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Ultrasound assisted cyanotoxin extraction for nematode inhibition in soil

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ABSTRACT

Root-knot nematodes are one of the plant damaging nematodes in agriculture causing a projected annual yield loss of ~12 % (~$160 billion) worldwide. Conventional solutions to control these plant-parasitic nematodes involve chemical nematicides. To reduce the use of harmful chemicals, microalgal extracts can be used as greener alternatives for nematode management. Microalgae produce valuable metabolites, including cyanotoxins which can aid in nematode suppression.

In this study, two microalgae species, *Trichormus variabilis* and *Nostoc punctiforme*, were treated with ultrasound for intensified recovery of secondary metabolites. Ultrasound results in cell wall disruption of the microalgal species, thus resulting in enhanced release of secondary metabolites. Microalgal biomass was treated with an ultrasound probe at 50 % amplitude, 20 kHz frequency, using water as the extraction medium, for 5–30 min. The extraction efficiency was determined in terms of the total chlorophyll (Chl) content of the extract. Microscopic images of the treated cells were also investigated to gain insight into the effect of the ultrasonication time on the cell morphology. Our results suggest that ultrasonication resulted in the intensified release of secondary metabolites, as established through the total chlorophyll content of the ultrasonicated microalgal samples as well as the microscopic images of the ruptured cells. The best extraction for *Trichormus variabilis* was achieved with 15 min extraction time where the Total Chl content increased by 29 times (compared to the non-ultrasonicated sample), and for the *Nostoc punctiforme*, 30 min extraction time gave the highest metabolite recovery of 6.4 times higher than the non-ultrasonicated sample.

Ultrasonicated algal extracts were then tested for their nematicidal potential against root-knot nematode, *Meloidogyne hapla,* in infested field soil samples. Experimental study was conducted using different concentrations of each microalga, *Trichormus sp*. and *Nostoc sp*., individually, as well as in combination. The nematode count for the treated soil was compared with that of the control (untreated soil). Ultrasonicated microalgal extracts showed 66% to 100% inhibition on root-knot nematodes in the soil samples tested.

1. Introduction

A vast variety of nematodes cause quantitative and qualitative losses of agricultural productivity with an estimated annual yield loss of ~12 % (~$160 billion) worldwide [53]. Root-knot nematodes are one of the plant-damaging plant-parasitic nematodes in agriculture reducing crop yield food quality. Root-knot nematode species can cause damage in a wide host range, including annual as well as perennial crops. Its infection causes the abnormal swelling of or on the root, known as root galls, in many crops and impedes required uptake of water and nutrients [46,19,27]. The conventional solutions to control these plant-parasitic nematodes involve chemicals, biological agents, as well as non-chemical alternatives [52,51]. For instance, for high-value crops including ornamental plants, chemical nematicides are often used [30], however, excessive use of these synthetic nematicides can lead to soil and water contamination. The inherent negative impact on the environment as well as inefficacy after year after year of continued use have led to a complete ban or constricted use of most nematicides, and leads to the pressing need for safe and potent alternatives [65,12]. Of the many possibilities being researched to screen and develop eco-friendly, chemical-free, greener, and sustainable alternatives for controlling nematode damage to plants, for example, soil management, soil solarization, using organic additives, and biological control [54,6]. Other scenarios for plant-parasitic nematode control include trap cropping, biofumigation, development of nematode-resistant crop varieties, and development of new soil amendments and natural products [60].

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Several new nematicides have been developed that have less impact on the non-target nematodes or the environment (as a result of low dosage application and readily degradable materials), such as fluazaindolizine, fluensulfone, and cyclobutrifluram [58,31]. Biological control is among the most promising sustainable alternatives, for example, *Bacillus firmus* [18]. The study of cyanobacteria that can suppress the plant-parasitic nematode is of great interest recently [27].

Cyanobacteria are microorganisms which produce a variety of bioactive compounds like pigments including carotenoids, lipids, proteins, polysaccharides, vitamins and hydrocarbons (phenolics) compounds which exhibit antioxidant, antimicrobial, anti-inflammatory, immunomodulation and anti-cancer activity [15,21,57]. Cyanobacteria evolved to generate secondary metabolites in response to environmental stress conditions including high temperature, high pH, and high nutrient loadings etc. [9,10,15,24,62]. These compounds can be categorized into non-toxic and toxic: phytohormones, siderophores and various UV-protective compounds such as mycosporine-like amino acids (MAAs) and scytonemin comprise the non-toxic secondary metabolites [21]; hepatotoxins (for example, microcystins) and neurotoxins (for example, anatoxin-a and saxitoxin) comprise the toxic secondary metabolites, named after the organs they target, liver and neuromuscular transmissions, respectively [3,32,36,37,61,35].

