditions is needed. Greater control was achieved in an experiment in which bulbils were attached to free-floating N. obtusa material (Pokrzywinski et al. 2021), suggesting that bulbils located higher in the water column and without overlying sediments are more susceptible to copper treatments. Research on control of asexual reproductive structures in vascular aquatic plants, such as Potamogeton crispus L. turions, has shown that contact herbicides have limited effectiveness on these structures once they are within lake sediments (Johnson et al. 2012). Understanding how application
of copper-based algaecides affects *N. obtusa* bulbils under typical field conditions (with overlying lake sediments) is crucial for identifying gaps in control strategies and developing realistic management goals.

Effective invasive plant management depends upon a foundation of laboratory and field studies to guide decision-making and better predict outcomes of management (Fernández and Irigoyen 1987; Cairns and Pratt 1989). In this foundational study, we tested the efficacy of commonly used copper-based algaecides on *N. obtusa* thalli (photosynthetic aboveground tissues) and bulbils in controlled laboratory experiments. We further evaluated whether sediment protects *N. obtusa* bulbils from algaecide exposure by conducting bulbil experiments with bulbils placed either below or above sediment. Efficacy was measured with respect to biomass reduction and discoloration (as an indicator of chlorosis and damage) of thalli, and viability of bulbils (measured as sprouting). We tested six different registered copper algaecide products that have been operationally implemented for *N. obtusa* control, at a series of copper concentrations up to the maximum legal labeled rate of 1.0 mg L\(^{-1}\) elemental copper. Based on available studies on *N. obtusa* treatment, we expected to see significant reductions of *N. obtusa* biomass compared to untreated controls. For *N. obtusa* bulbils placed above the sediment, we expected lower viability than controls, and for bulbils below the sediment, similar viability to controls.

**Materials and methods**

We collected live *N. obtusa* material for all experiments from Lake Koronis, in Stearns County, MN (USA) on 18 March 2019. We brought the material to the laboratory and carefully separated individual bulbils from rhizoids. Bulbils were kept refrigerated at 4 °C until the start of the experiments, which were conducted in the Minnesota Aquatic Invasive Species Research Center’s Containment Laboratory (MCL) at the University of Minnesota in Saint Paul, MN from April–June 2019.

**Thalli experiment**

**Preparation**

We filled 355 32-oz (946-mL) glass jars with 100 g of topsoil (Organic Valley® Topsoil, Garick LLC; Cleveland, OH, USA), covered with 35 g of sand (Nurserymen’s Preferred® White Play Sand, TCC Materials; Mendota Heights, MN, USA) to reduce soil mixing with the water column and maintain visibility of sprouted bulbils, and 800 mL of dechlorinated well water. The soil and sand mixture (sediment, hereafter) was approximately 2.5-cm deep. Water chemistry was similar to lakes where *N. obtusa* has been observed in its invaded range (Sleith et al. 2015; Midwood et al. 2016): pH = 8.65, conductivity = 253 μS cm\(^{-1}\), alkalinity = 159 mg L\(^{-1}\) as CaCO\(_3\), hardness = 145.4 mg L\(^{-1}\) as CaCO\(_3\), total phosphorus = 0.042 mg L\(^{-1}\), and
total nitrogen = 0.34 mg L⁻¹ (as recorded in Glisson et al. 2018). We planted four bulbils into each jar by gently pressing them into the sediment until fully covered (≤ 1 cm deep). We examined each bulbil prior to placement to ensure that bulbils were undamaged and similar in size (diameter ~ 2–6 mm). We placed the jars on a laboratory bench in the MCL and allowed *N. obtusa* bulbils to sprout and grow for a 40-d establishment period prior to treatment. Jars were maintained under a 16–8 h light-dark schedule with multi-spectrum lights (RX30, Heliospectra AB; Göteborg, Sweden) emitting photosynthetically active radiation (PAR) of 30 μmol photons m⁻²s⁻¹. We added water periodically to maintain stable water levels in all jars throughout the establishment period and the experiment. During the experiment, we monitored temperature and pH in jars containing sediment and water (but no *N. obtusa*) and jars containing water only, which were randomly placed among experimental jars; mean pH in these jars was 8.45 ± 0.06 (1 SE) and mean temperature was 20.5 ± 0.24 °C.

**Pre-treatment**

After the 40-d establishment period, we removed jars that did not contain any sprouted *N. obtusa*, and selected jars for the experiment that contained either multiple stems (i.e., main axes of the thallus; Bharathan 1983) or a single stem that filled most of the water column (> 8 cm tall). To characterize pre-treatment starting conditions in the selected jars, we counted the number of individual *N. obtusa* stems emerging from the sediment in all jars and measured biomass directly by removing thalli and bulbils from nine randomly selected jars. Upon removal, we placed bulbils and thalli in a wire mesh strainer, rinsed the material to remove any sediment, and weighed the material to the nearest 0.001 g (wet-weight biomass). The material was then placed in a drying oven at 65 °C for 7 d and weighed again (dry-weight biomass). The randomly selected pre-treatment *N. obtusa* had a mean wet weight of 0.315 ± 0.057 g (1 SE) and a dry weight of 0.030 ± 0.009 g. Jars contained 7.54 ± 0.17 stems prior to treatment.

