Algal Research 16 (2016) 368–375

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

# Determination of the time-dependent response of *Lemna trisulca* to the harmful impact of the cyanotoxin anatoxin-a

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ARTICLE INFO

*Article history:*
Received 9 December 2015
Received in revised form 7 March 2016
Accepted 3 April 2016
Available online 17 April 2016

*Keywords:*
Anatoxin-a
*Lemna trisulca*
Macrophyte
Stress response

ABSTRACT

Previous studies reported anatoxin-a (ANTX-a) accumulation and bioremediation by the aquatic macrophyte *Lemna trisulca*. In the present study, we determined that cultivation of this macrophyte in medium containing ANTX-a at a concentration similar to that occur in nature (1.0 $\mu g \cdot mL^{-1}$) did not cause any changes in the plant physiology. A much higher toxin concentration (12.5 $\mu g \cdot mL^{-1}$) reduced photosynthetic efficiency by 27% compared with the control, but had no significant effect on the respiration process. *L. trisulca* cultivated in medium containing 25.0 μg of ANTX-a $\cdot mL^{-1}$ reduced the $chl_a$ content by 40% on the 18th day of the experiment, the $chl_b$ content by 35% on the 14th day of the experiment and the total carotenoids by 11% on the 24th day of the experiment. ANTX-a at concentrations ≤12.5 $\mu g \cdot mL^{-1}$ did not cause any significant differences in the concentration of released anions ($Cl^-$, $NO_3^-$ and $SO_4^{2-}$) compared to the control. However, cultivation of *L. trisulca* with 5 μg of ANTX-a $\cdot mL^{-1}$ resulted in a 1.8-fold increase in the concentration of $K^+$ released into the medium compared with the control. The amount of $Na^+$, $Ca^{2+}$ and $Mg^{2+}$ released after plant exposure to 12.5 μg of ANTX-a $\cdot mL^{-1}$ was 1.3-fold, 1.6-fold and 1.7-fold higher, respectively, compared to the control. ANTX-a had a slight effect on the protein content and the oxidoreductase activity in the macrophyte. After 24 h, there were no significant differences in the enzymatic activity of the superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase and glutathione reductase, regardless of the ANTX-a concentrations in the media. Only polyphenol oxidase showed a slight increase in the activity in media containing ANTX-a. These findings confirmed that *L. trisulca* has a high tolerance to this toxin and a great potential as a phytoremediation agent in the aquatic environments.

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## 1. Introduction

Toxic cyanobacterial blooms represent from 25% to 95% of all blooms and are distributed in aquatic systems worldwide [3,30]. A higher frequency and intensity of these phenomena is caused by climate changes and the increasing eutrophication of water, which is primarily induced by anthropogenic activities such as agricultural run-off or industrial waste. A dangerous consequence of the growing number of toxic cyanobacterial blooms is the release of a wide range of cyanotoxins into the water that can be harmful to humans, animals, plants and microorganisms [6]. One of the most common freshwater cyanotoxin is the neurotoxic anatoxin-a (ANTX-a). There are numerous reports on the toxic effects of ANTX-a on various species of animals (i.e., bats, cattle, dogs, lesser flamingos, Mallard ducks, and mice) (for review; [25]). The most frequent symptoms of poisoning are: convulsions, muscle overstimulation, abdominal breathing, cyanosis and paralysis, possibly leading to death by asphyxiation when the respiratory muscles are affected [4]. ANTX-a in animals mimics the effect of the neurotransmitter acetylcholine, but ANTX-a is not degraded by acetylcholinesterase [4]. There are also some data on the effect of ANTX-a on plants. Macrophytes exposed to this toxin showed a significant increase in $H_2O_2$ production, activation of enzymatic defense systems, growth inhibition and changes in the total amount of photosynthetic pigments versus the controls [13,23]. Current studies on cyanobacteria are mainly concentrated on two aspects: 1) reducing the occurrence of cyanobacterial blooms by preventing eutrophication and 2) removing extracellular toxins dissolved in water [28,33,34,36]. Our study focuses on the biological inactivation of cyanotoxins released into the water because this approach is usually one of the cheapest, most accessible and environmentally friendly methods in monitored water bodies. In the previous studies, we examined the potential use of the macrophyte *Lemna trisulca* for the biodegradation and bioaccumulation of ANTX-a and to reduce the biomass of its producer, cyanobacterium *Anabaena flos-aquae* [17,18]. The impact of ANTX-a on macrophytes was observed over an extremely wide range of toxin concentrations from 50 pg of ANTX-a $\cdot mL^{-1}$ for *Ceratophyllum demersum* [12] to 25 μg of ANTX-a $\cdot mL^{-1}$ for *Lemna minor* [23]. In the present study, we have elucidated the concentration of ANTX-a that caused significant changes in *L. trisulca* and determined whether this plant could potentially be used for the toxin removal process. Here, we examined the content of photosynthetic pigments,

