

Article

Plant-Derived Products Selectively Suppress Growth of the Harmful Alga *Prymnesium parvum*

Mousumi A. Mary ¹, Shisbeth Tábora-Sarmiento ², Sarah Nash ², Gregory D. Mayer ² , Jordan Crago ² and Reynaldo Patiño ^{3,*} 

¹ Department of Biological Sciences and Texas Cooperative Fish and Wildlife Research Unit, Texas Tech University, Lubbock, TX 79409-2120, USA; mousumi-akter.mary@ttu.edu

² Department of Environmental Toxicology, Texas Tech University, Lubbock, TX 79409-1163, USA; shisbeth.tabora@ttu.edu (S.T.-S.); sarah.nash@ttu.edu (S.N.); greg.mayer@ttu.edu (G.D.M.); jordan.crago@ttu.edu (J.C.)

³ U.S. Geological Survey, Texas Cooperative Fish and Wildlife Research Unit and Department of Natural Resources Management, Texas Tech University, Lubbock, TX 79409-2120, USA

* Correspondence: reynaldo.patino@ttu.edu

Abstract: *Prymnesium parvum* is a harmful alga found in brackish waters worldwide whose toxins can be lethal to aquatic organisms. Established field methods to control blooms of this species, however, are unavailable. Earlier studies showed that various extracts of giant reed (*Arundo donax*) can suppress *P. parvum* growth and that ellipticine, an allelochemical present in giant reed, is a potent algicide against this species. The unintended effects of giant reed products on nontarget organisms, however, are not fully understood. This study determined the effects of giant reed leachate (aqueous extract of dried chips) and ellipticine on growth of *P. parvum* and the green microalga *Chlorella sorokiniana*; survival and reproduction of the planktonic crustacean *Daphnia pulex*; and hatching success, larval survival, and larval swimming behavior of the teleost fish *Danio rerio*. Leachate made with 3 g chips L^{−1} was lethally toxic to *P. parvum* and *D. pulex*, stimulated *C. sorokiniana* growth, and impaired *D. rerio* behavior. Leachate at 1 g L^{−1} fully suppressed *P. parvum* growth, had moderate effects on *D. pulex* reproductive output, and had no effects on *D. rerio*. Ellipticine at 0.01 mg L^{−1} irreversibly inhibited *P. parvum* growth, acutely but reversibly inhibited *C. sorokiniana* growth, slightly delayed *D. pulex* reproduction, and had no effects on *D. rerio*. These observations suggest that when applied at appropriate concentrations, natural products derived from giant reed can be used as tools to specifically control *P. parvum* growth with minimal effects on nontarget species.

Keywords: HAB control; algistatic activity; *Arundo donax*; aqueous extract; ellipticine; spectrum of activity; *Prymnesium parvum*



Citation: Mary, M.A.; Tábora-Sarmiento, S.; Nash, S.; Mayer, G.D.; Crago, J.; Patiño, R. Plant-Derived Products Selectively Suppress Growth of the Harmful Alga *Prymnesium parvum*. *Water* **2024**, *16*, 930. <https://doi.org/10.3390/w16070930>

Academic Editor: Haifeng Gu

Received: 1 March 2024

Revised: 19 March 2024

Accepted: 20 March 2024

Published: 23 March 2024



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1. Introduction

The geographic extent and intensity of harmful algal blooms (HABs) and their associated ecological and economic impacts have grown over the last several decades [1–3]. A major driver of these trends is the cultural eutrophication of aquatic habitats caused by inputs of nutrients from agricultural and developed lands [4,5]. *Prymnesium parvum* is an HAB-forming member of the phylum Haptophyta that is found in brackish waters worldwide and whose toxins can be lethal to aquatic organisms including fishes, amphibians, crustaceans, shellfish, and other algae [6]. Although *P. parvum* is responsible for some of the worst HAB-associated ecological catastrophes in inland waters [6], field methods to control its blooms are presently unavailable.

Much of the recent research on HAB control methods in standing waters has focused on natural, plant-derived products due to their high biodegradability, short half-lives, and effectiveness as inhibitors of algal growth [7–10]. However, little research has been carried out on the use of natural products to control *P. parvum* blooms. Earlier studies explored

the effects of barley straw (*Hordeum vulgare*) preparations on *P. parvum* growth, but the results obtained were not promising [11,12]. More recently, a methanolic extract of giant reed (*Arundo donax*) was shown to suppress *P. parvum* growth in a concentration-dependent manner [13]. A follow-up study reported that an indole alkaloid found in giant reed, ellipticine [14], has highly potent algicidal activity against *P. parvum* [15]. Various extract preparations from giant reed can also inhibit growth of other HAB species, such as the cyanobacterium *Microcystis aeruginosa* [14,16–18] and the marine dinoflagellate *Karenia brevis* [19]. These observations suggest that giant reed products may have the advantage over other natural products of being broadly applicable to the control of some of the most important prokaryotic and eukaryotic HABs.

There is some evidence indicating that, among microalgae, growth inhibition in the presence of giant reed extracts is preferentially observed in HAB species (i.e., *M. aeruginosa*) over green microalgae [16]; however, no information is available concerning the effects of giant reed products on aquatic fauna. This information is needed to evaluate the unintended environmental consequences of any bloom control method.

