PROTOCOL NOTE

Protocol to control the invasive alga *Avrainvillea lacerata* in a shallow Hawaiian reef flat

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Abstract

Premise: A novel control technique was developed to mitigate an invasive siphonous green alga, *Avrainvillea lacerata* (Dichotomosiphonaceae), within a shallow degraded reef flat in Oʻahu, Hawaiʻi.

Methods and Results: Replicated treatments of 3% and 10% hydrogen peroxide (H_2O_2) were administered into individual basal attachments of the bed-forming invasive seaweed on the Paikō reef, Oʻahu. Relative electron transport rate maxima ($rETR_m$) were measured using a Walz Diving Pulse Amplitude Modulated Fluorometer in two replicate 100-m^2 plots in 2020. Over the period of this short-term study, $rETR_m$ decreased following injections of either concentration of H_2O_2 in contrast with negative and positive controls.

Conclusions: Compared with existing techniques that have used oxidizing agents in the marine environment in localized areas, the protocol described here has the potential to successfully decrease macroalgal carbon gain, potentially leading to loss of biomass at larger scales.

KEYWORDS
Bryopsidales, Chlorophyta, hydrogen peroxide, oxidation, pulse amplitude modulation, siphonous

In Hawaiʻi, invasive species issues are at the forefront of conservation efforts aimed at protecting native species populations from extinction. As marine environments are also impacted by invasive species, a new protocol has been developed to deal with an invasive alga in the coastal regions of the island of Oʻahu, Hawaiʻi. *Avrainvillea lacerata* J. Agardh (Dichotomosiphonaceae) is a siphonous green alga that can attach to rock or in sand and is now prevalent on many shallow to mesophotic reefs (Veazey et al., 2019; Wade, 2019) in the Main Hawaiian Islands. Although this benthic species can form dense beds as the bulk of a reefʻs biomass, no grazer has been identified that readily and adequately keeps this species in check (Van Heukelem, 2016). Community organizations and coral reef managers are highly motivated to find new control methods for this perennial and persistent mound-forming organism. While previous efforts have removed millions of pounds of this alga from many hectares of impacted coral reef, there remains a need to develop new tools to control this

organism at scale. Therefore, we developed a novel protocol using discrete amounts of hydrogen peroxide (H_2O_2) to form reactive oxygen to denature the photosynthetic processes and cellular structure of this organism, thereby reducing its ability to sequester carbon via treatment-induced oxidation disrupting the light-harvesting photosynthetic reaction centers.

Siphonous green algae, in the order Bryopsidales, are a type of macroalgae composed of a single cell that is tubular in shape and elongated (Vroom and Smith, 2001). They grow via turgor-pressure-mediated cell expansion of the central vacuole and do not form cellular cross walls after each cell division. Even without the benefit of multicellularity, these cells exhibit complex and sophisticated structures for maximized light harvesting and holdfasts for anchoring. Their thalli can house millions of organelles that can move around the cell through a phenomenon known as cytoplasmic streaming. It is their simplistic yet streamlined design that allows many species within the order to

proliferate and become invasive. For example, *Codium fragile* (Suringar) Hariot subsp. *tomentosoides* (Van Goor) P. C. Silva is increasingly being seen as a threat to global biodiversity (Provan et al., 2005). Additionally, in the Mediterranean Sea, the spread of a distantly related siphonous green alga, *Caulerpa taxifolia* (M. Vahl) C. Agardh, is a primary concern to managers in that multi-nation region. The species has spread far and wide after its presumed accidental discharge in Monaco (Jousson et al., 1998). When *Caulerpa taxifolia* was found offshore of southern California, the chemical methods employed were successful and involved administering chlorine bleach in a localized area underneath a tarp. While the treatments were effective at eradicating the species at the population level, the treatments were found to be deleterious to animal life under the tarp in which it was contained (Williams and Schroeder, 2004; Anderson, 2005). In the future, residual chemical exposures of reactive products will be a primary concern of conservation managers when dealing with chemicals in sensitive areas such as delicate reefs and seagrass meadows.