**Treatment**

We randomly assigned nine replicate jars to each algacide treatment and to an untreated control. We tested the efficacy of six copper-based algacides at five concentrations that spanned from low to maximum labeled rates: Captain® XTR, Komeen®, and Nautique® (SePRO Corporation; Carmel, IN, USA), and Algimycin® PWF, Cutrine®-Plus, and Cutrine®-Ultra (Applied Biochemists; Alpharetta, GA, USA [acquired by SePRO Corporation in October 2020]), each at concentrations of 0.125, 0.25, 0.50, 0.75, and 1.0 mg L⁻¹ elemental copper, for a total of 30 unique treatment combinations (*n* = 270 treatment jars and 9 untreated control jars; Table 1). We selected these six products because they: 1) are commonly used for *N. obtusa* control (W. Glisson, University of Minnesota, unpublished data),
Table 1. Product name, active ingredient formulation, percent active ingredient, and percent metallic copper equivalent for algaecides used in *Nitellopsis obtusa* laboratory experiments. The maximum label rate of all algaecides is 1.0 mg L\(^{-1}\) elemental copper.

| Product \(^{a}\) | Active ingredient formulation | Active ingredient (%) | Metallic copper equivalent (%) |
|-----------------|-------------------------------|-----------------------|-------------------------------|
| Algimycin® PWF  | Chelates of copper gluconate   | 12.5                  | 5                             |
|                 | Chelates of copper citrate     | 12.9                  |                               |
| Captain® XTR    | Copper ethanolamine complex    | 28.2                  | 9.1                           |
| Cutrine®-Plus   | Copper ethanolamine complex    | 27.9                  | 9                             |
| Cutrine®-Ultra  | Copper ethanolamine complex    | 27.8                  | 9                             |
| Komeen®         | Copper ethylenediamine complex | 22.9                  | 8                             |
| Nautique®       | Copper ethylenediamine complex | 13.2                  | 9.1                           |
|                 | Copper triethanolamine complex | 14.9                  |                               |

\(^{a}\) Captain® XTR, Komeen®, and Nautique® manufactured by SePRO Corporation (Carmel, IN, USA); Algimycin® PWF, Cutrine®-Plus, and Cutrine®-Ultra manufactured by Applied Biochemists (Alpharetta, GA, USA), which was acquired by SePRO Corporation in October 2020, after experiments were conducted.

2) span a range of copper formulations and manufacturers (Table 1), and 3) are known to effectively control other algae (Bishop and Rodgers 2012; Bishop et al. 2018). Stock algaecide solutions were mixed within 1 hour of application and applied to jars via micropipette. To test for algaecide adherence to jar surfaces and sediment, we added each algaecide at 1.0 mg Cu L\(^{-1}\) to each of six jars containing 1) sediment and water as described above (but no *N. obtusa*), and 2) water only. These twelve jars were randomly placed among experimental jars.

To determine initial copper concentrations, we collected water samples (9.9 mL each) 10 minutes after algaecide application from one replicate jar for each of the 30 unique treatment combinations and untreated controls and from all of the algaecide-adherence test jars. Samples were preserved in a 1% v v\(^{-1}\) solution of 70% trace-metal grade nitric acid for concentration analysis. Copper concentration was measured using inductively coupled plasma-optical emission spectrometry (ICPE 9000, Shimadzu Corporation; Kyoto, Japan) with a matrix-matched calibration curve from serial dilution of a 1000 mg L\(^{-1}\) copper standard (SC194; Fisher Scientific, Inc.; Hampton, NH, USA; USEPA 1994). The limit of detection for copper was 5 µg L\(^{-1}\). We analyzed method blanks and routine QA/QC samples with each run to ensure there was no contamination or drift during sample preparation or analysis. We repeated this process at 14 DAT.

At 14 days after treatment (DAT), we recorded the thalli color for all replicates of each treatment and control. Thalli color was measured as the proportion (in 0.05 increments) of all visible thallus tissue (i.e., all internodes, nodes, and branchlets) that was: 1) green, 2) yellow (indicating chlorosis), or 3) clear (indicating cell damage and loss of cytoplasm). We additionally counted the number of *N. obtusa* stems as described above. Following the color and stem measurements at 14 DAT, six of the nine replicates were harvested to determine wet and dry weight as described above. We had initially anticipated substantial collapse of *N. obtusa* thalli by 14 DAT, and planned to evaluate whether there was subsequent recovery of thallus from bulbils in the remaining three replicate jars beyond
14 DAT. However, after we observed relatively minimal damage to thalli at 14 DAT, we concluded the experiment after one additional week (21 DAT) by harvesting the remaining three replicates and measuring stem counts, wet weight, and dry weight as described above. Because we observed little change in thalli condition from 14–21 DAT, and all treatment combinations had the same proportions of 14 and 21 DAT replicates (6 14-DAT replicates and 3 21-DAT replicates), we combined data from these two time points as our final stem and biomass measurements in subsequent analyses (hereafter, 14 DAT).