The goal of this study is to facilitate the development of natural and environmentally friendly methods of HAB control. The working hypothesis is that giant reed extracts and ellipticine will preferentially impair growth of *P. parvum* over selected nontarget species. We first determined the effect of giant reed dried chips (directly immersed in culture media) and leachate (aqueous extract) on *P. parvum* growth. We then determined the effects of leachate and ellipticine on the green microalga *Chlorella sorokiniana*, the planktonic crustacean *Daphnia pulex*, and the teleost fish *Danio rerio*. These nontarget species are widely used in basic and applied (including ecotoxicological) studies, and the large amount of information available for them facilitates inferences about environmental impacts based on laboratory studies.

2. Materials and Methods

2.1. General Culture Methods

Prymnesium parvum (UTEX-2797) was obtained from UTEX Culture Collection of Algae (The University of Texas at Austin, TX, USA). Stock cultures were maintained as previously described [15]. Briefly, nonaxenic cultures were maintained in modified UTEX artificial seawater medium at 5 psu and f/2 levels of nutrients (modified ASM) and placed in a plant germination rack with two full-spectrum LED lights. The photoperiod was set to 12:12 h light: dark with an average light intensity of $\sim 84 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and average daily temperature of $\sim 22^\circ\text{C}$. Culture flasks were gently swirled once daily for several seconds. Stock cultures in late exponential growth phase (day 15) were used to prepare new stock cultures and as source of inoculum for experimental cultures.

Chlorella sorokiniana (UTEX-1230) was obtained from UTEX Culture Collection of Algae. Stock cultures were maintained in BG-11 medium (UTEX) following culture conditions previously described [20]. Briefly, nonaxenic cultures were maintained on an orbital shaker at 120 rpm under two full-spectrum LED lights. The photoperiod was set to 16:8 h light: dark with an average light intensity of $\sim 100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and average daily temperature of 25°C . Stock cultures in exponential growth phase (day 3) were used to prepare new stock cultures and as source of inoculum for experimental cultures.

Daphnia pulex was acquired from Carolina Biological Supply Company (Burlington, NC, USA) and maintained as previously described [21]. Briefly, organisms were maintained in COMBO medium in a controlled Precision Low Temp BOD Refrigerated Incubator (VWR, Radnor, PA, USA). The photoperiod was set to 14:10 h light: dark with an average light intensity of $\sim 7000 \text{ lux}$ and an average daily temperature of 20°C . Stock cultures were maintained in glass jars, and organisms were gently transferred to new jars with fresh media once every 3 days to prevent accumulation of waste. Daphnids were fed with *Scenedesmus acutus* inoculated at a concentration of $10^5 \text{ cells mL}^{-1}$. They were allowed two months of acclimation prior to experimentation.

Adult, wild-type 5D-strain *D. rerio* were originally obtained from Robyn Tanguay's laboratory at Oregon State University and maintained as previously described [22]. Briefly, fish were maintained in a recirculating system (Aquaneering, San Diego, CA, USA) at a photoperiod of 14:10 h light: dark with an average light intensity of ~6900 lux, average daily temperature of ~28 °C, and dissolved oxygen of 80 percent saturation. Ammonia, nitrite, and nitrate levels were measured weekly and were consistently below 0.1 mg/L, 0.05 mg/L, and 2 mg/L, respectively. Fish were fed twice daily with commercially available dry food (Gemma Micro 300, Skretting, UT, USA).

For the purpose of *D. rerio* embryo collection, 3 adult males were paired with 5 adult females in each of three 6 L tanks equipped with false bottoms the night before breeding; these tanks were placed within the recirculating system. On the day of breeding, fertilized eggs were collected within 4 to 6 h post-spawn. Embryos were transferred to egg water media (60 mg of Instant Ocean Salt (Instant Ocean, Blacksburg, VA, USA) per liter of deionized water). Zebrafish procedures were conducted under Texas Tech University IACUC permit #20012-02.

2.2. Experimental Reagents

Giant reed with stems of approximately 2 m in height and without flowering plumes were selected for this study. Plants were collected from an urban stand in Lubbock (TX, USA), rinsed with tap water, and air-dried for 24 h. The stems and leaves were first chopped into small pieces with an electric garden chipper (GS70015, Earthwise, Indianapolis, IN, USA) and then cut into fine chips using a kitchen chopper (Nj100Gr, Ninja, Needham, MA, USA). Chips were placed in a heater at 60 °C for 48 h and the dried material (Figure 1) was stored at 4 °C in a desiccating chamber. For use in cultures, the appropriate amount of chips was placed in sterilized (autoclaved), drawstring tea filter bags and immersed in the experimental flasks. In initial experiments, cultures were started (inoculated) upon immersion of the bags in the flasks. In later experiments, chips were allowed to leach for 24 h at 4 °C and the leachate was collected and filtered sequentially with 0.45 and 0.22 µm syringe filters (SLHAM33S, SLGSR33SS, Sigma-Aldrich, St. Louis, MO, USA) before use. The latter procedure was applied to minimize the presence of debris and bacterial contamination in the cultures.