Owing to the ability to produce various toxic secondary metabolites, cyanobacteria has been studied to suppress nematodes in soil. Three species of cyanobacteria including *Anabena oryzae*, *Nostoc calcicola*, and *Spirulina sp.*, when used in a combination were able to mitigate root-knot nematode's (*Meloidogyne incognita*) gall numbers and egg masses that was infecting cowpea. Radwan et al. [42] examined the nematicidal potential of *Bacillus megaterium, Trichoderma album, Trichoderma harzianum* and *Ascophyllum nodosum* for controlling *M. incognita* in infected tomato plants, and found that the bioproducts made a significant reduction in the root galls and the numbers of nematode juveniles in the soil. Similarly, application of *Nodularia harveyana* on tomato plants also resulted in the reduction of root-knot nematode *M. incognita* [41]. Thus, it is evident that cyanobacterial secondary metabolites have the potential to suppress the nematode via causing disrupting hatching processes [16,26,49] or may serve as neurotoxins [4]. Even though they can suppress the plant damaging soil nematodes, cyanobacteria are known to be less toxic to plant beneficial nematodes [11]. Secondly, the phytohormones produced by cyanobacteria (for example cytokinins, auxins, indole acetic acid) play a vital role in supporting plant growth, cell division and nutrient release [48]. Application of microalgae to the soil has shown to improve plant growth in terms of fresh and dry weight of shoots, roots, length of shoots, and increase the number of root nodules thus improving the nitrogen fixing ability of the plant (cowpea) [64]. Hamouda and El-Ansary [19] investigated the nematicidal potential cyanobacteria species including *Aphanocapsa albida*,*Anabaena oryzae*, *Nostoc muscorum* and *Calothrix marchica* against *Meloidogyne incognita* in infected banana plants. All tested materials significantly reduced root-knot nematode numbers and remarkable increase in the plant growth parameters. Therefore, the secondary metabolites produced by the cyanobacteria should not be a concern for the plant or the beneficial nematodes.

The cell envelope of cyanobacteria consists of four distinct layers, the plasma membrane, the peptidoglycan layer, the outer membrane and in some cases the surface or S-layer, also known as the cell wall [13]. The secondary metabolites are enclosed within these layers, and therefore to enhance the nematicidal suppressing potential of the microalgae, it is necessary to break the layers and extract the secondary metabolites prior to application to the nematode infested soil. To aid the extraction, some researchers have used solvents such as methanol and hydrochloric acid for optimum extraction of the secondary metabolites [38,25], and/or ultrasonication bath. The main mechanism of ultrasonication includes the formation of ultrasound waves that pass through the liquid media (solvent), and in the process form expansion and compression cycles. These repeating cycles of expansion and compression created cavities that eventually grow in size and collapse violently upon reaching a maximum point. The localized temperature and pressure near the collapsed cavities increase and this phenomenon enhances mass transfer between the extractant media and the microalgae's inner content and consequent disruption of cell walls [43]. There are two major types of ultrasound equipment: a bath sonicator that provides indirect sonication, where the formation and distribution of the cavities are non-uniform and weak, hence resulting in a longer extraction time of the bio metabolites [45]. The second one is a probe/horn sonicator that provides direct sonication, where the formed cavities on collapsing result in intensified vibration as well as uniform homogenous matrix, hence reducing the extraction time and improving the overall efficiency of the process (as illustrated in Fig. 1). In a study conducted by Šic Žlabur et al. [50], they showed that an ultrasound probe resulted in efficient extraction and higher yields of metabolites in a shorter time as compared to an ultrasound bath.

The present study examines the nematicidal potential of two microalgal species: *Trichormus variabilis* and *Nostoc punctiforme* against a root knot nematode *Meloidogyne hapla*. These two species were selected based on screening cyanotoxins producedHaque et al. [21], *T. variabilis* is known to produce a neurotoxin, anatoxin-a, and *N. punctiforme* is known to produce a hepatotoxin, microcystin [38,55]. The main objective of this study is to investigate the effect of ultrasonication on the extraction of secondary metabolites from these two microalgae. Based on the literature search, as discussed in the previous paragraph, direct ultrasonication (using probe ultrasound) is better than indirect ultrasonication (using bath sonicator), therefore, direct sonication was chosen for this study. The purpose to extract these secondary metabolites is to check for their soil nematode suppressing potential, which is a secondary objective of this study. Therefore, after extraction, these metabolites were added to nematode-infested soil and the soil nematodes were counted before and after the experiment to determine the nematode inhibition by these extracts. The primary novelty of this work is the use of direct sonication employing an ultrasound probe to intensify the extraction of secondary metabolites from the microalgal cells and using water as the extractant, thus eliminating the use of chemicals such as methanol and hydrochloric acid. The process is intensified because chemical-free extractant, water, will be used as an alternative. As a result, the extract produced could be directly added to the soil because there will be no concern regarding adding harsh chemicals (methanol or hydrochloric acid) to the soil. To our knowledge, this study is the first of its kind use direct sonication to extract secondary metabolites from *Trichormus variabilis* and *Nostoc punctiforme,* especially providing a detailed information on the morphological changes to the cells as a result of cell damage due to sonication.

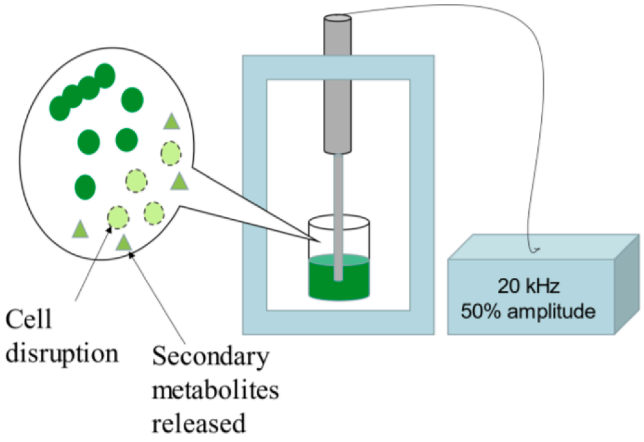

Fig. 1. Experimental setup for the disruption of the microalgal cells.