**Bulbil experiments**

**Preparation**

We used sprouting to assess the response of *N. obtusa* bulbils to algaecide treatment; hence, there was no establishment phase for the bulbil experiments. Two hours before treatment, we removed bulbils from cold storage and placed four bulbils each into 16-oz (473-mL) glass jars filled with 100 g of topsoil covered with 35 g of sand and 325 mL of dechlorinated well water. For jars in the below-sediment experiment, we planted four bulbils each as described above for the thalli experiment, and for jars in the above-sediment experiment, we placed four bulbils each on top of the sediment. The above-sediment experiment was intended to test whether sediment burial protected bulbils from algaecide exposure. As with the thalli experiment, we examined each bulbil prior to placement to ensure that bulbils were undamaged and similar in size.

We placed the jars for each experiment on a laboratory bench in the MCL and maintained them under the same light conditions as the thalli experiment. We monitored pH and temperature weekly in water-only jars placed among the experimental jars; mean pH was 8.48 ± 0.03 (1 SE) and mean temperature was 19.8 ± 0.25 °C. Stable water levels were maintained for the duration of the experiment by periodically adding water to jars.

**Treatment**

For each bulbil experiment, we randomly assigned five replicate jars (containing four bulbils each) to algaecide treatments and an untreated control using the same 30 algaecide × concentration treatments described for the thalli experiment (*n* = 150 treatment jars and 5 untreated control jars per experiment). Stock algaecide solutions were mixed within 1 hour of application and applied to jars via micropipette. We assessed initial and 14-DAT copper concentrations as described above for the thalli experiment.

To measure bulbil viability following treatment, we checked each jar for bulbil sprouting at 1 DAT and again every 7 d until 56 DAT. Bulbil sprouting was defined by the appearance of a green turgid shoot emerging from the bulbil (following Glisson et al. 2018, 2020). For below-sediment
bulbils, shoots emerging from the sediment were traced back to the sprouting bulbil. Sprouted bulbils were carefully removed upon observation to avoid duplicate counting and continued growth, which may have altered conditions for remaining unspouted bulbils (e.g., reduced light availability) and made it more difficult to observe newly sprouted bulbils.

**Statistical analysis**

All statistical analyses were performed using R version 4.1.0 (R Core Team 2021). For the thalli experiment, we measured the effects of algaecide treatments on *N. obtusa* biomass using wet weight, dry weight, and stem counts. We chose to focus on wet-weight biomass as the response variable in our models because it: 1) was the most direct measure of biomass, 2) was correlated with dry-weight biomass and stem counts (Pearson correlation coefficient \( r = 0.84 \) and 0.58, respectively), and 3) required less handling and processing of *N. obtusa* thalli, thereby avoiding potential loss of material. Nonetheless, we repeated the analysis described below with dry-weight biomass and stem counts and found similar patterns; the results from these analyses are presented in the supplementary material (dry-weight biomass: Supplementary material Table S1 and Figure S1; stem counts: Table S2 and Figure S2). Prior to analysis, we confirmed homogeneity of variance among treatments and controls using Fligner-Killeen tests (Conover et al. 1981). We then fit a linear model with wet-weight biomass as the response variable and the combination of algaecide type and concentration as the explanatory variable (i.e., all 30 combinations of algaecide type × concentration). We set the untreated control as the baseline level in the linear model and tested for the significance of an overall effect of algaecide type × concentration with analysis of variance (ANOVA). From the linear model, we report the significance of treatment combinations compared to the baseline of the untreated controls (treatment contrasts). As this was the first controlled laboratory trial of algaecides for the control of *N. obtusa*, we were primarily interested in whether treatments differed from controls (i.e., whether any treatments were effective), rather than how treatments differed from one another. Hence, we maintained \( \alpha = 0.05 \) for these direct comparisons of treatment combinations to controls (following Gotelli and Ellison 2013). To evaluate how each algaecide (across all concentrations) and concentration (across all algaecides) compared to the untreated controls, we also fit models in the same manner that included only algaecide type or concentration as the explanatory variable. For all models, we visually examined model residuals for normality and homogeneity of variance.

We assessed the effects of algaecide treatments on discoloration of *N. obtusa* thalli measured as the proportions of green, yellow, and clear tissue. We focused on the proportion of green tissue as the response
variable in our models because: 1) it was the easiest to visualize, 2) it was strongly and inversely correlated to the proportion of yellow tissue ($r = -0.96$), and 3) very few *N. obtusa* thalli displayed any clear tissue (17% of jars). Thus, we used the proportion of green tissue as an indicator of both chlorosis and cell damage, with proportionally more green tissue indicating less damage. For proportion of green tissue, values for all replicates in the Captain® XTR treatment group at 0.125 mg Cu L$^{-1}$ were 1.0, so we changed one value to 0.95 to ensure there was variation in the group, allowing us to make comparisons to the controls in our models. Additionally, we did not record a proportion of green tissue value for one replicate of the Nautique® treatment at 1.0 mg Cu L$^{-1}$; thus, this treatment group had only eight replicates for analysis. To fit assumptions of a linear model, we logit-transformed ($\log(y/\[1− y\])$) the proportional response data after subtracting 0.01 from all values to allow for log calculation (Warton and Hui 2011). Initial model fitting indicated heterogeneous variance in model residuals; in particular, higher-concentration treatments had greater residual variance than lower-concentration treatments. To address this, we used a generalized least squares (GLS) model that allowed for heterogeneity of variance for each concentration using the *gls* function of the *nlme* package in R (Pinheiro et al. 2020). We fit a GLS model with logit-transformed proportion of green tissue as the response variable and the combination of algaecide type × concentration as the explanatory variable. As with biomass, we set the untreated control to the baseline level in the model, assessed significance of an overall effect with ANOVA, and report the significance of treatment combinations compared to the untreated controls. Additionally, we fit models that included only algaecide type or concentration as the explanatory variable and we visually examined model residuals for normality and homogeneity of variance.