Figure 1. Dried chips of *Arundo donax*. Stems yielded smaller fragments than leaves.

Ellipticine (324,688, Sigma-Aldrich) was prepared following procedures previously described [15]. Dimethyl sulfoxide (DMSO) (D4540, Sigma-Aldrich) was used as carrier solution at a final concentration of 0.05% in experimental media.

2.3. Giant Reed Exposures

A range-finding test was conducted to determine the effective concentration of dried chips that inhibits *P. parvum* growth. Concentrations ranged from 0 to 16 g L⁻¹ in modified ASM. Algal cells were inoculated into 100 mL of modified ASM in 250 mL flasks at a starting density of 50,000 cells mL⁻¹, and one teabag containing the appropriate amount of chips was directly added to each flask. Ambient culture conditions were the same as described for stock cultures. Cells were counted at 24 h of incubation by hemocytometry [15]. Each exposure treatment was conducted in triplicate flasks.

Based on the estimated 24 h IC₅₀ (concentration that inhibits growth by 50%) for chips (see Results), leachate was prepared in modified ASM at concentrations of 0 and 3 g chips L⁻¹ (hereafter, g L⁻¹) in an initial experiment with *P. parvum*, and 0 and 1 g L⁻¹ in a subsequent experiment. The lower leachate concentration was used to serve as reference for experiments with *D. pulex* and *D. rerio*, where at least one of the endpoints measured was affected at 3 g L⁻¹ (see Results). *Prymnesium parvum* was inoculated in 100 mL of leachate, cultured, and counted as described above. Cell density was determined at 24 h [15].

For *C. sorokiniana*, leachate (in BG-11 media) was prepared at concentrations of 0 and 3 g L⁻¹. Cultures were inoculated in 20 mL of leachate at an initial density of 50,000 cells mL⁻¹. Ambient and culture conditions were the same as described for stock cultures. Cell density was determined at 24 h by hemocytometry, except cells were counted on a computer monitor using an Olympus DP74 camera mounted on a compound microscope. Each exposure treatment was conducted in triplicate flasks.

For *D. pulex*, leachate (in COMBO media) was prepared at concentrations of 0 and 3 g L⁻¹ in an initial experiment and 0 and 1 g L⁻¹ in a subsequent experiment. *Daphnia pulex* neonates (≤ 24 h) were placed in 24-well plates (one daphnid per well), with each well containing 2 mL of appropriate treatment medium. One full plate ($n = 24$) was assigned to each treatment concentration, and two independent experiments (trials) were conducted. Ambient culture conditions were the same as described for stock cultures. Cultures were continued until day 21 to determine exposure effects on long-term survival and on two reproductive indices: day of first brood production and cumulative brood size [23]. Survival was monitored every 24 h until the end of the culture. To minimize accumulation of waste and bacterial growth, *D. pulex* was gently transferred to new plates with fresh media and diet (2.2×10^5 *S. acutus* cells mL⁻¹) every other day with a transfer pipette.

For *D. rerio*, leachate (in egg water media) was prepared at concentrations of 0 and 3 g L⁻¹ in an initial experiment, and 0 and 1 g L⁻¹ in a subsequent experiment. Embryos were placed in 24-well plates (one embryo per well), each containing 1 mL of appropriate treatment medium. One full plate ($n = 24$) was assigned to each treatment concentration, and two independent experiments (trials) were conducted. Embryo cultures were maintained in a Fisher Scientific Isotemp Refrigerated Incubator (Fisher Scientific 3720, Hampton, NH, USA) for a total of 5 days at a temperature of 28.5 °C, photoperiod of 12:12 h light: dark, and light intensity of ~5100 lux. To minimize accumulation of waste and bacterial growth, culture media were replaced gently with a transfer pipette on days 2 and 4. Survival was checked every 24 h for 120 h under a stereomicroscope. Hatch rate was determined at 72 h, and eleutheroembryo (post-hatch) survival was recorded at 120 h. Swimming distance (startle response to light stimulation) was also determined at 120 h. For this measurement, well-plates containing the larvae were placed in a DanioVision™ (Noldus, Reston, VA, USA) and, after a 10 min acclimation period, larval behavior was videotaped for 20 min, during which larvae were stimulated with light for 10 min and kept in the dark for the next 10 min. Total swimming distance during this time period was estimated using Ethovision®XT software version 13 (Noldus, Reston, VA, USA).

2.4. Ellipticine Exposures

The 3-day IC₅₀ value for ellipticine in *P. parvum* was previously estimated at ~0.01 mg L⁻¹ [15], and in this study, ellipticine was prepared in modified ASM at concentrations of 0 and 0.01 mg L⁻¹. *Prymnesium parvum* was inoculated and cultured as

described earlier, and cell density was determined every 3 days for 9 days (exposure effects through day 21 were previously reported [15]). Each exposure treatment was conducted in triplicate flasks.

For *C. sorokiniana*, ellipticine was prepared in BG-11 media at concentrations of 0 and 0.01 mg L⁻¹. *Chlorella sorokiniana* was inoculated and cultured as described earlier, and cell density was determined every 3 days through day 21. Each exposure treatment was conducted in triplicate flasks.