2. Materials and method

2.1. Microalgal stock and cultivation

Strains of *Trichormus variabilis* (aka *Anabaena variabilis*; formerly *Anabaena flos-aquae*) CPCC 67 and *Nostoc punctiforme* CPCC 41 were procured from the Canadian Phycological Culture Centre (CPCC). 10 ml stock of each cyanobacteria species was subcultured every-three weeks by transferring to 50 ml of fresh BG11 liquid media. The growth conditions were maintained at pH 7.5, 22 ± 2 °C incubation growth temperature controlled by using 600 W MH light (approximately 1000 lx light intensity) for a photoperiod of 12 h each light and dark cycle. The cultures: media ratio was maintained at 5:25 (v/v) in Erlenmeyer cotton plugged flasks and incubated in a nine square feet Expert Growth Tent. These growth conditions were followed based on the instructions provided by the CPCC. For high-density cultivation of both cultures, 2.2 L photobioreactor (PBR) was used, maintained under the growth conditions as mentioned above. The growth of microalgae cells was determined by a UV spectrophotometer (Thermo Scientific Genesys 20) at an optical density of 730 nm for 750 nm for *Trichormus variabilis* and *Nostoc punctiforme*, respectively [1,44].

2.2. Cyanotoxin extraction

The microalgal culture (50 ml) were sampled from the liquid broth, and centrifuged (Thermo Scientific Sorvall ST 24) at 13000 × g for 20 min [22]. The residual cells were suspended in 200 ml of deionized water in a 500 ml beaker prior to ultrasonicating the liquid samples using a probe sonicator (1.3 cm diameter, VC 750 ultrasonic processor, Sonics & Materials Inc., 750 W, 20 kHz) at 50 % sonication amplitude (Fig. 1). At 5–30 min ultrasonication time intervals, 2 ml volume of samples were collected for further analysis [22].

2.3. Analysis

2.3.1. Total chlorophyll determination

The ultrasonicated samples were centrifuged at 13000×g for 20 min, and the absorbance of the supernatant were measured using a Thermo Scientific Genesys 20 spectrophotometer to determine the total chlorophyll (Chl) content using Arnon's formula (total chlorophyll $_{(a+b)}$ = 20.21 A_{645} + 8.02 A_{663})[40,66]. The blank used is the deionized water, since it was the material used as the extractant. The resulting Total Chl data at different times (5–30 min) were then compared to the one at T0 (before US treatment).

2.3.2. Microscopic observation

The changes in the microalgal cells, before and after the ultrasonication treatment, were observed under a compound microscope (Fisher) at 40 × and 100 × magnification [22], and the images were analyzed using the software Motic Images Plus 3.0.

2.4. Soil nematode suppressing test

The ultrasonicated microalgal extracts were tested for its nematicidal potential against root-knot nematode, *Meloidogyne hapla*. The soil was procured from a Research station of the University of Guelph, located at Holland Marsh, Ontario. The soil was rich in organic matter (45 %), and belonged to muck-type soil (a sapric soil that is waterlogged naturally [2]. The soil was collected in early May. In the previous growing season, the soil was used to grow tomatoes, and during the winter season, clover cover crops were planted. At this research station, the soil was inoculated with only *M. hapla* for their study, thus no other plant parasites were present, which was also confirmed by the Agriculture and Food Laboratory Services (Guelph, Canada) where the samples were sent for testing. To 1 kg of the soil, 200 ml of the extract was added, and the experimental setup was kept inside the growth tent for seven days. 200 ml of extract was used to maintain the soil moisture at 30 %, imitating the moisture content maintained at the Research station for growing their plants. The soil tests were carried out in beakers, thus ensuring no loss of water leachate of nematodes during the experiment. At the field scale, 200 ml of extract might seem unrealistic, however, before optimizing the appropriate application rate for agricultural field applications, we first wanted to verify if the extracts have the potential to suppress the soil nematodes. Therefore, based on the hatching test proposed by Ghareeb et al. [17], a modified soil nematode suppressing test was designed for seven days to investigate the impact of the extracts on the soil nematodes. Experimental set up included the following treatments: 200 ml of *Trichormus sp*. extract, 200 ml of *Nostoc sp*. extract, and a combination of 100 ml of *Trichormus sp*. extract and 100 ml of *Nostoc sp*. extract. A total of 4 replicates of each experimental set were maintained. A control setup was maintained where 200 ml of deionized water was added to the soil. The total soil nematode count was determined using the Baermann pan technique by sending the samples to Agriculture and Food Laboratory Services (Guelph, Canada).

2.5. Data analysis

All readings were taken in triplicates and results were represented along with standard deviation. The total chlorophyll content of the ultrasonicated microalgae were statistically analyzed using one-way analysis of variance (ANOVA) along with Tukey test. For soil nematode count, paired *t*-test comparisons were made between the treated soil and the non-nematized soil. Data analysis was done using IBM SPSS Statistics 26 software and $P < 0.05$ was used as the limit for statistical significance.

