Pharmacological Studies Confirm Neurotoxic Metabolite(s) Produced by the Bloom-Forming *Cylindrospermopsis raciborskii* in Hungary

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ABSTRACT: A rapid cyanobacterial bloom of *Cylindrospermopsis raciborskii* (3.2×10^4 filaments/mL) was detected early November, 2012, in the Fancsika pond (East Hungary). The strong discoloration of water was accompanied by a substantial fish mortality (even dead cats were seen on the site), raising the possibility of some toxic metabolites in the water produced by the bloom-forming cyanobacteria (*C. raciborskii*). The potential neuronal targets of the toxic substances in the bloom sample were studied on identified neurons (RPas) in the central nervous system of *Helix pomatia*. The effects of the crude aqueous extracts of the Fancsika bloom sample (FBS) and the laboratory isolate of *C. raciborskii* from the pond (FLI) were compared with reference samples: *C. raciborskii* ACT 9505 (isolated in 1995 from Lake Balaton, Hungary), the cylindrospermopsin producer AQS, and the neurotoxin (anatoxin-a, homoanatoxin-a) producer *Oscillatoria* sp. (PCC 6506) strains. Electrophysiological tests showed that both FBS and FLI samples as well the ACT 9505 extracts modulate the acetylcholine receptors (AChRs) of the neurons, evoking ACh agonist effects, then inhibiting the ACh-evoked neuronal responses. Dose–response data suggested about the same range of toxicity of FBS and FLI samples ($EC_{50} = 0.397$ mg/mL and 0.917 mg/mL, respectively) and ACT 9505 extracts ($EC_{50} = 0.734$ mg/mL). The extract of the neurotoxin-producing PCC 6506 strain, however, proved to be the strongest inhibitor of the ACh responses on the same neurons ($EC_{50} = 0.073$ mg/mL). The presented results demonstrated an anatoxin-a-like cholinergic inhibitory effects of cyanobacterial extracts (both the environmental FBS sample, and the laboratory isolate, FLI) by some (yet unidentified) toxic components in the matrix of secondary metabolites. Previous pharmacological studies of cyanobacterial samples collected in other locations (Balaton, West Hungary) resulted in similar conclusions; therefore, we cannot exclude that this chemotype of *C. raciborskii* which produce anatoxin-a like neuroactive substances is more widely distributed in this region. Environ Toxicol 00: 000–000, 2013.
Keywords: cyanobacteria; bloom sample; fish mortality; acetylcholine; *Helix pomatia*

INTRODUCTION

The freshwater cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya *et* Subba Raju (Noctocales, *Cyanophycae*) is one of the most invasive cyanobacterial species all over the world (Kling, 2009; Sinha et al., 2012). The originally tropical/subtropical nitrogen fixing filamentous organism with apical heterocysts seems to successfully accommodate to more temperate freshwater zones (Chorus and Bartram, 1999; Briand et al., 2004; Bonilla et al., 2012).

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Cyanobacteria produce a number of highly bioactive, structurally different metabolites, both toxic and non-toxic (Singh et al., 2005; Tidgewell et al., 2010), their invasive nature, therefore, also increases the risk of toxic strains to appear in the most populated areas of Europe or the United States (O'Neil et al., 2012). The major concern in water quality and public health perspective of *C. raciborskii* is due to its known ability to produce different, highly potent cyanotoxins, including the hepatotoxic alkaloid cylindrospermopsin (CYN), and saxitoxins, the neurotoxic paralytic shellfish poisons (PSP). The CYN producing *C. raciborskii* chemotypes were identified first in Australia (Griffiths and Saker, 2003) while PSP producer *C. raciborskii* strains were reported from the South-American continent (Lagos et al., 1999; Garcia et al., 2004) in connection with serious human poisoning incidents. Although more and more occurrences, water blooms were reported relating this species from European waters with variable toxicity [reviewed by Chorus and Bartram (1999)], the background mechanisms of the toxic effects or the chemical nature of the toxic cyanobacterial metabolites of European strains are still unidentified.

Nowadays *C. raciborskii* (together with *Microcystis* and *Planktothrix* genera) is one of the most frequent bloom-forming species in the Hungarian shallow lakes, often producing a rapid increase and accumulation of algal biomass in eutrophic lakes in summer months (Padisak, 1997; Vasas et al., 2010a, b). Bioassays (both vertebrate and invertebrate) demonstrated the toxicity of several *C. raciborskii* strains (Hiripi et al., 1998; Torokne et al., 2007; Ács et al., 2013), and also revealed biochemical, morphological, and pharmacological differences in their effects, probably resulted by different toxic components of the bioactive metabolic products, and modulation of different receptors (Antal et al., 2011; Vehovszky et al., 2012). However, neither analytical or molecular studies demonstrated the presence of any of the known cyanotoxins (CYN or PSP) in any strains isolated in Hungary (Torokne et al., 2007; Vasas et al., 2010a; Ács et al., 2013), already identified in other *C. raciborskii* isolates of tropical origin (Carneiro et al., 2009; Dittmann et al., 2012; Moreira et al., 2013). This contradiction strongly suggest the presence of not yet known toxic component(s) in the cyanobacterial matrix of the *C. raciborskii* isolates, but the structural identification of these new toxins is still the greatest challenge ahead.