The following protocol applies a novel field-based methodology to reduce the relative electron transport rate maxima ($rETR_m$) of the alga in situ to test for treatment efficacy. This is possible because $rETR_m$ is an indirect measure of photosynthesis. Hydrogen peroxide (H_2O_2) was the selected oxidation agent because of its efficacy in reducing photosynthesis among various algal groups (Dummermuth et al., 2003; Drábková et al., 2007). As a precautionary approach, we first tested if the treatment would be effective in isolated aquaria, followed by testing in outdoor mesocosms where the target organism was isolated from the surrounding reef biota. Once we confirmed that the treatments had a selective ability to decrease photosynthesis throughout the whole thallus, we began planning an applied experiment in a limited area to test field-use efficacy. Due to the proximity of our study organism to seagrass beds, corals, and other animal life, we decided to work with the chemical treatment method that seemed the most biodegradable and is already known to occur in coastal waters at low concentrations (Cooper and Zika, 1983).

The basic application method is simple and can be employed without the use of the physiological approaches discussed here. However, we offer the methods described below and detailed in Appendix 1 to demonstrate the speed of detectable response following administration and to encourage using a variety of methods and data acquisition to further improve investigations of best practices for reef biodiversity management. It is important to apply continuous monitoring and maintenance of native reef biodiversity and habitat when these types of chemical control methods are employed, both during and post-implementation. We chose to use $rETR_m$ as a relative approximate indicator of growth potential as previous studies on invasive algae have suggested that photosynthetic rates can be used to demonstrate loss of

carbon assimilation and as a proxy for growth (Smith et al., 2004). Although this study does not monitor long-term impacts on growth rate of the alga in question, characterizing the immediate impacts of the injections will have management potential when dealing with persistent and incipient populations that need to be addressed quickly.

METHODS AND RESULTS

The study was conducted at the shallow reef at Paikō, Maunalua Bay, on the southeast shore of Oʻahu. Paikō was selected because of the high abundance of *A. lacerata* in the shallow intertidal zone. At that site, the invasive alga competitively interacts with the native seagrass *Halophila hawaiiana* Doty & B. C. Stone (Peyton, 2009). The treatment project was carried out in the summer of 2020, for a 2-wk period from 19 June to 2 July. The benthos is shallow and accessible in the reef flat region and is a mix of sandy and rocky intertidal habitat (Battista et al., 2007). Fully developed adults in later growth stages were selected from the nearshore reef flat regions adjacent to the shoreline at Paikō. This site is part of the larger Maunalua Bay region, where one of the earliest detections of the alga in Hawaiian waters was noted in 1985 (Brostoff, 1989). Concurrently, Unabia (1984) noted the density of the native seagrass *Halophila hawaiiana* in the sand flat regions at Paikō numbered 15,000 leaves per square meter. Due to competitive interactions with invasive species and pollution, the density and abundance of seagrass meadows is in decline worldwide (Orth et al., 2006).

Two 100-m^2 plots were delineated with the assistance of the non-profit organization Mālama Maunalua (Honolulu, Hawaiʻi). They have been removing *A. lacerata* since 2005 and have had extensive experience in managing the abundance of this macroalga and galvanizing community support and engagement in removal efforts (Kittinger et al., 2016). As Mālama Maunalua already had estimates of percent cover in each plot within the reef flat, we used those estimates as a basis for plot selection to attain a minimum number of individuals in each plot. Plots with over 20% coverage of *A. lacerata* were selected (Mālama Maunalua, unpublished data); the plot centers correspond to the following GPS coordinates: 21°16′49.4754″N 157°43′56.4234″W and 21°16′49.1514″N 157°43′56.0274″W.

From the center of each plot, 5 m were measured south and then 5 m were measured west to delineate the southwest corner of each plot. From the southwest corner of each plot, one 10-m transect was placed along the western end of each plot for reference. Subsequently, five 10-m transects were laid out from west to east to help identify locations of individuals within each plot for repeated measure. Twenty individuals were marked and enumerated temporarily in each plot using a 1-cm-long ring cutout of 1/2″-diameter PVC, which was attached to the individual using a zip tie.

The 20 individuals were delineated in a haphazardly stratified pattern in each plot among two of the five east–west transects with the highest abundance of *Avrainvillea*, based on whether they overlapped with one of the five transect lines. Five were marked and delineated for treatment with 30 mL of 3% H_2O_2, and another five for treatment with 30 mL of 10% H_2O_2. Additionally, five were set aside for positive control (treated via injection with 30 mL of deionized water), and five were marked for negative control (no treatment). The order of treatments and controls along the east–west transects was randomized using a random number generator. A 2-oz bank lead weight sinker was then tied to a zip tie using fishing line to hold the marks in place and to minimize the marks from being lost with tidal fluctuations. All the marks were re-found and collected after a month-long period of post-implementation monitoring. There were a total of 40 individuals ($n = 10$ per treatment) within the two 100-m^2 plots. The protocols to prepare the various dilutions for treatment are described in Appendix 1. It takes about 20 s to administer the treatment into each thallus, allowing for the potential to scale these methods for treatment of up to 180 individuals per hour. It should be noted that at least two persons are required for implementation for field safety reasons.