For *D. pulex* and *D. rerio*, ellipticine was prepared in COMBO media and egg water media, respectively, at concentrations of 0 and 0.01 mg L⁻¹. All other experimental conditions and endpoints measured were the same as described earlier. One full plate ($n = 24$) was assigned to each treatment concentration for each species, and two independent experiments (trials) were conducted.

2.5. Data Analysis

The IC₅₀ of dried chips for *P. parvum* growth was estimated using a symmetrical–sigmoidal dose–response algorithm, as previously described [15]. In the leachate experiments with *P. parvum* and *C. sorokiniana*, differences in growth between control and respective treatment groups at 24 h were evaluated with Student's *t*-tests (two-tailed) using log-transformed values. In the ellipticine experiments, 2-way repeated measures (RM) ANOVA (treatment by culture time; RM factor, time; sphericity was not assumed, and the Geisser–Greenhouse correction was applied) was followed by Sidak's multiple comparison tests on algal counts at each sampling day using log-transformed values.

Kaplan–Meier's survival analysis was applied to *D. pulex* data, and comparisons between treatment and control groups were made using log-rank (Mantel–Cox) tests. Differences in *D. rerio* hatch rates were determined using Fisher's exact test. Post-hatch survival was 100% for *D. rerio* regardless of treatment (see Results), and larval survival analysis was unnecessary. All analyses were performed separately on each trial. Time to first brood production (time to first reproduction) and cumulative brood size in *D. pulex*, and larval behavior (swimming distance) in *D. rerio* were evaluated using nested *t*-test (trials nested into treatment). Prior to analysis, the ROUT test ($Q = 1$; ref. [24]) was used to identify and exclude outliers in behavior data, and the D'Agostino–Pearson test was used to assess normality of data distribution.

GraphPad Prism version 10.0 (GraphPad Software, Boston, MA, USA) was used for all analysis, and the α threshold for statistical significance was 0.05.

3. Results

3.1. Giant Reed Exposures

The range-finding test with direct immersion of dried chips into culture media showed that *P. parvum* growth is inhibited in a concentration-dependent manner, with an estimated 24 h IC₅₀ of 3.1 g L⁻¹ (± 0.8). Leachate at 3 g L⁻¹ showed algicidal activity (negative growth) against *P. parvum*; namely, cell density at 24 h was lower (38,333 cells mL⁻¹) than the initial density (50,000 cells mL⁻¹) and significantly lower than control values at 24 h (31 percent of control; *t*-test, $p < 0.0001$) (Figure 2). At 1 g L⁻¹, leachate showed algistatic activity (growth suppression); namely, cell density at 24 h was similar (52,500 cells mL⁻¹) to the initial density (50,000 cells mL⁻¹) but significantly lower than control values at 24 h (46 percent of control; *t*-test, $p < 0.0001$) (Figure 2). In *C. sorokiniana*, leachate at 3 g L⁻¹ stimulated growth; namely, cell density in the leachate cultures was 831% of the control value at 24 h (*t*-test, $p < 0.0001$) (Figure 2).

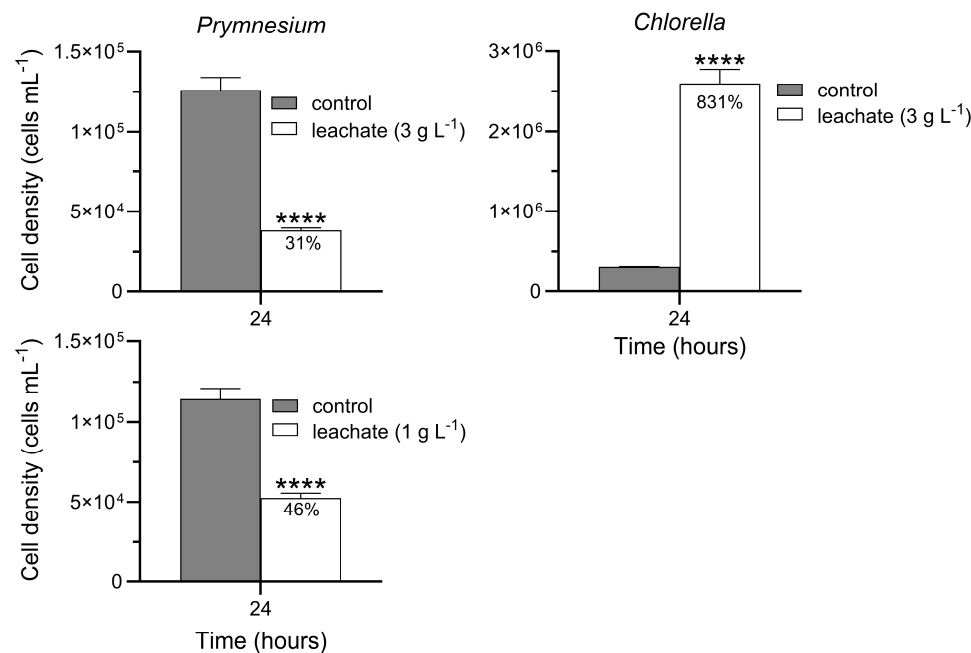


Figure 2. Effect of leachate from giant reed (*Arundo donax*) at 3 g L⁻¹ on growth of *Prymnesium parvum* and *Chlorella sorokiniana*, and at 1 g L⁻¹ on growth *P. parvum*, at 24 h. Bars represent the mean value of three replicate cultures (+SEM). Numbers within leachate bars indicate the percent value relative to the corresponding control group. ****, $p < 0.0001$ (t -test).

