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Uptake of the cyanobacterial neurotoxin, anatoxin-a, and alterations in oxidative stress in the submerged aquatic plant *Ceratophyllum demersum*

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ABSTRACT

The prevalence of cyanobacterial blooms in fresh water bodies worldwide has become a serious environmental problem. The blooms can increase the occurrence of cyanobacterial neurotoxin, anatoxin-a, and this toxin can interact with aquatic plants and other pivotal components of aquatic ecosystems. Despite this, several questions regarding the uptake of the toxin by aquatic plants and its association with toxic effects still remain. This study investigated the uptake of anatoxin-a in relation to alterations in oxidative stress, estimated by changes in lipid peroxidation and tocopherol contents (alpha-, beta-, gamma-, and delta-tocopherol), in the submerged aquatic plant, *Ceratophylum demersum*, at environmentally relevant concentrations. Exposure to five different concentrations of anatoxin-a (0.005, 0.05, 0.5, 5 and 50 $\mu g\, l^{-1}$) for 24 h increased concentrations in *C. demersum* in a dose-dependent manner. All four forms of tocopherols were elevated at low concentrations of anatoxin-a (0.005. 0.05. 0.5 and 5 $\mu g\, l^{-1}$). However, a decline in the four tocopherol forms along with a high level of lipid peroxidation was observed at 50 $\mu g\, l^{-1}$ exposure dose. During 336-h exposure to 15 $\mu g\, l^{-1}$ anatoxin-a, rapid toxin uptake during the first 24 h and subsequent steady accumulation of the toxin were observed. The four tocopherol forms increased in response to anatoxin-a uptake, attaining their maximum levels together with a significant increase of lipid peroxidation after 12 or 24 h. After 24-h exposure, the four tocopherol forms decreased gradually without recovery. The results clearly indicate that anatoxin-a uptake can cause a disturbance of the oxidative stress in the aquatic plant, and depending on the concentration and exposure duration, oxidative damage occurs.

1. Introduction

Cyanobacteria encompassing diverse photosynthetic prokaryotic micro-organisms exist in balanced aquatic environments at the proper level at which their frequency and abundance are naturally controlled (Paerl, 2000; Stockner, 1999). However, accelerated eutrophication and climate change facilitate them to excessively proliferate to the extent that they provoke a disturbance (e.g. high algal turbidity and oxygen depletion) in aquatic ecosystems and deteriorate water quality (e.g. offensive odor and taste) related to human well-being (Hart et al., 1999; Paerl and Huisman, 2009; Pitois et al., 2000; Smith, 2003). Furthermore, many of cyanobaterial genera such as *Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis and Planktothrix* are known to produce a wide range of toxic bioactive secondary metabolites designated as cyanotoxins. These toxins are principally released into the surrounding water during their senescence and decomposition (Carmichael, 1997; Codd, 1995). Anatoxin-a is one of the most potent cyanobacterial neurotoxins commonly detected all over the world (Ballot et al., 2004; Gugger et al., 2005; Park et al., 1998; Stevens and Krieger, 1988). It is a powerful cholinergic agonist competing with acetylcholine for nicotinic acetylcholine receptor in neuronal signal transmission (Gugger et al., 2005; Krienitz et al., 2003; Park et al., 1998; Thomas et al., 1993). The toxin is chemically defined as a low-molecular-weight semi-rigid alkaloid (MW = 165.26), labile under natural environmental conditions, especially sunlight and pH. Thus, anatoxin-a is reported to be easily degradable into two non-toxic compounds (i.e. dehydroanatoxin-a and epoxyanatoxin-a) and to have a half-life of several hours or days in strong sunlight and at high pH (Devlin et al., 1977; Smith and Sutton., 1993; Stevens and Krieger, 1991). Due to the distinctive chemical characteristics, low stability and persistence, as well as the notorious rapid neurotoxic mode of action, fatal to mammals, relatively little attention has been paid so far to the fate and impact of anatoxin-a in aquatic environments. However, considering the prevalence of the massive