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http://dx.doi.org/10.1016/j.algal.2016.04.004
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photosynthesis and respiration processes, the concentration of ions released from the macrophyte into the environment, the total protein content and the characteristics of the stress response. All experiments were performed under controlled laboratory conditions modeled on the ANTX-a concentrations present in the environment or higher.

## 2. Material and methods

### 2.1. Preparation of research materials

A sterile monoculture of *L. trisulca* (L.) strain LT-FR 1 (selected from the natural environment) was used in these experiments. ANTX-a was obtained from *A. flos-aquae* (Lyngb.) de Bréb. (*Dolichospermum flos-aquae*) strain SAG 30.87 purchased from the University of Göttingen (Germany). The macrophyte and cyanobacterium were separately cultivated in BG11 medium [31] in a phytotron (Bolarus S-711S/P, Poland) at 20 ± 1 °C with 80% humidity and 50 μmol $m^{-2}$ $s^{-1}$ photosynthetically active radiation (PAR) under a 12 h photoperiod. The containers with plants or cyanobacteria were regularly shaken. The toxin was extracted and purified from the cyanobacterial cells using the method described by Meriluoto and Codd [22]). ANTX-a concentrations in all samples were determined as described by Kaminski et al. [17]) at the beginning and at the end of each experiment. All of the experiments were performed under similar physical conditions, all of the reagents were of analytical or HPLC grade.

### 2.2. Photosynthesis and respiration processes

The measurements of the photosynthesis and respiration rates in *L. trisulca* were performed using an oxygen Clark electrode placed in a DW 2 chamber (Hansatech, UK) under a constant temperature of 20 °C with a CB1-D3 electrode control unit (Hansatech, UK). Electrode calibration was done according to the instructions before and after each series of analysis. Then, 0.040 ± 0.002 g fresh weight (fw) of *L. trisulca* was placed in 1 mL of media without toxin (control), with 1 μg of ANTX-a·$mL^{-1}$ or with 12.5 μg of ANTX-a·$mL^{-1}$. The photosynthesis and respiration processes were measured in two series of experiments. In the first series, a short-term influence of ANTX-a on the intensity of these processes for up to 120 min was studied. After 5 min of adaptation at the light intensity of 50 μmol·$m^{-2}$·$s^{-1}$ or in the dark, the plants were exposed to a light/dark/light cycle of 10/10/10 min. The changes in the oxygen concentration in the media were measured at 0, 30, 60, 90 and 120 min. In the second series of experiments, the long-term impact of ANTX-a was studied. The macrophytes were cultivated in media without toxin or with a concentration of ANTX-a equal to 1 μg·$mL^{-1}$ or 12.5 μg·$mL^{-1}$ for 4, 9, 14, 18 or 24 days. After the specified time, the plants were weighed and placed in fresh media in the electrode chamber, and the intensity of the photosynthesis and respiration rates were measured similar to the procedure described above.

### 2.3. The impact of ANTX-a on the photosynthetic pigment content

ANTX-a was dissolved in media to final experimental concentrations of 0.025, 2.5, 12.5, and 25.0 μg·$mL^{-1}$. *L. trisulca* was transferred to the ANTX-a solutions in a 1:20 ratio (fw/v). The sampling was performed immediately after the addition of the macrophyte to the media and after 4, 9, 14, 18 or 24 days of exposure. To determine the photosynthetic pigment content in the macrophyte, the fresh weight of *L. trisulca* was analyzed at the indicated time points, and then the macrophyte was homogenized in a manual homogenizer with an 80% aquatic solution of acetone (v/v) at 2 °C. The homogenate was centrifuged at 10,000 *g* for 60 s. The collected supernatant was transferred to a cuvette, and the absorbance was measured at $\lambda = 470$, 646 and 663 nm (spectrophotometer Jasco V-650, Japan). The photosynthetic pigments content was calculated using the equations developed by Wellburn [35]) and expressed per g fw of the macrophyte.