3. Results and discussion

3.1. T. variabilis and N. punctiforme growth and biomass yields in the PBR

High density cultivation of *T. variabilis* and *N. punctiforme* were obtained using a photobioreactor (Fig. 2). The 60-day growth period of cyanobacterial cultures showed typical lag, exponential, and stationary phases. The exponential growth of *T. variabilis'* peaked after 23 days (Fig. 2a), and the maximum production of 0.82 ± 0.05 gDW/L was also measured, comparable to the biomass density of 0.79 ± 0.01 gDW/L when *T. variabilis* was cultured in a 10*L* vertical polyethylene bag [1]. *N. punctiforme* reached the maximum growth in 26 days, comparable to the one reported by Lindo and Griffith [34] (Fig. 2f), and a biomass density of 0.44 ± 0.02 gDW/L. Microscopic images showed the long filamentous chains of both the cyanobacterial cultures (Fig. 2d, 2e, 2i, 2j). The secondary metabolites are enclosed within the cells and to release the active compounds, the structure needs to be broken down to allow the metabolites to escape.

3.2. Influence of ultrasonication on microalga extraction

3.2.1. Effect of ultrasonication on T. variabilis

The propagation of ultrasonic wave and cavitation is the key factor leading to the intensification of the extraction efficiency [59]. Therefore, the effect of extraction time on the yield of secondary metabolites, measured in terms of total chlorophyll (Total Chl), were investigated. Since, both the microalgae are green pigment, chlorophyll, producing species, and upon extraction, the extract produced is predominantly pigmented, therefore Total Chl is a good indicator for the cyanotoxin extraction, since Total Chl can be determined spectrophotometrically with ease. Fig. 3a shows the amount of Total Chl extracted from *T. variabilis* at different ultrasonication extraction time (0–30 min). As the extraction time increased, the Total Chl of the extracted sample also increased from 20 times to 30 times from 5 to 30 min, reaching a maximum at 15 min (T15). After 15 min, there was an overall increase

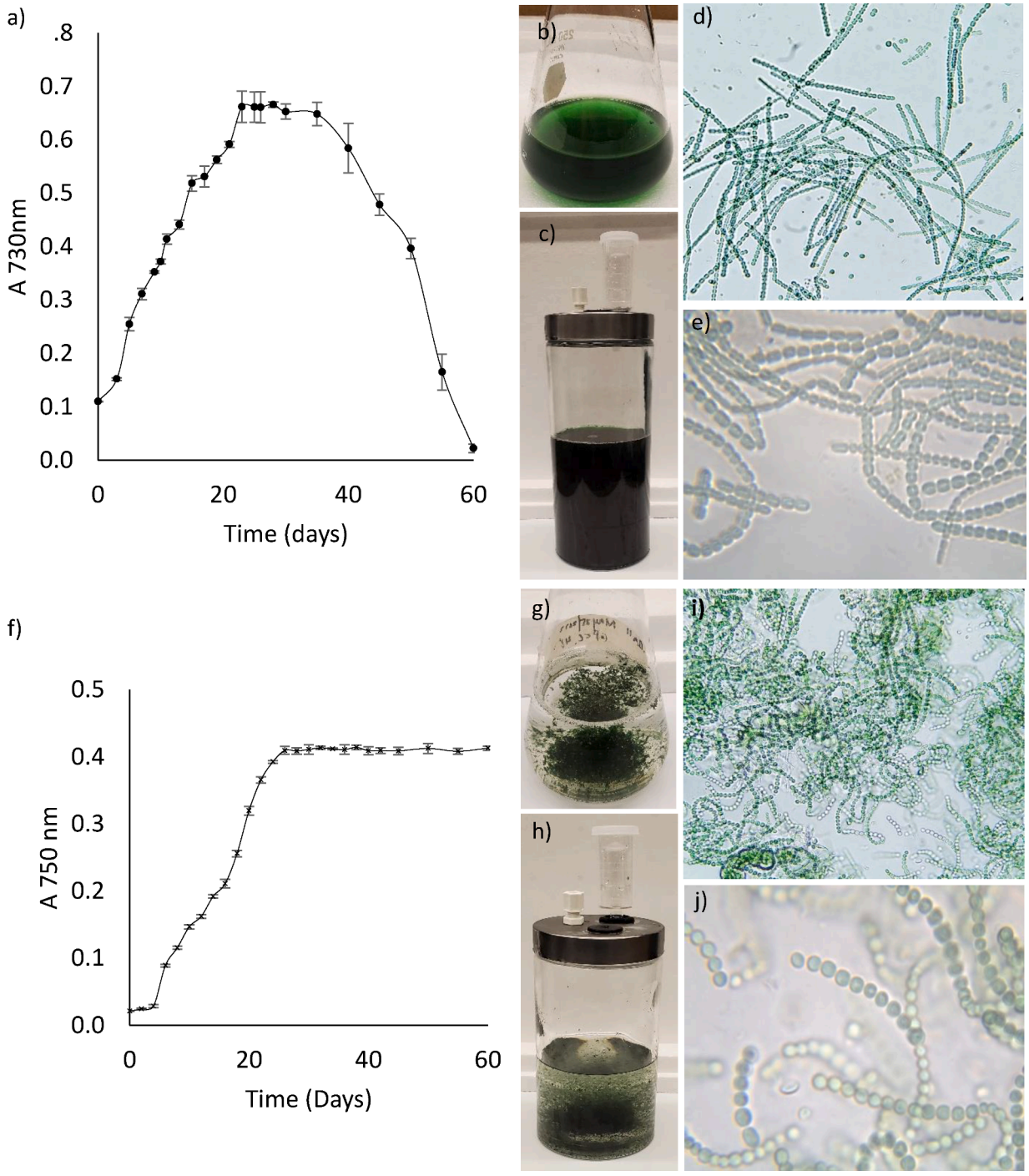

Fig. 2. Data for *Trichormus variabilis*: a) growth curve, b) bench scale growth system, c) high density cultivation in photobioreactor, c) microscopic image at 40×, d) microscopic image at 100 ×. Data for *Nostoc punctiforme*: a) growth curve, b) bench scale growth system, c) high density cultivation in photobioreactor, c) microscopic image at 40×, d) microscopic image at 100 ×.