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growth of cyanobacteria leading to increased presence and amount of cyanotoxins, knowledge about anatoxin-a uptake, metabolism and the resulting effects in aquatic ecosystems containing various living organisms (e.g. plant, zooplankton, mollusk and fish) needs to be improved (Carmichael, 2008; Glibert et al., 2005). Very few studies investigated the bioconcentration and depuration of anatoxin-a in particular aquatic organisms, mussel and fish, associated with the risk of human intoxication from anatoxin-a-contaminated dietary source (Osswald et al., 2011, 2008, 2007). Plants are generally regarded as major players in the transport and degradation of many well-known contaminants in aquatic environments through their uptake and metabolic detoxification (Dhir et al., 2009; Takamura et al., 2003). Moreover, they are also involved in the transfer of pollutants via bioaccumulation along the aquatic food chain as primary producers offering food to other higher organisms (Gobas and Morrison, 2000). Indeed, there have been several reports presenting that the powerful cyanobacterial hepatotoxins microcystins (MCs) can be taken up, metabolized and eventually accumulated to a certain degree in aquatic plants such as *C. demersum*, *Lemna gibba* and *Phragmites australia* (Pflugmacher et al., 2004, 2001; Saqrane et al., 2007). The publication from Esterhuizen et al. (2011a) also showed that the cyanobacterial neurotoxin β-methylamino-l-alanine (BMAA) can be transferred from water media into *C. demersum* and can possibly give rise to biomagnification through protein association following accumulation of the toxin. Simultaneously, phytotoxic effects (e.g. reduced photosynthetic pigment contents and altered enzymatic systems) have been observed and linked to uptake of these cyanotoxins (Esterhuizen et al., 2011b; Pflugmacher, 2004; Saqrane et al., 2007). Currently, there is some evidence indicating that anatoxin-a can also affect multiple levels of biological metabolism (e.g. growth, photosynthesis and enzymatic systems) of aquatic macrophytes, *Lemna minor* (*L. minor*) and *C. demersum* by inducing oxidative stress (Ha and Pflugmacher, 2013a, 2013b; Mitrovic et al., 2004). Previous studies demonstrated that the activity of antioxidative enzymes including superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR) and glutathione reductase (GR) was changed in response to the elevated formation of hydrogen peroxide (H_2O_2) in *C. demersum* exposed to anatoxin-a (Ha and Pflugmacher, 2013a, 2013b).

Therefore, the present study aimed to investigate the uptake of anatoxin-a in relation to alterations of the oxidative stress status in *C. demersum*, a world-wide distributed submerged aquatic plant. Oxidative stress refers to an imbalance between pro-oxidative reactions (e.g. generating reactive oxygen species (ROS)) and antioxidative defense (e.g. quenching ROS). Oxidative stress can lead to severe damage to all types of cellular molecules, DNA, proteins and lipids, depending on the degree of imbalance (Sies, 1997). Lipid peroxidation is described as the oxidative deterioration of polyunsaturated fatty acids (PUFAs), crucial components of cellular lipid membranes, and has been largely studied in the assessment of oxidative cell injury (Esterbauer et al., 1991; Gutteridge, 1995). It is well-established that plants possess effective antioxidative defense systems comprising various enzymes and non-enzymatic free-radical scavengers antioxidants like ascorbate (vitamin C), carotenoids, glutathione (GSH), and tocopherols (vitamin E) to mitigate various conditions of oxidative stress (e.g. controlling ROS production and relieving the resulting damage) (Gill and Tuteja, 2010; Mittler, 2002). Tocopherols, synthesized exclusively by plants and certain photosynthetic organisms, are believed to play important roles in the stabilization of lipid membranes by decomposing ROS and lipid peroxy radicals during the propagation of lipid peroxidation (Fryer, 1992; Munné-Bosch and Alegre, 2002). They exist in four different chemical forms, α-, β-, γ-, and δ-tocopherol, named according to the number and position of methyl groups on the chromanol ring. α-Tocopherol is known as the major form detected in green parts of plants. Accordingly, in this study lipid peroxidation and the corresponding antioxidant, four tocopherols (α-, β-, γ-, and δ-tocopherol), were used as indicators of changes in oxidative stress status in response to anatoxin-a uptake in *C. demersum*.