Heavy *C. raciborskii* bloom was detected early November, 2012 in the Eastern part of Hungary (Fancsika pond), resulting strong discoloration of the water accompanied by serious fish mortality (over a ton of fish was collected), moreover, even dead cats were observed on the shore of the pond. Chemical analysis (Micellar electrokinetic chromatography, Vasas et al., 2004) performed on the water samples from Fancsika pond (FBS) and the cyanobacterial (*C. raciborskii*) isolate FLI, excluded the presence of the well known cyanobacterial toxins (anatoxin-a, microcystin-LR, cylindrospermopsin), which could be responsible for the toxic effects. Preliminary electrophysiological tests also excluded the effect of saxitoxins, blockers of the voltage gated sodium channels, as no alteration of the amplitude or shape of action potentials was detected. However, the large scale destruction (fish and cat mortality) still raised the possibility of some, probably unidentified neurotoxic components in the water produced by the bloom-forming cyanobacteria (*C. raciborskii*). This assumption was supported by previous results concerning different *C. raciborskii* isolates from Lake Balaton, which displayed various neurotoxic effects of the cyanobacterial extracts on invertebrate excitable tissues, central nervous system (CNS), and heart (Kiss et al., 2002; Vehovszky et al., 2012).

A functionally linked approach, when the target recognition mechanisms are monitored, may provide an alternative method to detect (unidentified) toxic substances in a mixture of secondary metabolites (Van Dolah and Ramsdell, 2001). Changes of the potential targets may also indicate the background mechanisms behind the toxic effects, and characterize the specific toxin–target interactions of the bioactive components in the environmental samples (Devic et al., 2002; Kulagina et al., 2004; Coecke et al., 2006).

To establish the neurotoxic mechanisms of the cyanobacterial products in the Fancsika bloom sample (FBS) we applied this target-selective functional tool by characterizing the modulatory effects of cyanobacterial extracts on acetylcholine receptors (AChRs) of identified neurons in the CNS of *Helix pomatia*. First we electrophysiologically described the membrane effects of the cyanobacterial samples and the locally applied acetylcholine, then testing the responses in the presence of known cholinergic receptor antagonists (including the pure cyanobacterial neurotoxin, anatoxin-a) pharmacologically characterized the neuronal acetylcholine receptors (AChRs), the potential targets of the neurotoxic effects of cyanobacterial extracts. The pharmacological results were finally compared with the effects of the aqueous extracts of the FBS, and its laboratory isolate (FLI). As a reference, similar pharmacological experiments were performed with the aqueous extracts of the *C. raciborskii* ACT 9505 strain (its neurotoxic effects was established earlier, Vehovszky et al., 2012) and the *Oscillatoria* sp. PCC 6506 strain (known to produce anatoxin-a and homoanatoxin-a neurotoxins; Araoz et al., 2005).

MATERIALS AND METHODS

Blooming/Cyanobacterial Samples

Environmental samples from Fancsika pond (referred as Fancsika blooming samples, FBS) were collected from the water while blooming on the November 2, 2012 (see Fig. 1). The bloom-forming algal cells were harvested by a 5 μm membrane filter and the algal species identified by their morphological characteristics (using an inverted microscope, LEICA DMIL). The bloom sample (FBS) and also the

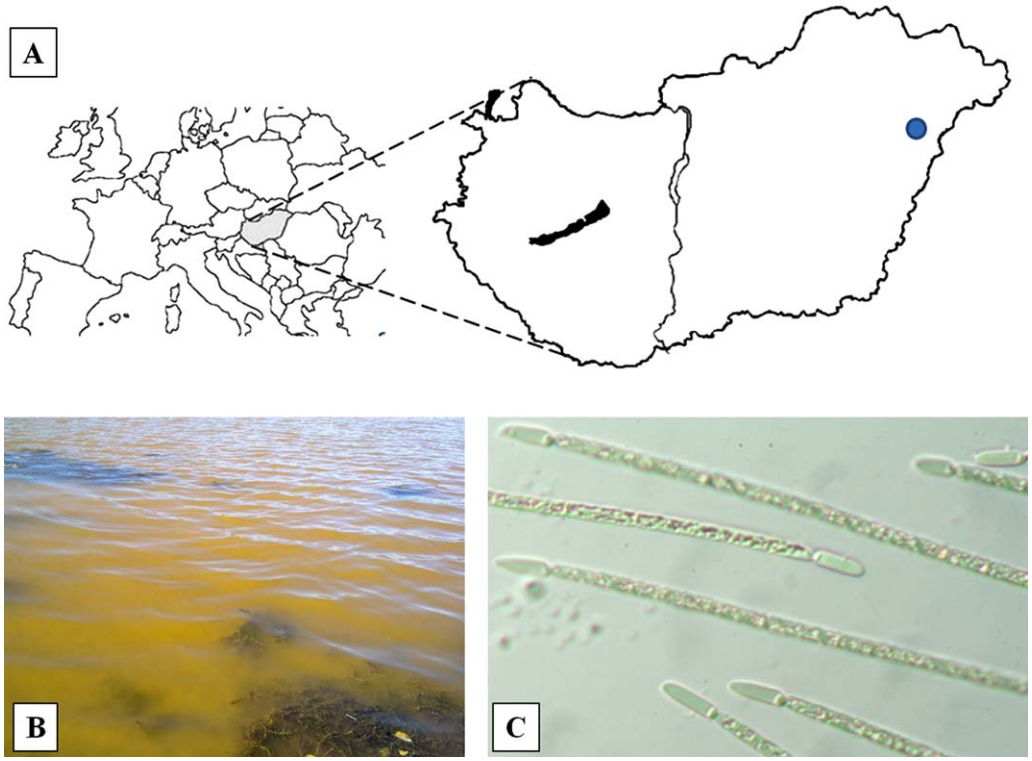

Fig. 1. Mass production of *C. raciborskii* resulting fish mortality in November, 2012. Location of the Fancsika pond in the Eastern part of Hungary, where the bloom samples were collected (A). Discoloration of the water turning orange caused by cyanobacterial blooming (3.2×10^4 filaments/mL) (B). The bloom-forming species identified as *C. raciborskii* by its characteristic morphological feature (terminal heterocyst) (C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

laboratory isolate of the bloom forming cyanobacteria (FLI sample) were filtered and lyophilized for further analysis. For references, the laboratory strains of *C. raciborskii* ACT 9505 (isolated from Lake Balaton, Hungary, during natural bloom in 1995), *C. raciborskii* AQS (donated by M.L. Saker), and the *Oscillatoria* sp. PCC 6506 strain (purchased from the Pasteur Culture Collection of Cyanobacteria, France) were used in identical experiments.