by 1 % at 30 min, the increase was not significantly different from the one obtained at T15 ($p < 0.05$). Thus, 15 min extraction time gives the best metabolites recovery, and the Total Chl content increased by 29 times compared to the non-ultrasonicated sample (T0). Fig. 3b and 3c shows the microalgal sample prior to ultrasonication and at the end of 30 min, and clear visual difference is observed.

Sub-samples centrifuged prior to ultrasonication are shown in Fig. 3d and the residual microalgae were observed under the microscope (Fig. 3e) to gain insight on the influence of ultrasonication treatment on the surface morphology of the *T. variabilis* cells. *T. variabilis* is filamentous in nature as seen in Fig. 2d and 2e before sonication and the metabolites are trapped within the enclosed cell walls. At T5, i.e. after 5 min of sonication, de-clumping of the cells occurs, and the filaments are broken into shorter segments or dispersed into individual cells (Fig. 3e). At T15, this de-clumping becomes more prominent. Breaking of cell walls, results in cell damage thus resulting in the release of metabolites. This correlates with the Total Chl content of the supernatant obtained after centrifuging the ultrasonicated sub-samples (Fig. 3a).

The change in the cell diameter was analyzed semi-quantitatively, and the cell diameter/ size of the cells decreased as the extraction time increased. It is also observed that the cell number decreased from T5 to T30, thus there might be a possibility that the cells reduced in size