We analyzed the bulbil sprouting data from the below-sediment and above-sediment experiments separately. To assess the viability of *N. obtusa* bulbils following algaecide treatment, we used Fisher’s exact tests with proportion of bulbils sprouted as the response variable and the combination of algaecide type × concentration as the explanatory variable. As with biomass, we set the untreated control to the baseline level in the model, assessed significance of an overall effect with ANOVA, and report the significance of treatment combinations compared to the untreated controls. Additionally, we fit models that included only algaecide type or concentration as the explanatory variable and we visually examined model residuals for normality and homogeneity of variance.

We analyzed the bulbil sprouting data from the below-sediment and above-sediment experiments separately. To assess the viability of *N. obtusa* bulbils following algaecide treatment, we used Fisher’s exact tests with proportion of bulbils sprouted as the response variable and the combination of algaecide type × concentration as the explanatory variable. We first tested for an overall effect of treatment combination, and then used individual Fisher’s exact tests to conduct direct comparisons between the controls and each treatment combination. We used Fisher’s exact tests in the same manner to compare the untreated controls to each algaecide type (across all concentrations) and concentration (across all algaecides).

**Results**

**Thalli experiment**

Measured initial copper concentrations for the thalli experiment were 88.0 ± 5.2% (1 SE) of targeted values across all algaecide treatment combinations,
and thus target treatment values were sufficiently achieved (complete concentration testing results for the thalli experiment are reported in Table S3). By 14 DAT, copper concentrations were 16.8 ± 1.2% of measured initial values across all algaecide treatment combinations and decreased by > 75% for each concentration level (Table S3). Copper concentrations in algaecide-adherence test jars without N. obtusa and sediment were 88.2 ± 6.8% of initial measured values by 14 DAT, indicating minimal copper adherence to jar surfaces.

For N. obtusa biomass, there was a significant effect of treatment combination (algaecide type × concentration; $F_{30, 248} = 1.81, P = 0.008$), and 2 of the 30 algaecide treatment combinations had significantly lower biomass than untreated controls at 14 DAT (Figure 1). Specifically, there were significant decreases in biomass between the controls and 1.0 mg Cu L$^{-1}$ Algimycin® PWF (34% decrease from controls) and Komeen® (40% decrease). We did not detect a significant effect of algaecide type on biomass ($F_{6, 272} = 0.70, P = 0.651$; Table 2). While there was a significant effect of concentration ($F_{5, 273} = 4.42, P = 0.001$), we did not detect any differences between untreated controls and the concentrations we examined (Table 2). We did, however, observe the greatest biomass reduction at the highest concentration (1.0 mg Cu L$^{-1}$; 22% decrease from controls; Table 2).

For discoloration of N. obtusa thalli, there was a significant effect of treatment combination ($F_{30, 247} = 6.28, P < 0.001$), and four of the six algaecides at concentrations of 0.75 and 1.0 mg Cu L$^{-1}$ significantly differed from the untreated controls at 14 DAT (Figure 2). Specifically, there were
Table 2. Mean *Nitellopsis obtusa* wet-weight biomass and standard error (SE), mean proportion of green *N. obtusa* tissue and standard error (SE), and results from linear (wet weight) and generalized least squares (proportion of green tissue) models for algaecide type and copper concentration. Proportion of green tissue was logit-transformed prior to analysis, *P*-values for individual factor levels (specific algaecides and concentrations) are based on contrasts with untreated controls. * indicates significant *P*-value (*α* = 0.05).

| Treatment              | Wet weight (g) |         |         | Proportion of green tissue |         |         |
|------------------------|----------------|---------|---------|---------------------------|---------|---------|
|                        | Mean           | SE      | *P*-value | Mean           | SE      | *P*-value |
| Control                | 0.601          | 0.078   | −       | 0.989          | 0.007   | −       |
| Algaecide              |                |         |         |               |         |
| Algimycin® PWF         | 0.517          | 0.032   | 0.316   | 0.901          | 0.024   | 0.064   |
| Captain® XTR           | 0.607          | 0.029   | 0.939   | 0.923          | 0.022   | 0.408   |
| Cutrine®-Plus          | 0.577          | 0.036   | 0.779   | 0.866          | 0.029   | 0.007*  |
| Cutrine®-Ultra         | 0.582          | 0.034   | 0.827   | 0.902          | 0.024   | 0.051   |
| Komeen®                | 0.562          | 0.038   | 0.645   | 0.971          | 0.007   | 0.455   |
| Nautique®              | 0.586          | 0.034   | 0.860   | 0.981          | 0.005   | 0.693   |
| Concentration (mg Cu L⁻¹) | 0.001*        |         |         | < 0.001*      |         |         |
| 0.125                  | 0.659          | 0.029   | 0.467   | 0.980          | 0.005   | 0.535   |
| 0.25                   | 0.582          | 0.029   | 0.816   | 0.986          | 0.004   | 0.842   |
| 0.5                    | 0.600          | 0.031   | 0.991   | 0.961          | 0.009   | 0.068   |
| 0.75                   | 0.553          | 0.030   | 0.547   | 0.921          | 0.012   | < 0.001* |
| 1.0                    | 0.467          | 0.032   | 0.093   | 0.768          | 0.033   | < 0.001* |