The culturing conditions of the strains for mass production and the harvesting procedures were the same as described earlier (Antal et al., 2011).

Preparation of Cyanobacterial Extracts

For electrophysiological/pharmacological studies aqueous crude extracts were produced both from the water of Fancsika pond (referred as FBS samples) and the laboratory strain of *C. raciborskii* isolated from Fancsika pond during blooming condition (FLI samples). Experiments were also performed applying the extracts of laboratory isolates of cyanobacteria, *C. raciborskii* ACT 9505, AQS, and *O. formosa* PCC 6506 strains. The cell-free crude extracts were produced from 100 mg dry weight of lyophilized environmental sample/algal biomass, resuspended in 5 mL *Helix* physiological saline. The cells were lysed by repeated freeze thawing and the supernatants collected after centrifugation ($12{,}000 \times g$, 10 min, 4°C), producing a stock solution of 20 mg dry weight of biomass per 1 mL physiological saline (20 mg/mL). During the experiments this stock was further diluted in normal saline and the final concentrations of the aqueous extracts of both the environmental sample and the cyanobacterial isolates were expressed as mg/mL.

Animals

Adult specimens of the terrestrial snail *H. pomatia* were collected locally (Tihany, Hungary), kept in outdoor cages and fed by lettuce *ad libitum*. The experiments were performed during spring and autumn (April–June and September–November) between 2012 (autumn) and 2013 (spring). The electrophysiological tests were performed in five to eight independent experiments on isolated CNS preparations. Moreover, when statistical methods were applied, the exact number of experiments is referred in the text (10–12). All together we used over 150 specimens of adult *H. pomatia*.

Electrophysiological Experiments

Electrophysiological experiments were performed by intracellular recording from identified (right parietal, RPas

cluster) neurons in the central nervous system of *H. pomatia* (Vehovszky et al., 1989). Single neurons were penetrated by two independent glass microelectrodes (12–14 MΩ tip resistances, filled with 4 M potassium acetate and 0.3 M potassium chloride) to either hold the membrane potential at a constant level while recording acetylcholine (ACh) evoked voltage responses (in bridge mode) or the ACh evoked membrane currents (in voltage clamp mode). For recording and storing electrophysiological data, we used an Axoclamp 2B microelectrode amplifier and the DasyLab 5.63 software through a PC 6035E (National Instruments) interface card.

Acetylcholine (0.1 M) was applied locally by microelectrophoretic injection through a micropipette located near the cell body, at 3 min intervals to prevent desensitization of the receptors involved. The amplitudes of the ACh injecting currents were selected in the linear range of the dose–response curve (between 50 and 500 nA, 0.2 s duration), when three repetitive applications of ACh resulted identical membrane responses. Local application of the algal extract (50 μL volume) was performed from a small tube positioned close to the CNS, while for pharmacological testing the aqueous extracts were applied by perfusion into the experimental chamber. All drugs were diluted in the standard physiological *Helix* saline (Vehovszky et al., 1992) and applied by perfusion providing a constant (1 mL/min) flow rate.

Drugs

All drugs were purchased from SIGMA, except anatoxin-a, which was bought from Ascent Scientific, UK.

Statistics

Dose–response relationship was analyzed to establish the Hill coefficients and EC_{50} values by multicomponent nonlinear fitting method using GraphPad Prism version 4 software (GraphPad Software)

RESULTS

Algal (Cyanobacterial) Blooming in the Fancsika Pond

The 70 ha Fancsika pond is situated in the Eastern part of Hungary [Fig. 1(A)] and one of the most popular recreational areas near Debrecen (the second largest city in Hungary), mainly used for angling and regularly stocked by fishes. The average water temperature of the pond is 12.8°C, while in summer months its temperature reaches 30°C, and in winter the water is usually covered by ice. On the November 2, 2012, a dramatic algal bloom was detected by a heavy orange discoloration of the water [Fig. 1(B)] accompanied by a serious fish kill, when more then a ton of different fish species [*Sander lucioperca, Hypophthalmichtys nobilis* R., *Ctenopharingodon idella* (Valenciennes, 1844) *Cyprinus carpio* L., *Silurus glanis* L., *Ameiurus nebulosus* (Lesueur), *Carassius carassius* L.] was collected from the water. In addition, three dead cats were also seen near the lake. In the blooming samples, 98% of the algal biomass was provided by *C. raciborskii* (3.2×10^4 filaments/mL), identified by its characteristic morphological feature, the terminal heterocyst [Fig. 1(C)], according to the recent classification system (Komárek, 2013). No other, potentially toxic cyanobacterial species were detected, while Eukaryotes were occasionally represented in a few samples by *Bacillariophyceae* and *Chlorococcales*.

Acetylcholine Responses of Identified RPas Central Neurons in the Helix CNS

Previous electrophysiological results demonstrated the neurotoxic effects of several *C. raciborskii* extracts (ACT isolates), primarily targeting the acetylcholine receptors (AChRs) in the central nervous system of both the pond snail *Lymnaea stagnalis* and the terrestrial snail *H. pomatia* (Kiss et al., 2002; Vehovszky et al., 2012). The mass production of *C. raciborskii* detected in the water samples of the Fancsika pond led us to assume, that similar neurotoxic effects were responsible for the devastating environmental consequences observed during Fancsika blooming. To establish the possible neuronal target and background mechanisms of the toxic effect of FBS and laboratory isolates (FLI), therefore, we applied similar electrophysiological and pharmacological experiments on identified neurons of the snail *H. pomatia* as before.