Leachate at 3 g L⁻¹ was lethally toxic to *D. pulex*. Survival was 95.8% in the control group and 41.7% in the leachate treatment at 24 h (chi-square, $p < 0.05$), and full mortality in the treated group was observed by 72 h in both trials. At 1 g L⁻¹, leachate had no effect on survival of *D. pulex* through day 21 (chi-square, $p > 0.05$) in both trials (average of 89.6% for control and treatment). The day of first brood production did not differ between the control and leachate groups (nested t -test, $p > 0.05$; trial did not influence results), but average brood size was reduced by 44.5% (nested t -test, $p < 0.05$; trial did not influence results) (Figure 3). Data from all treatments passed the normality test.

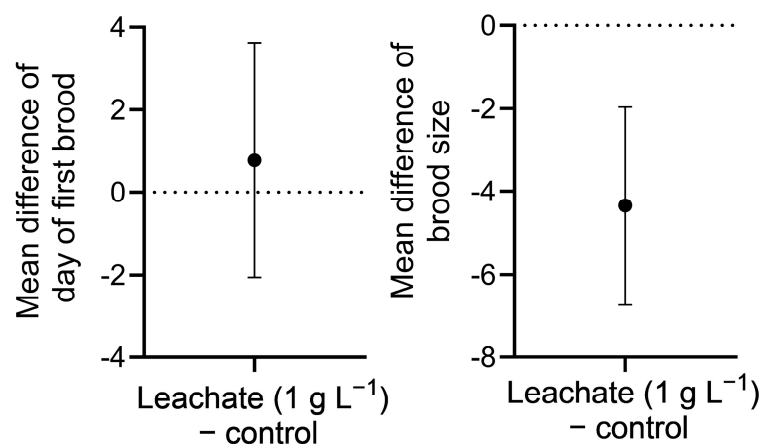


Figure 3. Effect of leachate from giant reed (*Arundo donax*) at 1 g L⁻¹ on day of first brood production and cumulative brood size at 21 days in *Daphnia pulex*. Plots show the difference between the leachate-exposed and the corresponding control group. Differences are significant when the 95% confidence interval does not cross the zero dashed line (nested t -test, $p < 0.05$). Plots represent the combined values of two trials ($n = 24$ per treatment group per trial). Control mean values for day of first brood and brood size are 14.2 days and 9.8 neonates, respectively.

Leachate at 3 g L^{-1} had no effect on *D. rerio* hatch rate (Fisher's exact test, $p > 0.05$; average of 91.7 and 85.4% for control and treatment) or post-hatch survival (100% for control and treatment) in both trials, but larval swimming distance in response to light stimulation was reduced compared to control values (nested t -test, $p < 0.05$; trial did not influence results) (Figure 4). At 1 g L^{-1} , leachate had no effect on hatch rate (Fisher's exact test, $p > 0.05$; average of 93.8% for control and treatment) or post-hatch survival (100% for control and treatment) in both trials, and swimming behavior was also not affected (nested t -test, $p > 0.05$; trial did not influence results) (Figure 4). No behavioral values were detected as outliers by the ROUT test, and data from all treatments passed the normality test.

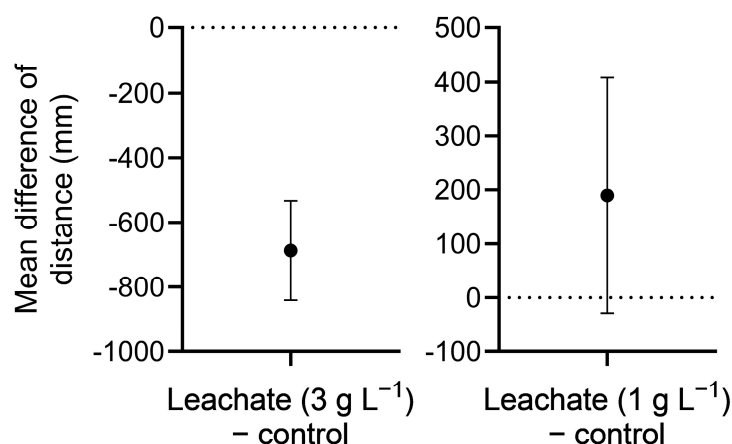


Figure 4. Effect of leachate from giant reed (*Arundo donax*) at 3 and 1 g L^{-1} on swimming distance (startle response of larvae to light stimulation) in 5-day-old *Danio rerio* larvae. Plots show the difference between the leachate-exposed and the corresponding control group. Differences are significant when the 95% confidence interval does not cross the zero dashed line (nested t -test, $p < 0.05$). Plots represent the combined values of two trials ($n = 24$ per treatment group per trial). Control mean values for swimming distance at 3 and 1 g L^{-1} are 920 and 942 mm over a 20 min period, respectively.