Figure 2. Mean proportion of green *Nitellopsis obtusa* tissue following 14-day exposure to six copper-based algaecides at five concentrations of elemental copper. Error bars are ± 1 SE. * indicates *P* ≤ 0.05 and ** indicates *P* < 0.001 compared to untreated controls (Ctrl) based on a generalized least squares model with logit-transformed data.

Significant decreases in the proportion of green tissue between the control and 0.75 and 1.0 mg Cu L⁻¹ Algimycin® PWF, Captain® XTR, Cutrine®-Plus, and Cutrine®-Ultra. There was a significant effect of algaecide type on discoloration (*F*_6, _271_ = 2.64, *P* = 0.017) and one algaecide, Cutrine®-Plus, had significantly lower proportion of green tissue than untreated controls; the proportion of green tissue for Cutrine®-Ultra was marginally lower.
than controls (Table 2). The effect of concentration on proportion of green tissue was also significant ($F_{5, 272} = 17.02$, $P < 0.001$); the two highest concentrations, 0.75 and 1.0 mg Cu L$^{-1}$, had significant reductions in green tissue compared to untreated controls (Table 2).

**Bulbil experiment**

Measured initial copper concentrations for the bulbil experiments were 86.2 ± 4.9% and 102.4 ± 4.7% of target values across all algaecide treatment combinations for the below- and above-sediment experiments, respectively; thus, treatments were consistent with target values (complete concentration testing results for the bulbil experiments are reported in Table S4). By 14 DAT, copper concentrations were 41.6 ± 5.4% and 28.1 ± 1.0% of measured initial values across all algaecide treatment combinations for the below- and above-sediment experiments, respectively (Table S4). Copper concentrations in algaecide-adherence test jars were 86.1 ± 7.8% of initial measured values by 14 DAT, indicating minimal copper adherence to jar surfaces.

For the below-sediment *N. obtusa* bulbil experiment, there was a significant effect of treatment combination (algaecide type × concentration; $P = 0.031$); however, none of the 30 algaecide treatment combinations we tested reduced *N. obtusa* bulbil viability compared to untreated controls by 56 DAT (Figure 3). The proportion of sprouted bulbils was significantly higher than in controls for three different algaecides at 0.25 mg Cu L$^{-1}$: Captain® XTR, Komeen®, and Nautique®. Captain® XTR at 0.25 mg Cu L$^{-1}$ was the only treatment combination that had 100% bulbil sprouting (Figure 3).
Table 3. Proportion of *Nitellopsis obtusa* bulbils sprouted in below- and above-sediment experiments, and results from Fisher’s exact tests for algaecide type and copper concentration. *P*-values for individual factor levels (specific algaecides and concentrations) are based on contrasts with untreated controls. * indicates significant *P*-value (*α* = 0.05).

| Treatment          | Proportion sprouted below sediment | Proportion sprouted above sediment |
|--------------------|-----------------------------------|------------------------------------|
|                    | Mean     | *P*-value | Mean     | *P*-value |
| Control            | 0.60     | < 0.001*  | 0.20     | 0.012*    |
| Algimycin® PWF     | 0.77     | 0.160     | 0.02     | 0.007*    |
| Captain® XTR       | 0.86     | 0.011*    | 0.13     | 0.481     |
| Cutrine®-Plus      | 0.71     | 0.426     | 0.12     | 0.468     |
| Cutrine®-Ultra     | 0.67     | 0.609     | 0.10     | 0.248     |
| Komeen®           | 0.89     | 0.004*    | 0.07     | 0.086     |
| Nautique®         | 0.82     | 0.039*    | 0.14     | 0.499     |
| Concentration (mg Cu L⁻¹) |          |            |          |            |
| 0.125             | 0.74     | 0.280     | 0.13     | 0.477     |
| 0.25              | 0.88     | 0.004*    | 0.08     | 0.092     |
| 0.5               | 0.78     | 0.102     | 0.09     | 0.231     |
| 0.75              | 0.76     | 0.171     | 0.08     | 0.092     |
| 1.0               | 0.78     | 0.102     | 0.12     | 0.291     |

There was a significant effect of algaecide type on bulbil sprouting (*P* < 0.001), and Captain® XTR, Komeen®, and Nautique® had significantly higher sprouting than controls (Table 3). The effect of concentration was also significant (*P* = 0.019); concentrations of 0.25 mg Cu L⁻¹ had significantly higher sprouting than controls (Table 3).