To measure the effect of treatments in situ and under diurnal, moderate low tides, a red actinic Diving Pulse Amplitude Modulated Fluorometer (D-PAM; Heinz Walz GmbH, Pfullingen, Germany) was used to calculate the $rETR_m$ between photosystems, as an indirect measure for photosynthesis (Longstaff et al., 2002). A GoPro Hero 7 (GoPro Inc., San Mateo, California, USA) camera was used to capture photography and videography of the reaction as it occurred over time. A 100-mL-volume automatic repeating syringe was used to administer the different treatment types. The solution was contained in a modified hydropack connected to plastic tubing that fed into the back of the automated syringe. Only a 10-mL volume was able to be aliquoted at a time from the automatic syringe. We used

FIGURE 1 Underwater photography exhibiting controlled individuals and individual impacts from the lower-concentration (3% H_2O_2) treatment. (A) Negative control individual before treatment. (B) Negative control individual after treatment. (C) Individual before 3% H_2O_2 treatment. (D) Individual after 3% H_2O_2 treatment. The photographs remain unedited due to variations in sunlight and turbidity based on tidal fluctuations. Discoloration was observed in the margins of the 3% H_2O_2 treatment (D) within 1 h post-injection.
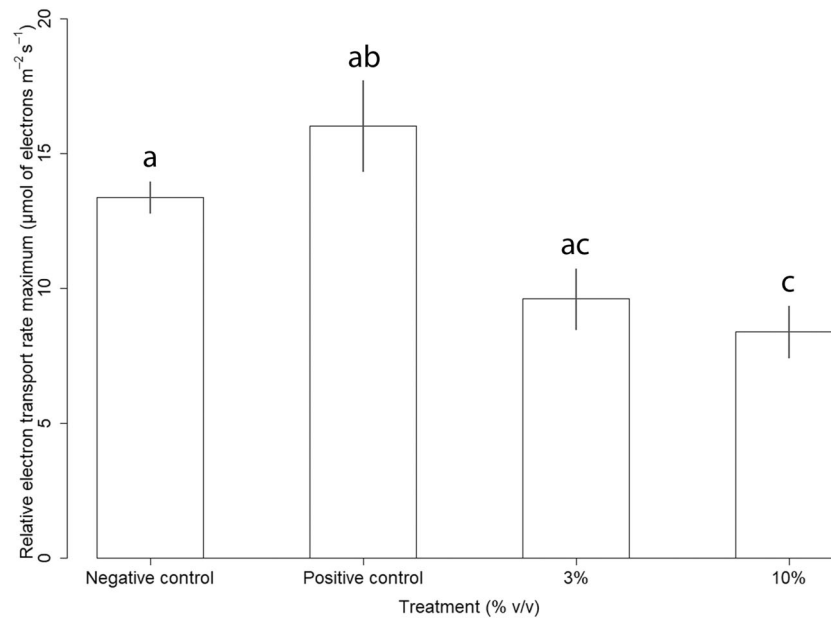
FIGURE 2 Effect of H_2O_2 (% v/v) concentration on the relative electron transport rate maximum (μmol of electrons $m^{-2}s^{-1}$) in *Avrainvillea lacerata* in situ in the shallow water reef flat at Paikō, Maunalua Bay, Oʻahu, Hawaiʻi. Treatments are listed from left to right: negative control, positive control, 3% H_2O_2, and 10% H_2O_2 ($n = 10$ per treatment). Values represent means and standard errors, with different superscript letters indicating significant difference ($P < 0.05$).

multiple pumps of the syringe to aliquot 30 mL into a single basal holdfast for each organism. A volume of 30 mL was selected for the treatment types based on previous laboratory experiments that showed 10 mL had a negligible effect. After 30-mL aliquots of each treatment were collected, a video of the post-injection period was taken over 3-min durations and still photographs were taken before and after each treatment (Figure 1). The D-PAM was used on each marked alga before each treatment, then used on the same individuals 1 h after treatments to determine the immediate effect of the treatment types. The most mature blade unobscured by epiphytes that was closest to the central axis was chosen on each individual alga to perform the fluorescence measurements. Before-and-after photographs were taken to characterize damage to the immediate surrounding benthic cover and epiphyte load around each marked individual.