Local application of the aqueous extract of FBS (50–100 μL volume, 5–10 mg/mL concentration) resulted an excitatory response on RPas neurons with increased frequency of the spontaneous action potential generation [Fig. 2(Ai)], or membrane depolarization, when hyperpolarizing current was injected by the second electrode [Fig. 4(A)]. The same excitatory intracellular responses (depolarization and frequent potential generation) were also evoked by local application of the cyanobacterial extracts: *C. raciborskii* ACT 9505 and PCC 6506 strains [Figs. 2(Aii, Aiii), 6(Ai), 7(Ai)]. Acetylcholine (ACh) applied locally by ionophoretic injection evoked a similar excitatory effect on the same neurons [Fig. 2 (Aiv)], suggesting the involvement of the same target (the acetylcholine receptor, AChR) in the membrane responses of both the environmental sample (FBS) and the cyanobacterial extracts (ACT 9505 and PCC 6506). To further specify the neuronal target of cyanobacterial samples next we characterized the AChRs by recording the acetylcholine (ACh) responses of neurons and analyzing their kinetic and pharmacological characteristics.

The amplitudes of the ACh evoked responses displayed a linear relationship with the recording potential while shifting the membrane potential level to more negative values. The amplitudes of both the depolarizing responses [Fig. 2(Bi)] and the ACh-evoked inward membrane currents were

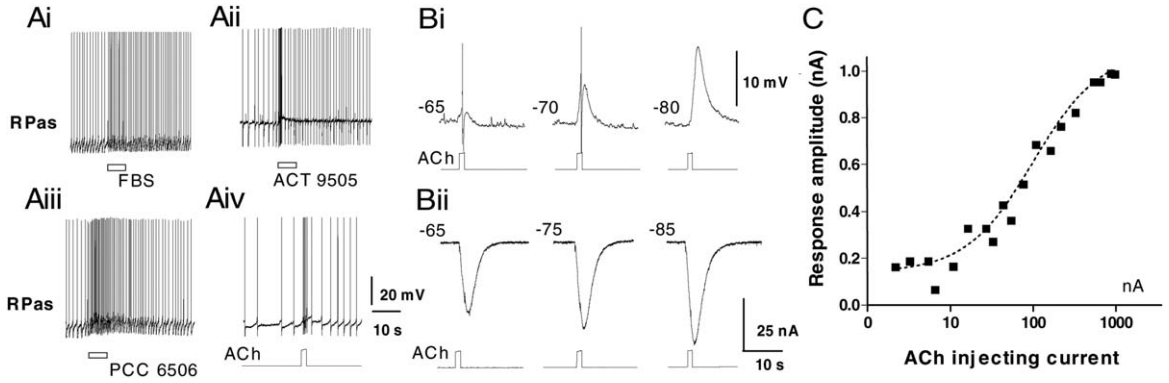

Fig. 2. Acetylcholine-agonist effects of cyanobacterial extracts on the RPas neurons. Excitatory membrane effects are recorded after local application of the aqueous extract of the FBS (Ai) the crude extract of ACT 9505 strain (Aii), and extract of PCC 6506 strain (Aiii). Local acetylcholine (ACh) application evokes similar excitatory response (increased firing frequency) on RPas neuron (Aiv). After local (iontophoretic) application of ACh the amplitudes of both the depolarizing responses (Bi) or the ACh-evoked membrane currents (Bii) are increasing while shifting the holding potential towards more negative values (−65 to −85 mV). Summarized data of 19 experiments produce a sigmoidal dose–response curve of ACh evoked current responses at −75 mV holding potential (C), with a calculated Hill coefficient value of 1.17 ± 0.144. Local application of the Fancsika sample (FBS), ACT 9505, and PCC extracts (50 μL, 10 mg dry weight/mL) are marked below the individual current records [Fig. 1(Ai, Aii, Aiii)]. Bottom traces on Aiv, and Bi, Bii records display the iontophoretic currents used for ACh application.

gradually increasing as the holding potential was set to more negative values [Fig. 2(Bii)]. The equilibrium potential of responses (the extrapolated membrane potential values, where either the depolarization or inward current amplitudes decreased to zero or reversed) was around 0 mV value (4.90 ± 7.3 mV, Mean ± SD, $n = 12$).

Recording the ACh responses on a constant (−75 mV) holding potential level, a sigmoidal dose–response relationship was established between the holding potential and the amplitudes of the inward membrane currents [Fig. 2(C)]. Cumulative data revealed a Hill coefficient around 1 (1.17 ± 0.144; Mean ± SD, $n = 19$), suggesting a single site of substrate coupling on the ACh receptors.

Inhibitory Effects of Cholinergic Antagonists on Acetylcholine Responses

To pharmacologically characterize the acetylcholine receptors of the RPas neurons involved in cyanotoxin effects the best known nicotinic AChR antagonist, d-tubocurarine (dtc) was tested first, using voltage clamp mode for recording ACh evoked membrane currents. Similar experiments were performed with the muscarinic antagonist atropine and the pure cyanobacterial neurotoxin, anatoxin-a as well.

The excitatory effect of local ACh application (ACh-evoked inward currents) on RPas neurons was inhibited all by dtc [Fig. 3(Ai)], atropine [Fig. 3(Aii)], and anatoxin-a [Fig. 3(B)], showing a dose-dependent blocking effect [Fig. 3(C)]. Application of dtc resulted reversible inhibition, as the reduced ACh responses started to recover during washing out with normal saline [Fig. 3(Ai)]. Atropine, generally regarded as muscarinic antagonist, was found to be a more effective inhibitor of the ACh responses starting from lower (around 0.5 μM) threshold, while higher (100 μM) concentration of atropine nearly irreversibly blocked the ACh responses [Fig. 3(Aii)]. Anatoxin-a, the pure cyanobacterial neurotoxin, was found to be the most effective drug tested, decreasing the ACh responses below 0.005 μM threshold concentration, while above 0.5 μM it already reached its maximal inhibitory effect [Fig. 3(B,C)]. The relative inhibitory potencies (EC_{50} values) of the above antagonists were assessed by fitting the dose–response curves using GraphPad (see Materials and Methods). Summarized data clearly showed that anatoxin-a was the most, about 2 magnitudes more effective antagonist ($EC_{50} = 0.072$ μM), while d-tubocurarine was even less effective ($EC_{50} = 35.5$ μM), than the muscarinic antagonist atropine ($EC_{50} = 8.82$ μM).