For the above-sediment *N. obtusa* bulbil experiment, we did not detect a significant effect of treatment combination (*P* = 0.334), indicating that none of the treatment combinations significantly differed from the untreated controls by 56 DAT (Figure 4). We did not detect a significant effect of concentration on bulbil sprouting (*P* = 0.372), but there was a significant effect of algaecide type (*P* = 0.012); there was significantly less sprouting for Algimycin® PWF treatments than untreated controls (Table 3). We observed universally low sprouting for above-sediment bulbils, possibly due to inhospitable sprouting conditions. Only 20% of above-sediment control bulbils sprouted compared to 60% for below-sediment controls, which was a significant decrease (*P* = 0.022, Fisher’s exact test; Table 3). This low sprouting in the controls resulted in a lack of statistical power to detect differences between controls and treatment combinations with lower sprouting (i.e., < 20%). Nonetheless, we report these results to inform future research addressing *N. obtusa* bulbil control.

Discussion

To our knowledge, this is the first study to report on the efficacy of copper-based algaecides for *N. obtusa* treatment using a controlled, well-replicated, laboratory experiment. For *N. obtusa* thalli, we observed significant biomass reduction from some algaecides at the highest concentration evaluated, i.e., the maximum label rates of 1.0 mg L⁻¹ elemental copper, but we did not detect
significant reductions across all algaecides at this concentration, nor did we observe significant reductions at concentrations < 1.0 mg Cu L\(^{-1}\). The highest concentrations of copper, 0.75 and 1.0 mg L\(^{-1}\), resulted in significant discoloration of *N. obtusa* tissue for several algaecides. In contrast, sprouting of *N. obtusa* bulbils placed below the sediment was not decreased by any of the algaecides or copper concentrations we assessed; in fact, sprouting significantly increased after exposure to 0.25 mg L\(^{-1}\) copper concentrations relative to controls for three of the six algaecides we evaluated. Sprouting of above-sediment bulbils was universally lower than bulbils placed below the sediment, but we observed this pattern across algaecide treatments and untreated controls alike. These findings provide a needed baseline for future improvement of chemical treatment options for *N. obtusa* and reinforce the difficulty of achieving sustained *N. obtusa* control.

We followed generally accepted guidelines for the design and implementation of our experiment (Netherland and Richardson 2016; Richardson and Haug 2018). However, a caveat of our study is that there is limited information on laboratory conditions favorable for *N. obtusa* growth (but see Pokrzywinski et al. [2020b], which was published after we began our experiments) and it is possible that conditions in our experimental jars may have affected our results. For example, the low sprouting we observed for above-sediment bulbils may be because bulbils are simply less likely to sprout above the sediment under natural conditions, or because
we did not provide adequate conditions for above-sediment sprouting in the laboratory. Gottschalk and Karol (2020) observed high sprouting rates for *N. obtusa* bulbils placed on top of sediment and Pokrzywinski et al. (2021) observed high viability of bulbils attached to rhizoids and placed in water. These findings suggest that conditions in our experiment may indeed have been inhospitable. While starting conditions for jars appeared favorable, we noticed nuisance algae growth in 66% of above-sediment bulbil jars by 34 DAT (Figure S3). This nuisance algae growth typically surrounded the bulbils sitting on top of the sediment. There was no correlation between bulbil sprouting in above-sediment jars and the presence of nuisance algae ($r = 0.10$, $P = 0.20$), but the prevalence of nuisance algae may have reduced bulbil sprouting overall in this experiment, possibly by reducing light or nutrient availability. Future algaecide trials for bulbils placed above the sediment should consider alternative water, substrate, and light conditions (e.g., those used by Gottschalk and Karol 2020; Pokrzywinski et al. 2020b) to promote sprouting and reduce nuisance algal growth.

Conditions for the thalli experiment may also have been suboptimal; specifically, we noticed growth of a thin, translucent film of brown epiphytic algae on some thalli and the sides of some jars before and during the experiment (31% of jars on day 0 and 41% on day 14; data not shown). This growth did not impede our ability to observe *N. obtusa* color or assess biomass, and there was no correlation between these response measures and the presence of the nuisance algae at 14 DAT (|$r| < 0.1$ and $P > 0.20$ for both). However, epiphytic algae growth might have reduced the effectiveness of the algaecides we examined due to some portion of the elemental copper being sorbed by the nuisance algae, with a commensurate reduction of *N. obtusa* exposure. Growth of nuisance algae is a common issue in macrophyte laboratory experiments (Richardson and Haug 2018), but continued research on optimal growth and environmental conditions for *N. obtusa* (e.g., Pokrzywinski et al. 2020b) could help reduce this issue in future algaecide trials.

Copper-based algaecides are widely used for algae control (Lembi 2014), thus we expected the algaecides we examined, particularly at maximum label rates, to have greater effects on *N. obtusa* than we observed. Moreover, we evaluated relatively small individual plants under static conditions, a putative "best case" scenario for treatment (Richardson and Haug 2018), especially compared to the dense *N. obtusa* beds often found *in situ*. Similar laboratory studies of copper-based algaecides, including those we examined, have reported greater effectiveness for controlling algae (Hallingse and Phlips 1996; Murray-Gulde et al. 2002; Bishop and Rodgers 2012; Calomeni et al. 2015; Bishop et al. 2018). However, findings for blue-green and planktonic green algae assessed in prior studies have limited transferability to macroalgae like *N. obtusa*, which differs substantially
from other algae both structurally and evolutionarily, and is closely related to land plants (embryophytes; Karol et al. 2001; Judd et al. 2002; Ruhfel et al. 2014). Indeed, in the only other experimental evaluation of copper algaecides for *N. obtusa* control that we are aware of, Pokrzywinski et al. (2021) observed similar reductions in wet-weight biomass (≤ 45%) – and this despite their study lacking sediments and *N. obtusa* being free-floating rather than planted, i.e., conditions under which control may be easier to achieve.