All statistical analyses were carried out using R version 4.1.1 (R Core Team, 2021). We used a one-factor analysis of variance to test whether relative electron transport rates were affected by H_2O_2 concentrations with the aov function in the stats package (Chambers et al., 1992). The data were assumed to be normal based on the Shapiro–Wilk normality test ($P > 0.05$) using the shapiro.test function in the stats package (Royston, 1982).

The average ETR_m for the negative and positive control in the field-manipulated study was 13.367 ± 0.582 SE and 16.023 ± 1.697 SE, respectively, $n = 10$ (Figure 2). Both the 3% and 10% treatments had a significant reduction in the electron transport rate compared to the controls ($F = 9.11$, $P = 0.0001$). The 3% treatment had an average ETR_m value of 9.605 ± 1.118 SE, while the 10% treatment had a similar reduced average value of 8.387 ± 0.951 SE. These data suggest that there was approximately a 40% reduction in physiological capacity for photosynthesis in treated organisms within 1 h post-treatment, regardless of the H_2O_2 concentration.

Two weeks after the post-treatment implementation, one individual mortality was observed in the 10% treatment group after the basal holdfast started to detach from the substrate; the detaching individual was whitish gray in color. Similarly, the rest of the treated individuals showed signs of discoloration two weeks after both the 3% and 10% treatments, showing that there was a considerable impact to these perennial mounds over an extended time frame.

CONCLUSIONS

We found the injection of H_2O_2 treatments at both 3% and 10% to be an effective means of reducing macroalgal $rETR_m$ throughout the thalli in a field-use setting. As an applied field method, we have found considerable value for further investigation of H_2O_2 as a tool for widespread control of this invasive mat-forming benthic macroalgae. Photographic and observational evidence also verified that there was a noticeable impact on coloration and pigmentation throughout the organism.

Reagent-grade H_2O_2 is an affordable treatment agent at a cost of US\$26 for 500 mL at 30% concentration at the time of this study. Furthermore, it is relatively easy to prepare dilutions with volumetric measurements and to administer treatments (Appendix 1). In cases where laboratory-grade H_2O_2 is unavailable, store-bought H_2O_2 should suffice to

replace the laboratory-grade dilution, although it will likely cost more per milliliter. For the utility of this method's implementation at a larger scale, participants, practitioners, and managers must be able to discern *Avrainvillea* species from other siphonous green algae genera that are similar in appearance, such as *Halimeda* J. V. Lamouroux and *Udotea* J. V. Lamouroux. We used the field guide *Hawaiian Reef Plants* (Huisman et al., 2007) to identify the algae in our plot system to species level. New volunteers and working groups will require regular training to prevent targeting the wrong organisms in the field.

There is considerable ability to scale up the treatment methods implemented in this study, but regular input from a trained and dedicated team and community members will be needed. State and federal agencies and partners interested in dealing with large amounts of algal biomass at larger scales can employ dedicated teams for shallow water snorkeling and SCUBA-certified divers to better tackle the extent of the problem in deeper waters. Teams of five to 10 people can administer treatment and subsequently monitor treatment effectiveness at a wider scale than was implemented in this study. Using multiple injectors will allow field teams to work in multiple plots simultaneously during appropriate weather conditions to allow for maximum output of mitigation efforts. Furthermore, roles that are diversified within the teams to deal with application, monitoring, field safety, and boat operation will be useful for implementation at larger scales.

Further chemical and biological questions that arise from these types of field treatments include how H_2O_2 could be used to oxidize pollutants in natural waters (e.g., hydrogen sulfide in surface waters), as this may concurrently improve water quality dynamics (Millero et al., 1989). In biological systems, H_2O_2 is an important cellular signaling mechanism for a variety of functions including pathogen resistance, senescence, and cell elongation and differentiation (Petrov and Van Breusegem, 2012). To date, this is one of the only studies to investigate the ability of extracellular H_2O_2 to cause discoloration in macroalgae associated with a reduction of photosynthetic physiological capacity in a field-based setting.