Inhibitory Effects of Cyanobacterial Extracts on the Acetylcholine Responses

Local application of the crude aqueous extract of FBS (50 μL, 5–10 mg/mL) evoked excitatory (ACh agonist) effect on RPas neurons, either as increased frequency of spontaneous activity [Fig. 2(Ai)] or large (up to 15 mV) membrane depolarization [Fig. 4(A)]. Simultaneously, the ACh-evoked responses were decreased after FBS application and slowly recovered during wash out [Fig. 4(B,C)].

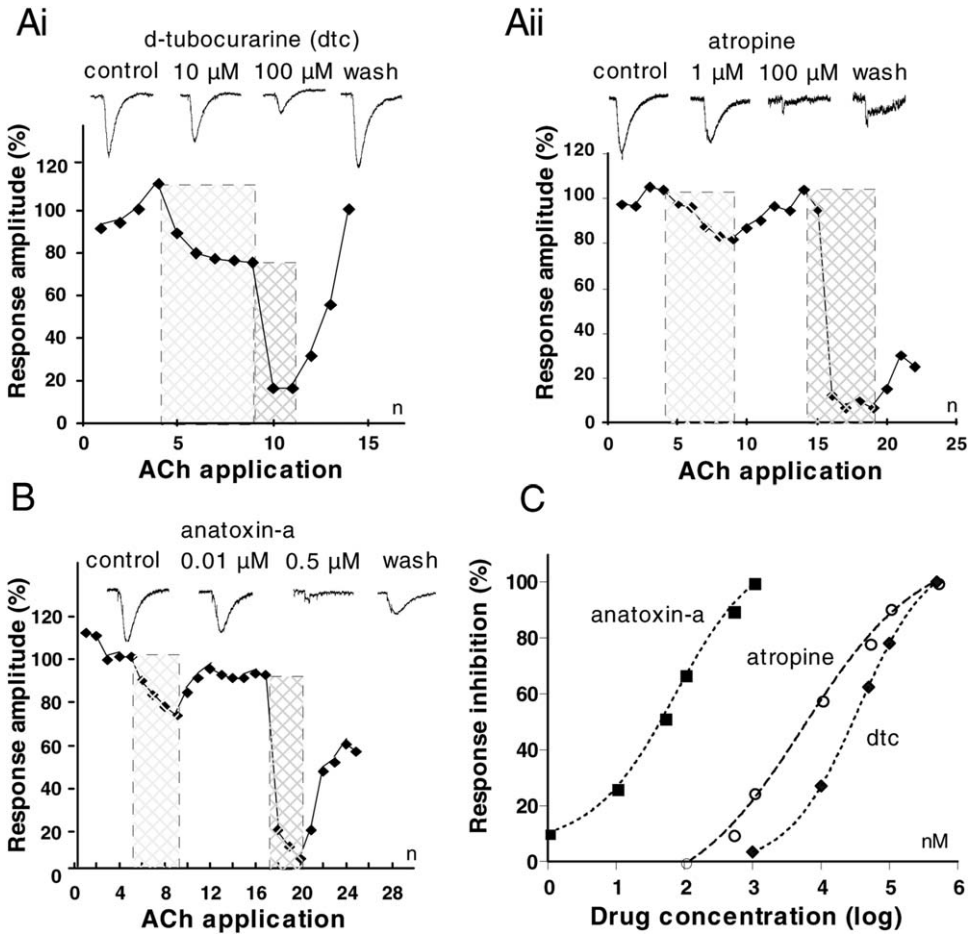

Fig. 3. Cholinergic antagonists inhibit the ACh effects on RPas neurons. Dose-dependent decrease of the ACh evoked membrane responses by the nicotinic antagonist d-tubocurarine (Ai), the muscarinic antagonist atropine (Aii), and the pure cyanotoxin, anatoxin-a (B). Graphs show the relative amplitudes (percent values of control) of ACh evoked currents during the course of individual, representative experiments, while applying ACh locally in 3 min intervals. Hundred percent of control was calculated as mean of four to six ACh-evoked current amplitudes in normal saline. Shaded areas mark the antagonist applications, and the traces above the graphs show selected records of ACh-evoked current responses (inward current traces). Dose–response graphs of summarized data (10–12 experiments) for each putative antagonists show a substantial shift of the graph demonstrating anatoxin-a, the most effective inhibitor of ACh responses (EC_{50} = 0.072 μM) compared with atropine (EC_{50} = 8.82 μM) and d-tubocurarine (EC_{50} = 35.5 μM).

FBS applied by perfusion reversibly decreased the ACh responses from 0.05 mg/mL concentration, and above 1 mg/mL almost reached its maximum inhibitory effect [Fig. 5(A)]. Summarized dose–response data demonstrated a substantial inhibitory effect of FBS, with an EC_{50} value of 0.397 mg/mL [Fig. 5(B)]. A similarly produced extract of the laboratory isolate of *C. raciborskii* from Fancsika pond (FLI) also had, although somewhat lower inhibitory effect on the ACh responses (EC_{50} = 0.917 mg/mL, n = 4), suggesting the same target, the ACh receptors are involved in both experiments [Fig. 5(C)]. To provide further data for quantitative assessment of the relative (cholinergic) toxicity of the Fancsika samples (FBS, FLI), we tested the effects of two already established laboratory isolates, the ACT 9505 and *Oscillatoria* sp. PCC 6506 strains in similarly designed series of experiments.