The complex multi-cellular structure of *N. obtusa* likely makes it less susceptible to copper-based contact algaecides than single-celled planktonic algae. This difference in structure is reflected in algaecide labels, which generally recommend higher copper concentrations for benthic or macrophytic algae (SePRO Corporation 2014). From an aquatic plant management perspective, the challenges of *N. obtusa* control thus have parallels with management of vascular plants. For example, for the vascular invasive macrophyte *Hydrilla verticillata* (L. f.) Royle., susceptibility to copper-based products alone is generally low, particularly at the concentrations examined in our experiments (Turnage et al. 2015). More typically, copper-based products are used in combination with other herbicides to increase their effectiveness for vascular macrophytes (Gettys et al. 2014). However, Pokrzywinski et al. (2021) evaluated this treatment approach for *N. obtusa* and found that combining copper-based products with non-copper herbicides (carfentrazone, diquat, and endothall) did not increase reductions in *N. obtusa* biomass over copper products alone. Nonetheless, evaluations of additional product combinations and chemical treatment approaches more typical of vascular aquatic plants should be considered in future studies.

Field-based studies of treatment outcomes for *N. obtusa* and confamilial *Chara* spp. have demonstrated substantial reductions in biomass following copper-based algaecide application (McIntosh 1974; Guha 1991; Glisson et al. 2018). These findings may contrast with those from our thalli experiment for two reasons. First, degradation rates of injured algal biomass and chlorophyll *a* content (i.e., discoloration) may be increased in the field following algaecide application (Bishop and Rodgers 2011) due to multiple and interacting factors, such as bacterial degradation, altered light intensity, and natural physical disturbance (Hurley and Armstrong 1990; Belova 1993). Additionally, the dosage of copper (i.e., ratio of copper mass to algal biomass) could be substantially higher in some field settings than in our experiments. Because copper concentration is dependent on water volume, targeted concentrations for sites with relatively little *N. obtusa* relative to the volume of water (e.g., sites with greater water depth), can result in applications of much more algaecide per unit *N. obtusa* biomass than in scaled laboratory studies where water volume and biomass are more closely matched (Bishop et al. 2015).
Given the differences in conditions between laboratory and field algaecide treatments, and the differences in depth, *N. obtusa* biomass, and other environmental conditions among treated locations within lakes, findings from multiple field studies are needed to draw conclusions about the effectiveness of *N. obtusa* algaecide treatments *in situ*. Findings from applications to a single management unit within a lake (e.g., Glisson et al. 2018) may not be broadly representative of *N. obtusa* algaecide treatment outcomes. Indeed, analysis of monitoring data from 15 *N. obtusa*-invaded lakes in Wisconsin and Minnesota indicated that treatments with copper-based algaecides did not reduce frequency of occurrence or relative abundance (rake density) of *N. obtusa* within treated areas (W. Glisson, University of Minnesota, *unpublished data*). Further research using larger-scale laboratory and mesocosm studies, as well as continued evaluation of within-lake treatments with paired untreated reference areas, could help clarify the effectiveness of individual algaecides and treatment combinations (e.g., algaecides paired with non-copper herbicides, mechanical harvesting, etc.).

As with biomass, we only saw substantial discoloration in *N. obtusa* at the highest concentrations evaluated (Table 2). Discoloration has been documented following contact algaecide treatments of other characean algae (Burkhart and Stross 1990; Kelly et al. 2012). Compared to observed discoloration, measurement of chlorophyll *a* content or fluorescence are more rigorous means to quantify the effectiveness of treatment for algae (Bishop and Rodgers 2012; Bishop et al. 2018; Pokrzywinski et al. 2021), but these methods were not employed in the present study. Nonetheless, we considered reductions in the proportion of visibly green *N. obtusa* tissue to be a reasonable indicator of chlorophyll and cellular damage, consistent with copper’s mode of algaecidal action (Cid et al. 1995). Indeed, our results for discoloration of green *N. obtusa* tissue were broadly consistent with the fluorescence analysis results of Pokrzywinski et al. (2021) for the algaecides shared between experiments. Specifically, for Nautique® and Komeen® (granular formulation) at 1.0 mg Cu L⁻¹, Pokrzywinski et al. (2021) recorded high fluorescence and overall photosynthetic yield values, and we observed little discoloration. For Captain® XTR at 1.0 mg Cu L⁻¹, Pokrzywinski et al. (2021) recorded low fluorescence values, and we similarly observed significant discoloration. While reductions in photosynthetic activity are indicative of algal damage and decay, the extent to which such damage impedes growth and reproduction in *N. obtusa* remains uncertain. Injured tissue could potentially recover from bleaching where cells remain intact (as observed for other macrophytes; Wilson and Koch 2013), or undamaged healthy portions of the thalli may continue to grow.