### 2.4. Ions released from macrophytes under ANTX-a treatment

The macrophytes were cultivated for 5 days in MilliQ water containing ANTX-a at concentrations of 0, 2.5, 5.0, 12.5 or 25.0 μg·$mL^{-1}$. *L. trisulca* was transferred to the ANTX-a solutions in a 1:20 (fw/v) ratio. After a predetermined amount of time, 5 mL of medium was collected from each sample and the concentration of anions ($Cl^-$, $NO_3^-$, and $SO_4^{2-}$) and cations ($Ca^{2+}$, $K^+$, $Mg^{2+}$, and $Na^+$) were analyzed on the DX600A ion chromatograph (Dionex, USA). The media were filtered through 25TF PURADISC™ membrane filters (Whatman, USA) with a diameter of 0.45 μm. Using the AS40 autosampler, solutions were introduced into IonPac analytical columns. The AS9-HC column (4 × 250 mm) and a liquid phase consisting of a 9 mM solution of $Na_2CO_3$ were used for the determination of anions. The concentrations of the cations were determined using an CS-12A column (4 × 250 mm) and 20 mM methanesulfonic acid (MSA, Sigma-Aldrich) as the eluent. The flow rate of the eluent through the column in both cases was 1 mL·$min^{-1}$. Separated ions were identified by the ED50 detector, and the ion concentrations were analyzed using the PeakNet program.

### 2.5. Characterization of the stress response

#### 2.5.1. Electrical conductivity

The initial electrical conductivity value ($C_i$) was measured for the ANTX-a solution at concentrations of 0, 5, 12.5, or 25 μg·$mL^{-1}$ (LF 323 conductometer, WTW, Germany). Then, *L. trisulca* (1:20, fw/v) was added to each solution. The electrical conductivity measurements of each sample ($C_s$) were made immediately after the plant addition and at 1 h intervals for 4 h. After the last measurement, the solutions were boiled for 5 min. Then, the samples were cooled to room temperature, the evaporated volume of the solutions was supplemented with MilliQ water and the theoretical total conductivity ($C_t$) of the aqueous plant extract was measured. The indicator of cell membrane damage ($M_d$) in time was calculated according to the equation described below:

$$M_d = \left[\frac{(C_s - C_i)}{(C_t - C_i)}\right] \cdot 100\%.$$

#### 2.5.2. Lipid peroxidation

Lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA), which is the final product of the peroxidation of unsaturated fatty acids constituting biological membranes, using the method described by Heath and Packer [15]). *L. trisulca* was cultivated for 5 h or 5 days in media supplemented with ANTX-a at concentrations of 0, 5, 12.5, 25 or 50 μg·$mL^{-1}$. The mass ratio of the plant to the medium in each sample was 1:20 (fw/v).

#### 2.5.3. Plant material preparation

*L. trisulca* was placed in media (1:20, fw:v) with ANTX-a at concentrations of 0, 1.0, or 12.5 μg·$mL^{-1}$. The culture was placed in the phytotron for 24 h with parameters similar to those described in Subsection 2.1. After this time, the plants were washed three times with MilliQ water, transferred to a manual homogenizer, frozen with liquid nitrogen, pre-crushed and homogenized at 2 °C in 6 mL of 50 mM phosphate buffer solution (pH 7.0) containing 1% polyvinylpyrrolidone (PVP) and 0.2 mM ascorbic acid. The homogenate was centrifuged at 1000 *g* for 10 min (MPW-351R, Poland). Supernatants were collected and frozen at −20 °C prior to the determination of the protein content and activity of the individual enzymes.

#### 2.5.4. Determination of the total protein content and oxidoreductase activity

The total protein content in the extracts was determined by the method described by Bradford [2]).

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