The aqueous extract of ACT 9505 also evoked strong excitatory (ACh agonist) effect on RPas neurons as mentioned above [Figs. 2(Aii), 6(Ai)], and simultaneously, the ACh-evoked responses were decreased after ACT 9505 application [Fig. 6(Aii)]. ACT 9505 extract applied by perfusion similarly inhibited the ACh responses as seen by decreased amplitudes of the ACh-evoked inward currents [Fig. 6(B)]. The inhibitory effect of the ACT 9505 extract

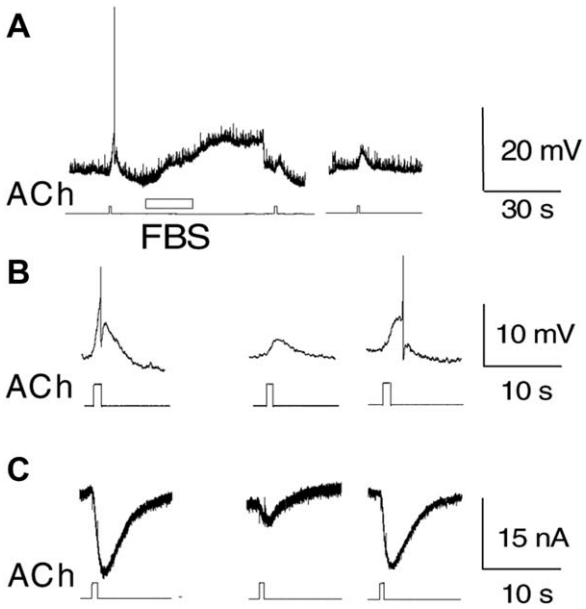

Fig. 4. FBS modulates the ACh responses of the RPas neurons. Locally applied extract of FBS (50 μL, 10 mg dry weight/mL) evokes excitatory (depolarizing response) of the membrane (A) and reversibly inhibits the ACh-evoked effects by decreasing the amplitude of both the ACh depolarizing response (B) or the corresponding ACh-evoked inward current (C). Bottom traces on A, B, and C records mark the iontophoretic currents used for ACh application, and local application of the FBS is also marked below (A).

between 0.1 and 2 mg/mL concentrations resulted a sigmoidal dose–response relationship [Fig. 6(C)] giving an EC_{50} value of 0.734 mg/mL ($n = 12$).

The extract of PCC 6506 similarly evoked a strong ACh agonist (excitatory) effect resulting increased firing frequency [Fig. 2(Aiii)] or depolarization when the RPas membrane was hyperpolarized [Fig. 7(Ai)]. Additionally, PCC 6506 application decreased the amplitude of the ACh responses of the RPas neuron [Fig. 7(Aii)]. The inhibitory effect of the PCC 6506 extract applied by perfusion had (about a magnitude) lower threshold (below 0.025 mg/mL) compared with the similarly produced aqueous solution of ACT 9505 (below 0.2 mg/mL). Higher (0.1 mg/mL) concentration of PCC 6506 extract reversibly inhibited the ACh responses by more than 50% [Fig. 7(B)]. Cumulative data of PCC 6506 applications between 0.01 and 5 mg/mL concentrations displayed a dose-dependent inhibitory effect [Fig. 7(C)] and resulted an EC_{50} value of 0.073 mg/mL ($n = 14$).

The similarly produced aqueous extracts of *C. raciborskii* AQS strain (reference for cylindrospermopsin-producing cyanobacterial sample from Australia); however, when applied locally up to 20 mg/mL concentrations did not evoke repeatable or dose-dependent effects on either the spontaneous activity pattern or the ACh-evoked membrane responses (data not shown).

DISCUSSION

Characterization of Acetylcholine Receptors of Identified Neurons in Helix CNS

Natural neurotoxins often target the nicotinic acetylcholine receptors (Daly, 2005), therefore, those neuronal processes, which use acetylcholine as a neurotransmitter or neuromodulator either in the CNS or periphery, are seriously damaged by intoxication leading to severe symptoms, often death of the animals or human involved.

In the snail CNS, the role of acetylcholine (ACh) as a neurotransmitter and modulatory substance mediating both

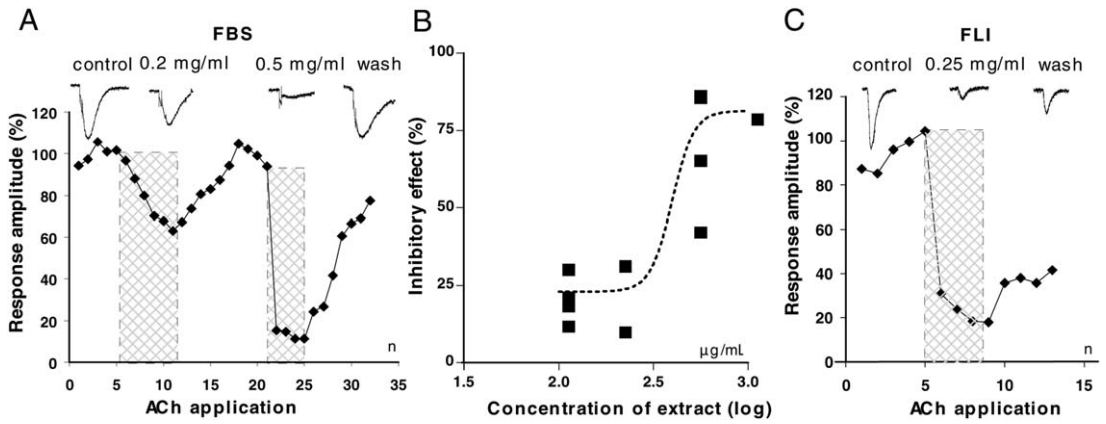

Fig. 5. Fancsika samples antagonize the ACh effects on RPas neurons. The aqueous extract of FBS reversibly inhibits the ACh evoked membrane currents (A), and summarized data ($n = 12$) show a sigmoidal dose–response relationship (B) with $EC_{50} = 0.397$ mg/mL value. The cyanobacterial extract of *C. raciborskii* isolate from the Fancsika pond (FLI) similarly inhibits the ACh-evoked membrane currents (C). Shaded areas mark the presence of Fancsika extracts (FBS and FLI, respectively) while applying ACh locally in 3 min intervals. Traces above the graphs show selected records of ACh-evoked current responses (inward current traces).