Given that *N. obtusa* bulbils were covered with sediment in the below-sediment experiment, it is unsurprising that the copper-based contact algaecides we examined did not reduce sprouting. Copper can rapidly bind with sediments (Rader et al. 2019), which typically constitute the ultimate
sink of less-bioavailable forms of copper applied in aquatic systems (Huggett et al. 1999; Willis and Bishop 2016). We observed the process of copper binding to sediments in our experiments; copper concentration decreased substantially more in algaecide-adherence test jars with sediment than in those without sediment by 14 DAT (Table S3). Hence, the sediment likely formed a protective barrier for bulbils. In addition, more favorable conditions might have led to greater sprouting overall in the below-sediment experiment; compared to above-sediment bulbil jars, those for the below-sediment experiment had less nuisance algae (66% versus 40% of jars, respectively; Figures S3, S4). However, as with above-sediment bulbils, there was no evidence of a relationship between sprouting and the presence of nuisance algae in the below-sediment experiment ($r = 0.09, P = 0.29$). Indeed, untreated control bulbils had the lowest sprouting rate in the below-sediment experiment, despite the lack of any nuisance algae (i.e., putatively favorable sprouting conditions; Figure S3).

Growth and reproduction can be stimulated in plants and algae following herbicide treatment at low concentrations (Cedergreen et al. 2007; Calabrese and Blain 2009; Velini et al. 2010). Given that the sediment formed a physical barrier and that most of the copper applied in our experimental jars without $N. obtusa$ was taken up by the sediment (Table S3), it is possible that low concentrations reached bulbils under the sediment and caused a stimulatory response. Low-dose stimulation of growth may also be responsible for the increased bulbil density following treatment with Cutrine®-Plus observed by Glisson et al. (2018). Importantly, possible stimulation of bulbil sprouting at low copper concentrations does not preclude inhibition of sprouting at higher concentrations, i.e., a hormetic response where low doses stimulate growth but higher doses inhibit growth (Calabrese and Baldwin 2002). Further research is needed to investigate the possibility of a stimulatory response of $N. obtusa$ bulbils to copper algaecide treatment.

Our assessment of six commonly used copper-based algaecides provides a foundation for future work on $N. obtusa$ control. The scaled laboratory bioassay we conducted allowed for replicated comparison of untreated $N. obtusa$ to multiple treatments in a controlled environment. Such experiments can improve predictions of efficacy for field applications to algal infestations (Bishop et al. 2015; Kinley-Baird et al. 2021). Nonetheless, translation of efficacy data to field sites is critical for validation of laboratory results, as many environmental variables may differ from a laboratory setting (e.g., temperature, light intensity, exposure duration, sediments, and water dynamics). Our study can inform future laboratory trials and be scaled up to more realistic mesocosm and field evaluations that better account for environmental conditions (Netherland and Getsinger 2018; Richardson and Haug 2018). For example, relatively well-performing copper algaecides could be examined in combination with other herbicides.
and adjuvants (e.g., Sutton et al. 1970; Pennington et al. 2001; Turnage et al. 2015). Most within-lake *N. obtusa* treatments consist of multiple products, particularly copper algacides in combination with flumioxazin, endothall, or 2,4-D (Larkin et al. 2018; W. Glisson, University of Minnesota, unpublished data); hence, such combination treatments should be evaluated in future studies. Repeated applications of the algacides we evaluated should also be assessed, as laboratory-based trials indicate that repeat treatments with the same or different products can be more effective than single treatments (Calomeni et al. 2015). Lastly, other products, copper-based or otherwise, should be evaluated to determine if they might be more effective than the products we evaluated.

Despite this being the first reported laboratory trial of algacides for *N. obtusa* control, the reality is that management of *N. obtusa* has been ongoing for decades (Pullman and Crawford 2010), with little assessment of treatment effectiveness. While copper-based algacides can be effective for *N. obtusa* biomass control in some situations (e.g., Glisson et al. 2018), our laboratory findings indicate that these algacides may have limited effectiveness and could potentially stimulate *N. obtusa* bulbil sprouting under certain conditions. Based on our laboratory findings and those of Pokrzywinski et al. (2021), some copper-based products at 1.0 mg Cu L⁻¹ may provide moderate control of *N. obtusa*, but repeat treatments are likely needed because much of the biomass remains intact following treatment. Alternative treatments may be more effective for reducing bulbil density and viability. For example, Glisson et al. (2018) observed lower bulbil density and viability when *N. obtusa* was first mechanically harvested, and then treated with copper algacide. Hence, management actions such as mechanical removal or hand removal by divers may be a promising complement or alternative to algacide treatment, particularly when infestations are small. These approaches should be implemented cautiously, however, as they could allow fragments to spread beyond the infested area. Analysis of current treatments and scaled-up mesocosm and within-lake experiments are needed to further advance treatment effectiveness for *N. obtusa*.

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Authors’ contribution

DJL: research conceptualization; WJG, RC-R, WMB, DJL: study design and methodology; WJG: investigation and data collection; WMB: analytical support; WJG and RC-R: data analysis; WJG, RC-R, WMB, and DJL: data and results interpretation; WJG and RC-R: writing – original draft; WJG, RC-R, WMB, and DJL: writing – review and editing.

Conflict of interest/Declaration of interests

WMB is an employee of SePRO Corporation.

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