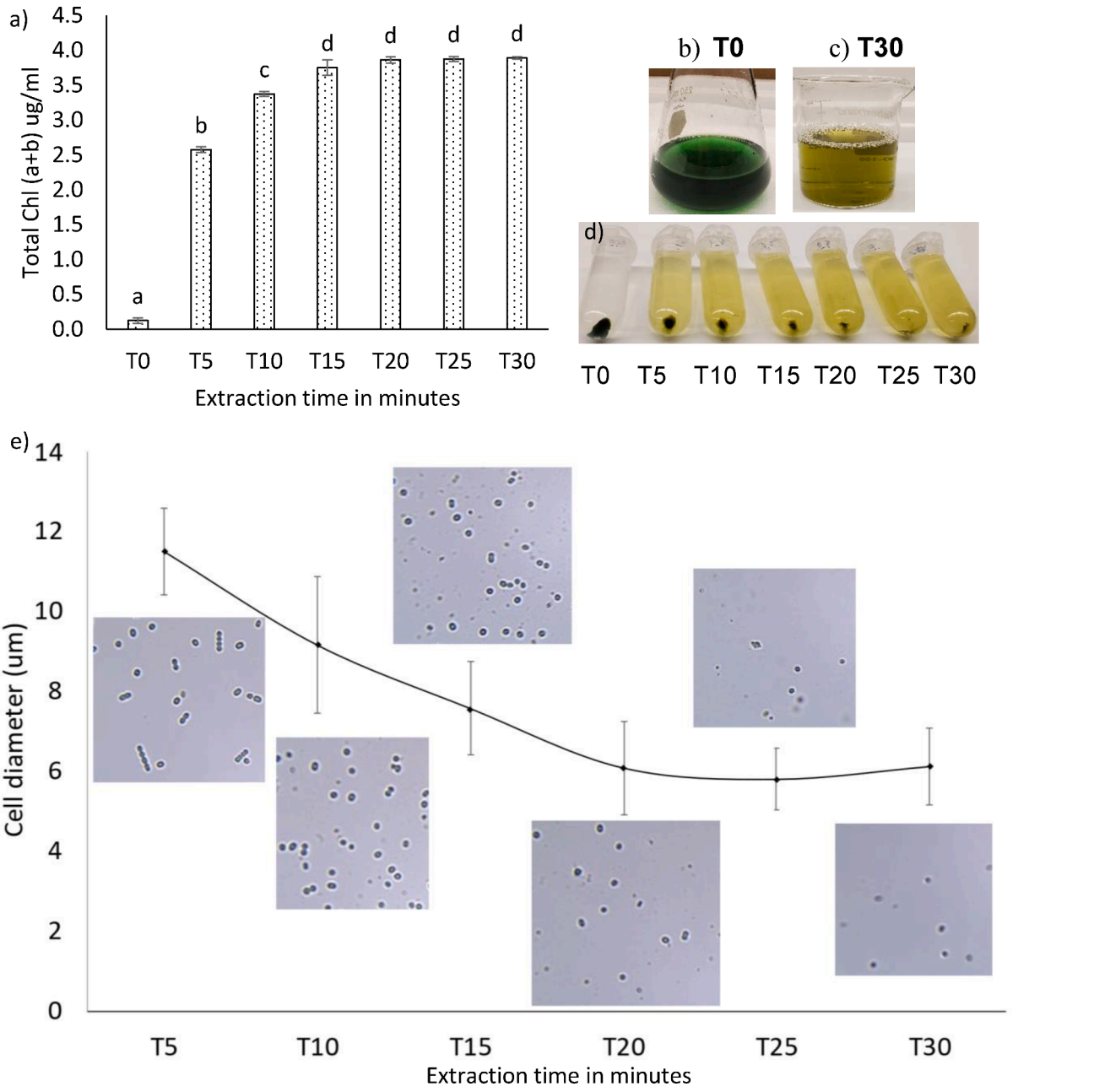

Fig. 3. a) Total Chl content of the ultrasonicated *T. variabilis* samples; *T. variabilis* sample b) before ultrasonication (denoted as T0), c) after 30 min ultrasonication (denoted as T30); d) centrifuges sub-samples of *T. variabilis* after extraction time 0–30 min (denoted as T0-T30); e) semi-quantitative analysis of the *T. variabilis* cells as a result of ultrasonication, along with the microscopic images at 40 ×.

and eventually degraded in the solution, as evident from the microscopic images of the residual microalgae (i.e., diminished cell size and cell number) and the increased Total Chl data (implying the release of metabolites). As the cavitation bubble created as a result of ultrasonication grows in size, and when it is closer to the microalgal cells, upon bursting it can rupture the cell wall, thus intensifying the release of trapped metabolites from inside the cell wall into the extractant [47]. A decrease in cell number of *T. variabilis* after ultrasound treatment was reported by Peng et al. [39], who also provided a SEM image of the ultrasonicated cells treated at 740 kHz for 5 min using an ultrasound bath. Before ultrasonic treatment, the *T. variabilis* cells demonstrated regular filamentous chain structures, with smooth cell wall surface, and after the ultrasonic treatment, wrinkles were observed on the surface of the cells along with a small number of broken fragments. However, in their study, Peng et al. [39] report that the microalgal cells remain intact as filamentous chains, whereas in this study the filaments are seen dispersed into individual cells. This is primarily due to the ultrasound probe horn used in this study, in contrast to the ultrasound bath used by Peng et al. [39]. A bath sonicator provides indirect sonication, therefore the formation and distribution of the cavities are non-uniform and weak, that explains why the filamentous chains remained intact in the study reported by Peng et al. [39]. On the other hand, a probe/horn sonicator, used herein, provides direct sonication, where the formed cavities on collapsing result in intensified vibration as well as a uniform homogenous matrix [22], that could have resulted in the defragmentation of the long chains into smaller filamentous chains. Additionally, the mild operating conditions of ultrasonication treatment, and faster extraction time, do not modify the metabolites, rather one of the main advantages of using ultrasonication is that it prevents the deterioration of the metabolites [56].

3.2.2. Effect of ultrasonication on N. Punctiforme

Before the ultrasonic treatment, at T0 as shown in Fig. 4b, the microalgal cells are suspended in the water (extractant), and immediately after sonication, at 5 min (T5), the cell suspensions appears more uniform and dispersed than the initial sample for *N.punctiforme*. The extraction time displayed a positive linear effect on the total Chl content, increasing from 1.6 to 6.4 times from 5 to 30 min (Fig. 4a) Extraction time beyond 30 min was not investigated since a longer processing time induces localized hot spots due to ultrasonic cavitation and may lead to degradation of the metabolites extracted. Secondly, longer extraction time is less energy efficient for scale up studies.

Similar to *T. variabilis*, *N. punctiforme* is also filamentous in nature (Fig. 2i and 2j), and upon sonication, the cells separate, and disrupt (Fig. 5b-d, T5-T15). At T10, some white empty cells are observed (Fig. 5c), indicating cell wall disruption. Cell damage might be due to the oxidation of lipids present on the cell surface by the hydroxyl radicals produced by high frequency ultrasound [39]. In addition, these cells can also be destroyed when their resonance radius and bubbles radius are the same [39,33,20]. In our previous studies, we used ultrasound to extract astaxanthin from encysted *Haematococcus pluvialis* microalgae, and we observed the breaking of double cell walls of *H. pluvialis*, and white damaged cells similar to the one found in this study [22,23].

In summary, extraction of secondary metabolites is enhanced due to ultrasound treatment. high localized energy leads to localized heating and free radicals (H· and OH·) formation which are considered to be the primary causes of ultrasound interaction with microbial cells [28]. The imploded bubble produces high shear forces in the solvent to physically damage the cell wall/membrane. The free radical can attack the chemical structure of the microalgal cell wall and weaken it to the point of disintegration [63], as evident from the microscopic images reported in this study. Another point that the authors would like to emphasize is that in other studies, researchers have made use of different solvents such as methanol and hydrochloric acid for optimum extraction of the secondary metabolites [38,25]. Given the aim of this work is to apply microalgal extracts to soil for suppressing the nematodes to support healthier plant growth, only water is used as the extractant instead of potentially harmful chemicals.