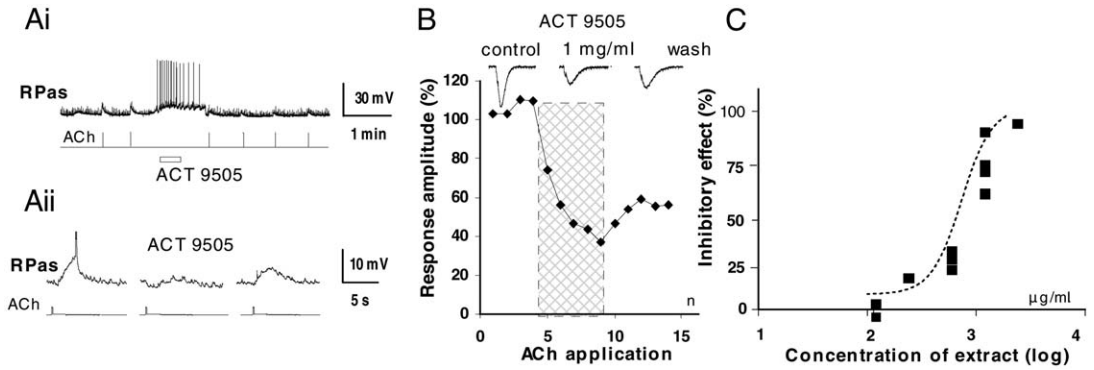

Fig. 6. Effects of *C. raciborskii* ACT 9505 extract recorded on RPas neurons. Excitatory response (burst of action potentials) evoked by local application (5 mg/mL, 50 μL) of cyanobacterial extracts (Ai), while the ACh-evoked depolarization strongly inhibited (Aii). Bottom traces show the iontophoretic current used for local ACh application. The amplitudes of ACh evoked currents (shown as percent of control responses) are decreased during perfusion of 1mg/mL ACT 9505 extract (shaded area marks the extract application, while traces above the graph show selected records of ACh-evoked inward current responses at −75 mV holding potential level). Dose–response graph of summarized data (12 experiments) demonstrates the ACT 9505 extract as an effective inhibitor of the ACh evoked acetylcholine responses with the $EC_{50} = 0.734$ mg/mL (dry weight of lyophilized algal mass/mL physiological saline).

excitatory and inhibitory neurotransmission is already well established (Walker et al., 1996). Although the snail ACh receptors (AChRs) are generally regarded as structurally similar ones to the vertebrate nicotinic ACh receptors [reviewed by Nierop et al. (2005)], their basic subtypes can be further distinguished by ion selectivity and pharmacological characteristics (Kehoe and McIntosh, 1998; Vulfius et al., 2001, 2005). Nicotinic acetylcholine receptor blockers including d-tubocurarine and anatoxin-a may further select between different subclasses of acetylcholine receptors based on different structural features (Daly, 2005).

The serotonergic RPas cluster neurons in the CNS of *H. pomatia* with identical pattern of spontaneous activity, chemical sensitivity, and synaptic inputs (Vehovszky et al., 1989) also showed similar ACh responses by their electrophysiological and pharmacological features. The equilibrium potential of the ACh-evoked responses (around 0 mV) suggests the involvement of both Na^+ and K^+-dependent membrane channels, and the Hill coefficient (around 1) suggests a single site for receptor binding of ACh as a substrate.

Dose–response data of pharmacological studies on the RPas neurons revealed at least 2 magnitudes higher sensitivity of the AChRs to the pure cyanobacterial neurotoxin, anatoxin-a ($EC_{50} = 0.072$ μM) than either the muscarinic antagonist atropine or the nicotinic blocker d-tubocurarine ($EC_{50} = 8.82$ μM and 35.5 μM, respectively). Anatoxin-a

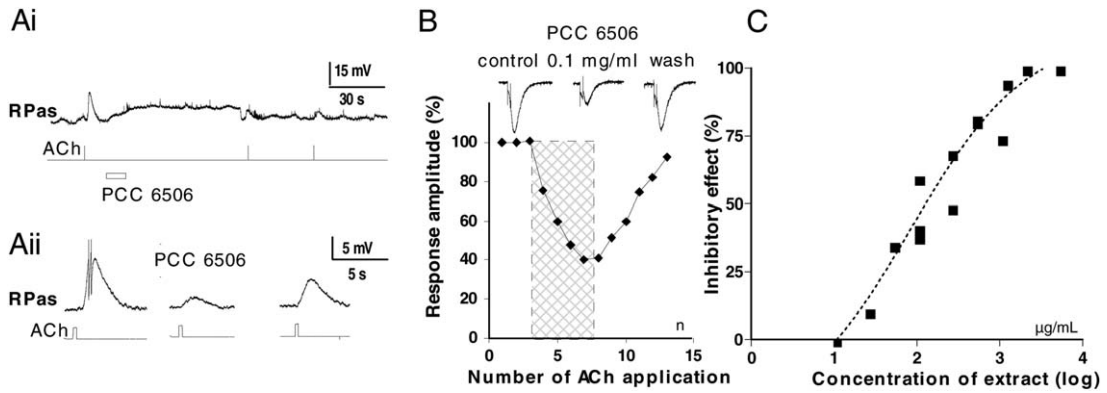

Fig. 7. Effects of *Oscillatoria sp*. PCC 6506 extract recorded on RPas neurons. Depolarization evoked by local application (5 mg/mL, 50 μL) of cyanobacterial extracts (Ai), while the ACh-evoked depolarization is strongly inhibited (Aii). Bottom traces on Ai, Aii: record show the iontophoretic currents used for local ACh application (Ai, Aii). Reversible inhibition of the ACh evoked currents by about 60% of control responses in the saline containing 0.1 mg/mL PCC 6506 extract (shaded area marks the extract application, while traces above the graph show selected records of ACh-evoked inward current responses at −75 mV holding potential level). Summarized data of 14 experiments demonstrate the dose-dependent effect of PCC 6506 extract antagonizing the ACh evoked acetylcholine responses ($EC_{50} = 0.073$ mg/mL).