3.2. Ellipticine Exposures

Ellipticine exposure (two-way RM ANOVA; $F_{1,4} = 35.82$, $p = 0.0039$) and culture time ($F_{2,8} = 491.3$, $p < 0.0001$) had significant effects on growth of *P. parvum*; interaction and matching effects were not significant ($p > 0.05$). Cell density was similar to the control group on day 3 (Sidak's multiple comparison test; $p > 0.05$), but growth was suppressed by ellipticine on days 6 and 9 ($p = 0.0350$ and 0.0085 , respectively) (Figure 5).

Ellipticine exposure (2-way RM ANOVA; $F_{1,4} = 77.02$, $p < 0.0001$) and culture time through day 9 ($F_{2,8} = 2395$, $p < 0.0001$) had significant effects on growth of *C. sorokiniana*; matching effect was not significant ($p > 0.05$), but interaction was significant ($p < 0.0001$). Growth was suppressed by ellipticine on days 3 and 6 (Sidak's multiple comparison test; $p = 0.0255$ and 0.0098 , respectively), but cell density was not significantly different from the control group ($p > 0.05$) on day 9 (Figure 5). Thereafter, cell density remained similar to the control group through day 21 (Figure S1).

Ellipticine at 0.01 mg L^{-1} had no effect on *D. pulex* survival (chi-square, $p > 0.05$) in both trials (average of 89.6% for control and 87.5% for treatment). Ellipticine did not affect brood size (nested t -test, $p > 0.05$; trial did not influence results), but delayed the day of first brood production (nested t -test, $p < 0.05$; trial did not influence results) (Figure 6). Data from all treatments passed the normality test.

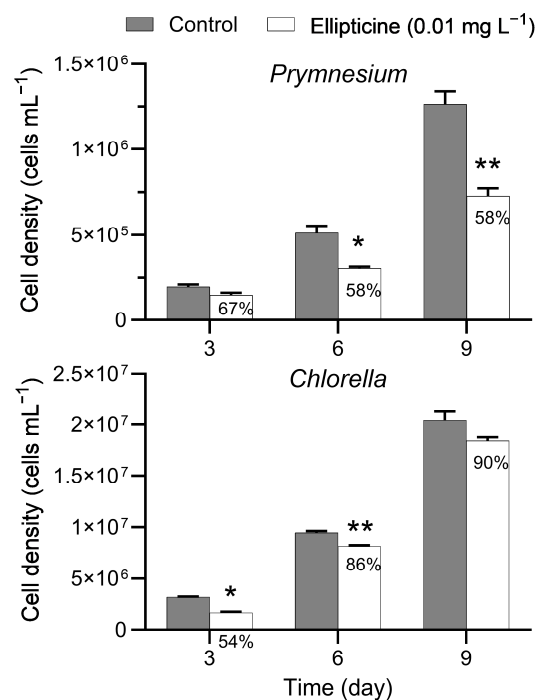


Figure 5. Effect of ellipticine at 0.01 mg L⁻¹ on growth of *Prymnesium parvum* and *Chlorella sorokiniana* on days 3, 6, and 9. Bars represent the mean value of three replicate cultures (+SEM). Numbers within the ellipticine bars indicate percent value relative to the corresponding control group. * $p < 0.05$; ** $p < 0.01$ (Sidak's multiple comparison test).

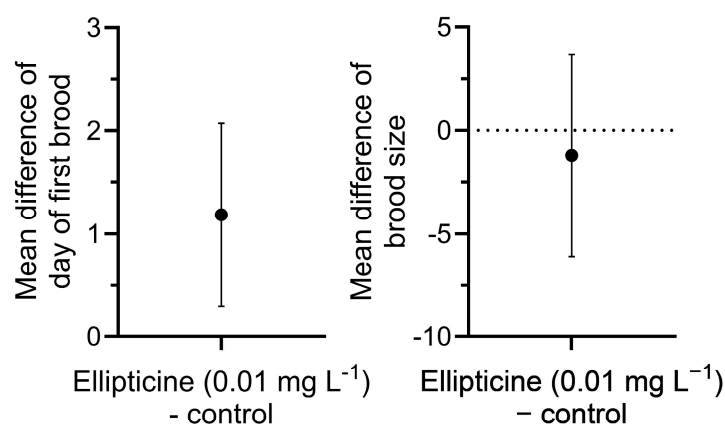


Figure 6. Effect of ellipticine at 0.01 mg L⁻¹ on day of first brood production and cumulative brood size at 21 days in *Daphnia pulex*. Plots show the difference between the ellipticine-exposed and the corresponding control group. Differences are significant when the 95% confidence interval does not cross the zero dashed line (nested t -test, $p < 0.05$). Plots represent the combined values of two trials ($n = 24$ per treatment group per trial). Control mean values for day of first brood and brood size are 13.7 days and 11.1 neonates, respectively.