3.3. Nematode suppressing property

Effect of microalgal extracts on soil nematodes, *M. hapla* population is summarized in Table 1. The efficiency of the treatments was demonstrated by the final nematode number in soil. The nematized control soil contained 80 nematodes per kg soil, and upon addition of *T. variabilis* extracts (TV), 100 % nematodes are reduced. With *N. punctiforme* extract (NP) addition, 66 % nematode inhibition is observed. For a combination of the two microalgal extracts (TV + NP), 91 % nematode inhibition is observed. It is important to note that the paired *t*-test indicate the number of nematodes in microalgal extract treated soil are significantly different ($p < 0.05$). Thus implying that the microalgal treatment could result in nematode suppression. Nematode suppression may be attributed to the secondary metabolites released by these two microalgae. The two main cyanotoxins produced by these species are anatoxin-a, which is a neurotoxin; and microcystins, which is a hepatotoxin [14,38,55]. Nematized soil contained 80 nematodes per kg of soil, which might seem low if agricultural soil is considered, however for high-value crops such as ornamental plants, even a single nematode can be detrimental to its market value [7]. In this context, the chemical-free- intensified solution of using microalgae extracts in water can be used as a soil amendment to suppress nematodes.

The higher inhibition for *T. variabilis* treatment can be attributed to the concentration of the cyanotoxin present in the extract, the Total Chl content of *T. variabilis* is four times higher than that of N. *punctiforme* (Fig. 3a and 4a). Secondly, the biomass density of *T. variabilis* produced in this study is twice that of *N. punctiforme*, this would result in higher cyanotoxin present in the extract of T. variabilis in comparison to *N. punctiforme*. Another reason is the strength of the cyanotoxins produced by the two species, as well as other bioactive secondary metabolites produced. Both *T. variabilis* and *N. punctiforme* are known to produce anatoxin-a and microcystin, however the most predominant toxin produced by *T. variabilis* is anatoxin-a and by N. *punctiforme* is microcystin, hence the ratio of these metabolites in the extract dominates the nematode suppressing strength. In this study, *T. variabilis* extract results in higher nematode inhibition, thus implying that anatoxin-a might be more effective in suppressing *M. hapla* than compared to microcystins produced by *N. punctiforme*. The anatoxin-a analysis of the *Trichormus* spp.'s extract using liquid chromatography-tandem mass spectrometry (LC-MS/MS) shows the presence of anatoxin-a in the extract (~0.11 ± 0.1 pg/cell) [5]. For *Nostoc punctiforme*, an enzyme-linked immunosorbent assay (ELISA) test was conducted using Ridascreen™ saxitoxin ELISA kit (R-Biopharm, Darmstadt, Germany) for saxitoxin and Microcystin Plate kit (EnviroLogix Inc., Portland, USA.) for microcystin. These tests confirmed the presence of saxitoxin (up to a detection limit of 0.01 ppb of the ELISA kit) and microcystin (LR, LA, RR, YR), and nodularin (combined concentration of 0.056 ppb) [55]. There is a possibility that other additional phytohormones are being produced by these species that are supplementing the produced cyanotoxin to inhibit the root-knot nematode, for example, auxins, IAA, cytokinins, asetamide, hexamethyl, methoxyphenyl, phenol, and others, are reported to be produced by these microalgal

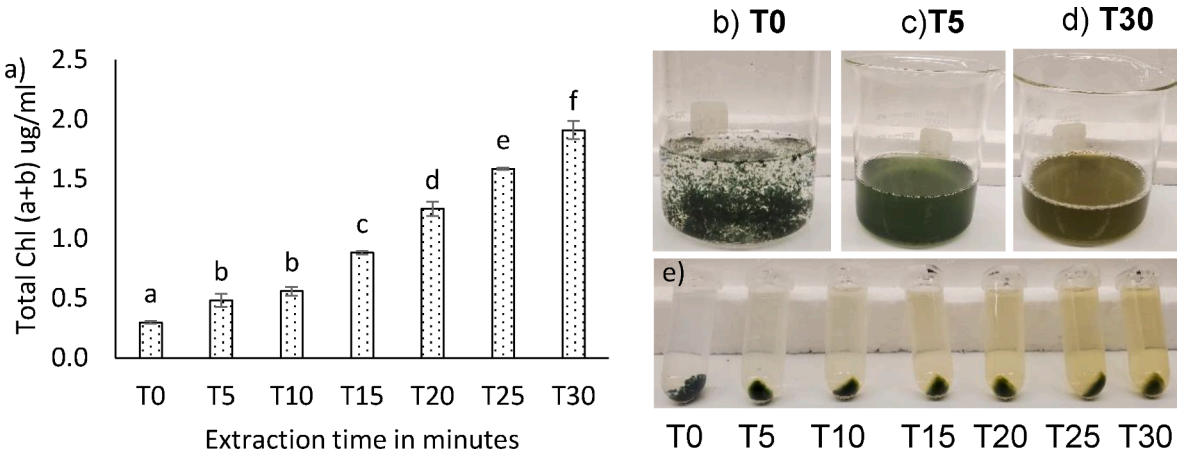

Fig. 4. a) Total Chl content of the ultrasonicated *N. punctiforme* samples; *N. punctiforme* sample b) before ultrasonication (denoted as T0), c) after 5 min ultrasonication (denoted as T5), d) after 30 min ultrasonication (denoted ad T30); e) centrifuges sub-samples of *N. punctiforme* after extraction time 0–30 min (denoted as T0-T30).