The exceptional ability of some marine macroalgae such as *Avrainvillea* species to form extensive, dense patches of beds, and their subsequent impact on the decline in reef biodiversity, has been extensively documented (Littler et al., 2004; Williams and Smith, 2007; Foster et al., 2019). Future studies should investigate novel ways to improve the applicability of the methods mentioned above in a larger study area and in different sites and environments, such as more exposed and rockier shoreline habitat. It is likely that an increase in water flow will cause greater dilution of the oxidant and thus make the reaction time shorter. However, it is suggested that the regions exposed to minimal bottom currents are most susceptible to invasion (Veazey et al., 2019).

The 3% and 10% H_2O_2 concentrations were found to be similarly effective in reducing $rETR_m$ in a 2-wk-long short-term field experiment. We recommend using the lower concentration in most treatment applications to avoid deleterious effects to the biological composition of the water column. If treatment is deemed to be unsuccessful in future applications in areas with higher water flow, we recommend using repeated applications rather than increasing the treatment concentration. Future work on this and other target siphonous algae should test the effectiveness of low concentrations of H_2O_2 at greater depth ranges and in different habitats with varying levels of water motion. It is likely that a decrease in available ultraviolet wavelengths and an increase in water pressure may decrease the efficacy of the treatments employed in this study.

AUTHOR CONTRIBUTIONS

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Appendix 1. Required materials and protocol to perform the mitigation treatments on the invasive macroalga *Avrainvillea lacerata*.

Materials

1. Nitrile/neoprene gloves
2. Eye protection: tight-fitting safety goggles or glasses
3. Protective clothing to prevent skin contact
4. 30% Hydrogen peroxide (H_2O_2)
5. Deionized (DI) or distilled water
6. 100-mL and 500-mL beakers
7. Hydropack bladder
8. 0.5 m of 1-mm-diameter plastic tubing
9. Automatic repeating syringe (minimum 30 mL)
10. Luer Lock needles (10 gauge)
11. Numbered tagging system (1-cm-long ring cutout of 1/2″ diameter PVC, zip tie)
12. 2 transect tapes (minimum of 10 m) or 1-m^2 quadrats
13. 0.5-mm-diameter rod for point-intercept method
14. GoPro (GoPro Inc., San Mateo, California, USA) or other camera with video capabilities
15. GPS unit
16. Diving Pulse Amplitude Modulated Fluorometer (Heinz Walz GmbH, Pfullingen, Germany)
17. 556 Handheld Multiparameter Instrument (Yellow Springs Instruments [YSI], Yellow Springs, Ohio, USA)

Procedure

Step 1: Set up the applicator by connecting the 1-mm-diameter tubing to the outlet tube on the hydropack.

Step 2: Connect the other end of the tubing to the intake tube on the automatic repeating syringe.

Step 3: Create the dilutions for treatment. Refer to the equations below for the various concentrations. Mix in a well-ventilated area and use appropriate personal protective equipment (as listed in the materials).

- 300 mL of 3% H_2O_2 (% v/v) = 30 mL of 30% H_2O_2 + 270 mL of DI or distilled water
- 300 mL of 10% H_2O_2 (% v/v) = 100 mL of 30% H_2O_2 + 200 mL of DI or distilled water

- Store this mixed solution in a shaded larger container or in dark plastic bottles to extend the potency of the solution during transport.

Step 4: Find a field location where *Avrainvillea lacerata* is prevalent. This can be done via observational or ecological survey techniques using transects or quadrats.

Step 5: Fill the hydropack bladder with 300 mL of the solution for application. Before sealing the water-tight lid, make sure to expel any additional air from the hydropack.

Step 6: Attach the 10-gauge dispensing needle to the end of the automatic repeating syringe.

Step 7: Repeatedly squeeze the automatic repeating syringe until the liquid solution is drawn up to the dispersing end of the needle.

Step 8: Identify an individual for treatment. Mark the individual using the numbered tagging system and record its location on the GPS unit.

Step 9: Take a photo of the individual using the GoPro before treatment.

Step 10: Using the YSI multiparameter instrument, start continuous measurement with the probe in the water column about 10 cm above the treatment individual.

Step 11: Inject the solution into the holdfast (base) of the individual thallus underneath the substrate level.

Step 12: Stop the YSI continuous reading 5 min after the treatment.

Step 13: Measure the $rETR_m$ using the D-PAM light-curve function 30 min after the treatment.