Ellipticine at 0.01 mg L⁻¹ showed no effect on *D. rerio* hatch rate (Fisher exact test, $p > 0.05$; average of 93.8% for control and treatment) or post-hatch survival (100% for control and treatment) in both trials. In addition, ellipticine had no effect on swimming distance (nested t -test, $p < 0.05$; trial did not influence results) (Figure 7). One swimming distance value from one of the control trials was identified as an outlier by the ROUT test and was excluded from the dataset before analysis. Data from all treatments passed the normality test, including the one control trial after the removal of the outlier value.

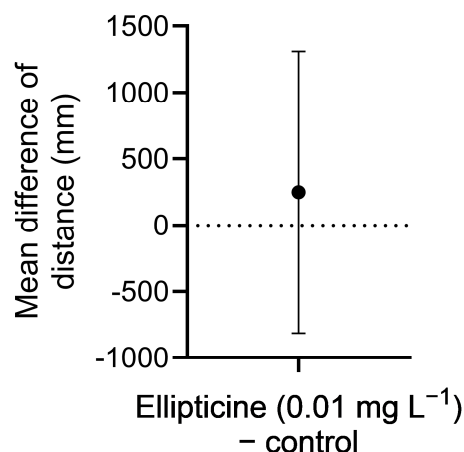


Figure 7. Effect of ellipticine at 0.01 mg L^{-1} on swimming distance (startle response of larvae to light stimulation) in 5-day-old *Danio rerio* larvae. Plots show the difference between the leachate-exposed and the control group. Differences are significant when the 95% confidence interval does not cross the zero dashed line (nested *t*-test, $p < 0.05$). Plots represent the combined values of two trials ($n = 24$ per treatment group per trial). Control mean value for swimming distance is 667 mm over a 20 min period.

It was previously shown that DMSO (carrier solution used for ellipticine) at 0.05% has no effect on *Prymnesium* [15] and *Chlorella* [25] growth. In the present study, DMSO at 0.05% did not affect any of the endpoints measured in *D. pulex* and *D. rerio* compared to medium alone (results not shown).

4. Discussion

Earlier studies reported that methanolic extracts of giant reed suppress growth of *P. parvum* [13] and *M. aeruginosa* [17], that volatile oil extracts inhibit growth of *M. aeruginosa* [18], and that aqueous extracts (leachate) have algicidal activity against *M. aeruginosa* [14,16]. This study found that giant reed leachate suppresses growth of *P. parvum* and additionally characterized the unintended effects of leachate and ellipticine—a potent anti-*P. parvum* allelochemical found in giant reed [15]—on nontarget aquatic organisms. The results obtained indicate that when applied at the appropriate concentrations, giant reed products can inhibit growth of *P. parvum* with little or no harm to nontarget species.

When directly immersed in medium at the time of inoculation, giant reed chips acutely inhibited growth of *P. parvum* with a 24 h IC_{50} of $\sim 3 \text{ g L}^{-1}$. When prepared over a 24 h period prior to inoculation, leachate seemed to have a stronger effect; namely, compared to control cultures, 3 g L^{-1} of leachate inhibited growth of *P. parvum* by $\sim 70\%$. One reason for the stronger effect of preprepared leachate may be that allelochemicals are already present in culture media—and at relatively high concentrations—from the moment the cultures begin. The potency of giant reed leachate against *P. parvum* growth seems somewhat stronger than against *M. aeruginosa*, where 5 g L^{-1} achieved $\sim 30\%$ growth inhibition at 24 h [14].

Giant reed leachate at 3 g L^{-1} was not toxic to *C. sorokiniana*. On the contrary, leachate enhanced the growth of this species by a large margin compared to control cultures. Giant reed leachate reportedly also stimulates growth of at least two other species of freshwater green algae [16]. Although it seems perplexing that giant reed leachate can suppress the growth of harmful species such as *P. parvum* (haptophyte) and *M. aeruginosa* (cyanobacteria) but enhance the growth of some green algal species, the phenomenon of species specificity is typical of allelopathy [8]. Selective inhibition of cyanobacterial growth, sometimes but not always coupled with stimulation of green algal growth, has been reported for extracts and allelochemicals derived from various aquatic macrophytes [8,26]. One advantage of giant reed leachate over other natural or synthetic products as an HAB control tool seems to be that, based on current knowledge, it selectively suppresses growth of harmful prokaryotes (*M. aeruginosa*) and harmful eukaryotes (*P. parvum*).

Leachate at 3 g L^{-1} considerably reduced survival of *D. pulex* at 24 h and was fully lethal by 72 h. Although no significant effects of leachate at 3 g L^{-1} were observed on hatching or survival of *D. rerio*, swimming behavior (distance) in 5-day-old larvae in response to light stimulation was significantly reduced. At the lower concentration of 1 g L^{-1} , leachate retained full algistatic activity against *P. parvum* at 24 h but had no effects on survival or age of first reproduction of *D. pulex*, and only moderately reduced its brood size. In addition, leachate at 1 g L^{-1} had no effects on *D. rerio* hatching, survival, or swimming behavior. It appears, therefore, that giant reed leachate at 1 g L^{-1} could be used to control *P. parvum* blooms without major impacts to the survival and various key indices of performance of nontarget species.