2. Material and methods

2.1. Plant material

C. demersum, obtained from Extraplant (Extragroup GmbH, Münster, Germany), was cultivated non-axenically in a glass tank (60 cm × 60 cm × 60 cm) filled with modified Provasoli's medium containing de-ionized water, 0.2 g l^{-1} $CaCl_2$, 0.103 g l^{-1} $NaHCO_3$ and 0.1 g l^{-1} sea-salt. The culture was conducted under cool white fluorescent light (35 μE m^{-2} s^{-1} irradiance) with a day/night cycle of 14/10 h at 22–24 °C.

2.2. Experimental design

(±) Anatoxin-a fumarate was provided by Tocris Bioscience (Strathmore Road Natick, MA, USA). The toxin was dissolved to 1 g l^{-1} in 70 percent (v/v) methanol and further diluted with culture medium to the final exposure concentrations. The environmentally feasible concentrations of anatoxin-a were derived from monitoring studies conducted in German fresh water bodies (Bumke-Vogt et al., 1999; Sächsisches Staatsministerium für Soziales, 2009). The plant, comprising of shoot apical meristem, main stem and leaves, was cut one week before the experiments to avoid any physical stresses resulting from cutting. After this pre-culture for one week, 4.5 ± 0.5 g fresh weight (FW) of plant material was exposed to anatoxin-a in a volume of 100 ml under the above mentioned light and temperature conditions for plant culture.

Two different static exposure systems were set to investigate concentration- and time-dependent anatoxin-a-uptake in connection with subsequent changes in the levels of lipid peroxidation and tocopherols in *C. demersum*. Controls consisting of the plant and culture medium without anatoxin-a were prepared and sampled in parallel with the toxin treatment in order to consider the influence of the exposure conditions on the plant. Three independent replicate experiments were performed for each exposure system. In the first exposure system, plants were treated with 0.005, 0.05, 0.5, 5 and 50 μg l^{-1} anatoxin-a for 24 h. In the second experiment, plants were exposed to 15 μg l^{-1} anatoxin-a reflecting the highest concentration of the toxin in the environment for 4, 8, 12, 24, 48, 168 and 336 h. Additionally, the pre-cultured plants that were not introduced into the exposure systems were directly used as 0 h controls. Exposure medium was daily refilled with culture medium without anatoxin-a to the initial volume to avoid changes in anatoxin-a concentration due to evaporation.

After exposure, plants were washed twice with de-ionized water to remove any surface-associated toxin and snap-frozen in liquid nitrogen. The frozen plant material was stored at −80 °C until toxin, tocopherol and LPO analysis, respectively. In addition to plant sampling, the exposure medium containing anatoxin-a in the time-dependent exposure system was sampled at each exposure time to determine the remaining toxin in the medium over the period of exposure.

2.3. Anatoxin-a analysis

2.3.1. Extraction of anatoxin-a

Anatoxin-a was extracted from plant tissue according to Rellán et al. (2007) with slight modifications. The frozen plant material was ground in liquid nitrogen to a fine powder. Anatoxin-a was extracted by mixing 0.2 g of pulverized plant tissue in 1 ml of acidified 70 percent methanol (MeOH) containing 0.1 percent trifluoroacetic acid (TFA). Tissue samples were continuously shaken in the dark for 3 h at room temperature using overhead shaker, Intelli-Mixer RM-2 (Neolab, Heidelberg, Germany). Extracts were then centrifuged at 10,600*g* for 10 min at 4 °C and the supernatant was adjusted to pH 7.0 ± 0.2 with 1 M sodium hydroxide (NaOH) before solid-phase extraction (SPE) using a weak cation-exchange material in a vacuum manifold processor (SPE-12G, JT Baker, Großgerau, Germany). SPE columns, Strata WCX, 3 ml with 200 mg silica gel-based packing (Phenomenex, CA, USA), were conditioned with equal volumes of MeOH and water. Sample loading and elution with 100 percent MeOH containing 0.2 percent TFA were carried out under gravity. Afterwards, eluates were completely dried under constant nitrogen flow. The final residue was reconstituted in 200 μl of 70 percent MeOH and anatoxin-a content was immediately determined by LC–MS/MS.

Water samples were centrifuged at 5000*g* for 15 min at 4 °C and transparent water was settled at pH 7.0 ± 0.2 with 1 M NaOH. The remaining anatoxin-a in the exposure media was extracted and concentrated with Strata WCX SPE columns using the same procedure as described above. Solid phase-extracted water samples were immediately processed for the toxin analysis via LC–MS/MS.