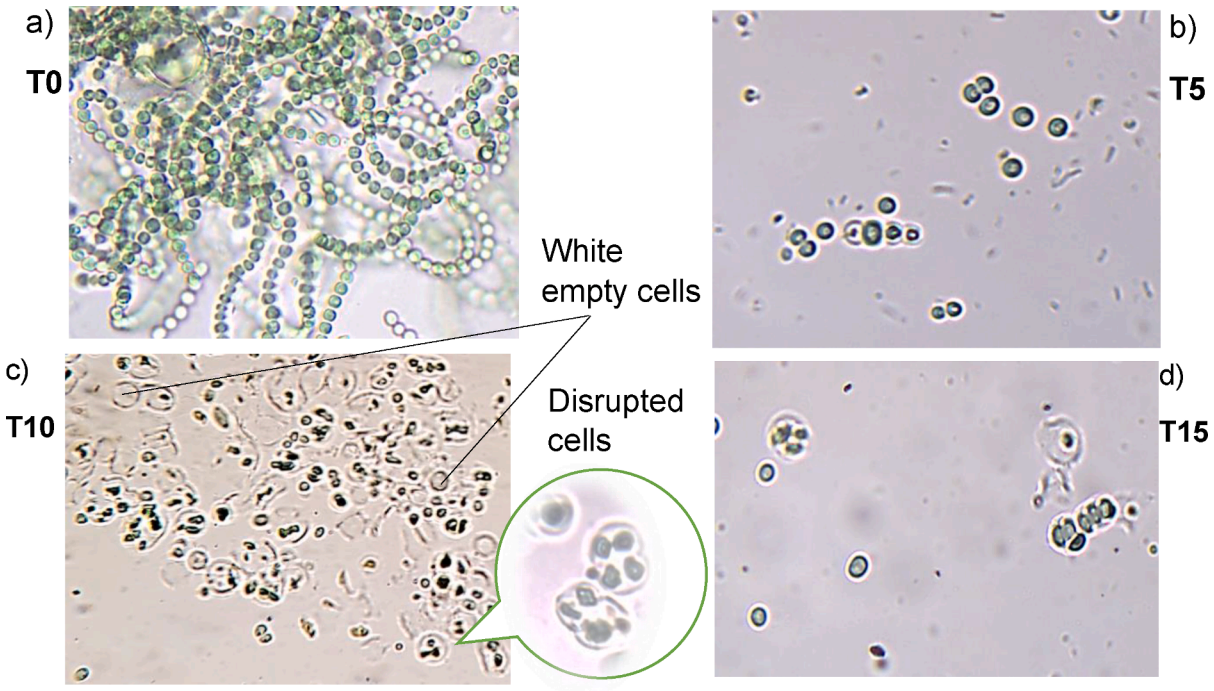

Fig. 5. Microscopic images (at 40 ×) of *N. punctiforme* a) before ultrasonication (denoted as T0), b) after 5 min ultrasonication (denoted as T5), c) after 10 min ultrasonication (denoted as T10), d) after 15 min ultrasonication (denoted as T15).

Table 1
Effect of microalgal extracts on soil nematodes.

Sample	#/kg soil	% Inhibition
Nematized soil	80	–
T. variabilis (TV) extract	0*,[1]	100
N. punctiforme (NP) extract	27*,[2]	66.25
TV + NP	7*,[3]	91.25

*signifies that the paired *t*-test shows the results are significantly different ($p < 0.05$).

[1] p value could not be computed because the difference in standard error was zero.

[2] $p = 0.015$.

[3] $p = 0.008$.

species [25,57,29,8].

4. Conclusion

In this study, we investigated the effect of direct sonication using ultrasound probe for extracting secondary metabolites from microalgae. Our results suggest that ultrasonication resulted in intensified release of secondary metabolites, as established through the total chlorophyll content of the ultrasonicated microalgal samples as well as the microscopic images of the ruptured cells. The best extraction for *Trichormus variabilis* was achieved with 15 min extraction time where the Total Chl content increased by 29 times (compared to the non-ultrasonicated sample), and for the *Nostoc punctiforme*, 30 min extraction time gave the highest metabolite recovery of 6.4 times higher than the non-ultrasonicated sample. Secondly, this study confirms that nematode suppression in soil samples using microalgal extracts could be feasible alternative to the chemical approach. The two microalgal species, *Trichormus variabilis* and *Nostoc punctiforme*, both significantly reduced the root knot nematode in soil samples.

Encouraged by the positive results obtained from this study, i.e., the intensified extraction of metabolites from *Trichormus variabilis* and *Nostoc punctiforme* using direct ultrasonication, and its nematode suppressing potential, the future research has been outlined as follows: investigate the mechanism of inhibition of these cyanotoxins; optimizing the operating conditions (for example, frequency, time, extractant volume, pulse rate, amplitude) of ultrasonication considering response surface methodology; quantification of the extract to determine the application rate required to kill nematodes, especially juvenile (J2) eggs to prevent their hatching; and lastly, to further assess the nematode suppressing potential of these microalgal extracts field studies on nematode-infested soil under cropped conditions using the host plants for *M. hapla*, namely, tomato and lettuce would be instrumental.

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CRediT authorship contribution statement

Fatima Haque: Conceptualization, Funding acquisition. **Mahendra Thimmanagari:** Conceptualization, Resources. **Yi Wai Chiang:** Conceptualization, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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