Ellipticine is a plant-derived indole alkaloid found in giant reed leachate [14]. An earlier study of *P. parvum* reported a 3-day IC_{50} on growth of 0.01 mg L^{-1} [15], which is among the highest known allelochemical potencies against harmful algae. In the present study, ellipticine at 0.01 mg L^{-1} suppressed growth of *P. parvum* in a time-dependent manner. Cell density was similar to the control group on day 3 but growth was significantly reduced compared to the control group from day 6 through day 9. This finding is consistent with results of the earlier study with *P. parvum* [15], which found that growth suppression by ellipticine is time-dependent and irreversible. Although ellipticine inhibited growth of *C. sorokiniana* on day 3 and day 6, the magnitude of the inhibition was lower on day 6 than on day 3 and, moreover, growth inhibition was no longer observed from day 9 through day 21. These observations indicate that although ellipticine has an acute effect on the growth of *C. sorokiniana*, the effect is short-lived. In addition, ellipticine at 0.01 mg L^{-1} had no major adverse effects on *D. pulex* and *D. rerio*—only a slight delay in the day of first reproduction of *D. pulex* was recorded (~ 1 day). Overall, this study confirmed the high potency of ellipticine against *P. parvum* growth reported earlier [15], and also established its specificity against *P. parvum* relative to various nontarget organisms.

Mechanisms of action were not examined in this study. However, ellipticine was previously shown to reduce chlorophyll content, increase cell volume, and impair swimming behavior in *P. parvum* [15]. Reduction in chlorophyll content and increased cell swelling have also been observed in *M. aeruginosa* exposed to various plant-derived allelochemicals [27–29] and bacterial toxins [30]. In *Chlamydomonas reinhardtii*, a synthetic derivative of ellipticine reduced growth, perhaps by disrupting DNA topoisomerase activities [31]. The chemical composition of leachate is likely to be complex, and so the cellular and physiological mechanisms responsible for its growth-inhibiting activity against *P. parvum* are likely to be more complex than those of ellipticine. A study of the transcriptomic response of *P. parvum* to giant reed leachate suggested that pathways and processes associated with photosynthesis and cell division are likely involved in the suppression of growth [32].

Water managers may need to weigh the benefits of an HAB control treatment against its unintended ecological impacts. Treatment cost and application feasibility are also important considerations. The present study found that giant reed leachate at a concentration of 1 g chips L^{-1} and ellipticine at a concentration of 0.01 mg L^{-1} blocked or reduced growth of *P. parvum* with minimal effects on nontarget aquatic flora and fauna. Ellipticine seemed to have the fewest side effects but, at the present time, its availability and cost may be discouraging for field application purposes. Leachate, on the other hand, is easier and less costly to prepare. It should be mentioned that giant reed leachate impairs growth of not only *P. parvum* (present study) but also the cyanobacterium *M. aeruginosa* [16], two of the most important HAB species found in inland waters [6]. Growth of the harmful marine dinoflagellate *K. brevis* can be suppressed with a pyrolytic extract of giant reed [19], but the effects of leachate on this species are unknown.

Although the present laboratory results indicate that giant reed products have promise as tools for HAB control in standing inland waters, mesocosm and field trials would be useful to better understand the products' limitations and adaptively adjust treatments. Future research on ellipticine could explore its potential use in combination with allelochemical sustained-release technologies to enhance its HAB control efficiency at low additional

cost [10]. Research to determine the risk of giant reed establishment in non-native habitats may be necessary before directly using dried chips in the field. The presence of an intact stem node seems necessary for the vegetative propagation of this highly invasive plant [33] but, to our knowledge, the ability of dried node fragments (part of the material used in the present study; Figure 1) to sprout has not been examined. The use of leachate or leachate derivatives may be preferable. For large water bodies where treatment application over their entire surface is not feasible or practical, knowledge of the spatial sources and seasonal timing of propagule production would facilitate spot treatment strategies to stop blooms from forming and spreading. Spot treatments could also be used to create refugia for fishes and other aquatic organisms during active toxic blooms. The potential of giant reed products to control the growth of multiple HAB species may serve as incentive for further exploration.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w16070930/s1>, Figure S1: Effect of ellipticine at 0.01 mg L⁻¹ on growth of *Chlorella sorokiniana* from days 3 to 21.

Author Contributions: M.A.M.: data curation, formal analysis, investigation, methodology, visualization, and roles/writing—original draft; S.T.-S.: methodology, resources, and writing—review and editing; S.N.: methodology, formal analysis, resources, writing—review and editing; G.D.M.: resources and supervision; J.C.: formal analysis, resources, and supervision; R.P.: conceptualization, formal analysis, project administration, resources, supervision, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was not supported by grant funding.

Data Availability Statement: Research data are available upon request to the corresponding author.

Acknowledgments: The Texas Cooperative Fish and Wildlife Research Unit is jointly supported by the U.S. Geological Survey, Texas Tech University, Texas Parks and Wildlife Department, The Wildlife Management Institute, and U.S. Fish and Wildlife Service. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Conflicts of Interest: The authors declare no conflicts of interest.

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