was found to be one of the most effective agonist/antagonist of the nAChRs in vertebrate systems as well, with measured EC_{50} value similarly in the nanomolar range (Thomas et al., 1993; Dar and Zinder, 1998; Kaiser and Wonnacott, 2000). On fish embryos about the same dose (0.4 μg/mL) of anatoxin-a modulates heart rate (Oberemm et al., 1999).

In snail central nervous system, both nicotine and muscarine can antagonize the acetylcholinergic responses of the same neurons both in *H. pomatia* and *Lymnaea stagnalis* (e.g., Witte et al., 1985; Elliott et al., 1992) suggesting different subclasses of ACh receptors than established for vertebrates (Nierop et al., 2005). The physiological and pharmacological heterogenity of acetylcholine receptors throughout the snail central nervous system seems to be a general feature as mentioned above (Kehoe and McIntosh, 1998; Vulfius et al., 2001, 2005; Nierop, 2005), and also underlines the importance of identification of neurons while testing the electrophysiological or pharmacological mechanisms of toxic effects.

Based on our summarized electrophysiological data, we may conclude that the RPas cluster neurons in the *Helix* CNS possess a kinetically and pharmacologically homogenous population of ACh receptors. This conclusion also encourages us to use the ACh responses of the RPas neurons as a suitable model to further characterize the target-specific interactions between the ACh receptors and the neuroactive components in the cyanobacterial extracts.

Neurotoxic Effects and Toxicity Assessment of Cyanobacterial Samples

Freshwater cyanobacterial neurotoxins are classified by their background mechanisms specific for the neuronal functions as blockers of the voltage sensitive Na^+ channels (saxitoxin), inhibitors of the neurotransmitter (ACh)-activated membrane receptors (anatoxin-a), or the enzymatic breakdown of ACh in the synaptic cleft [anatoxin-a(s), as reviewed by Araoz et al. (2010)]. Chemically, these toxins are often represented by a whole family of structurally related compounds, for example, about 20 homologs of the alkaloid saxitoxin act on the voltage dependent Na^+ channels (Catterall et al., 2007), and anatoxin-a also has its homologue (homoanatoxin-a) and their metabolites (Mann et al., 2012). The presence of cholinergic neurotoxins: anatoxin-a, or its related alkaloids, although they were identified in many cyanobacteria as *Anabaena flos-aquae, Anabaena* spp. (*flos-aquae-lemmermannii* group), *Anabaena planktonica*, *Oscillatoria*, *Aphanizomenon*, and *Cylindrospermum* (reviewed by Chorus and Bartram, 1999), were never established in any *C. raciborskii* strains yet.

Our presented data, however, suggest that the toxic effect of the FBS is based on the cholinergic inhibitory action of some water soluble neuroactive components. The same ACh receptor blocking effects were also recorded by applying the extract of *C. raciborskii* isolate from Fancsika pond (FLI). It is very likely, therefore, that some yet unidentified component(s) of the secondary metabolites of this particular strain of *C. raciborskii* are responsible for the acetylcholine response blocking (anatoxin-a like) neurotoxic effects. Previous electrophysiological studies of different *C. raciborskii* ACT strains also demonstrated inhibitory cholinergic effects (Kiss et al., 2002; Vehovszky et al., 2012), so we cannot exclude the presence of the same, or chemically related substances in all the *C. raciborskii* (ACT and Fancsika) samples collected from Hungary. This neurotoxic feature may characterize some, but definitely not all the strains of *C. raciborskii*, as the cylindrospermopsin producing strain AQS, for example, did not display the cholinergic (ACh agonist or AChR blocking) effects on our neuronal model.

Dose–response data of pharmacological experiments established the same range of toxicity of the FBS and FLI samples ($EC_{50} = 0.397$ mg/mL and $EC_{50} = 0.917$ mg/mL) and the ACT 9505 strain ($EC_{50} = 0.734$ mg/mL), and all about 10 times lower, than was measured for the anatoxin-a (homoanatoxin-a) producer reference *Oscillatoria* sp. PCC 6506 ($EC_{50} = 0.073$ mg/mL). Comparative results, therefore, made it very likely, that the effective yet unidentified neurotoxin content of both *C. raciborskii* strains studied (isolated from Fancsika blooming or from Lake Balaton, some years ago) may represent a low, but still significant threat for the environment during their mass production.

We also have to be aware that toxicological tests—either of environmental samples or isolates—hardly give proper data for evaluation of the intoxication risk and the environmental effects of cyanobacterial blooms, as they often result large aggregates (colonies) or wind-driven accumulation (surface scum) of the algal mass. Consequently, the toxic algal components may be locally concentrated, resulting 1–3 magnitudes higher toxicity then would be established from laboratory experiments when homogeneously distributed algal mass is tested (Chorus and Bertram, 1999).

Finally, the synergistic effects of (toxic and non-toxic) bioactive substances in the environmental samples cannot be excluded either, as suggested by those studies, which compared the effects of the pure cyanotoxins with cyanobacterial extracts (Kiviranta et al., 1991; Oberemm et al., 1999; Metcalf et al., 2002; Osswald et al., 2009, etc.). During natural blooming situations, when a whole range of active components of the cyanobacterial matrix is released into the water, such interactions most likely happen.

C. raciborskii in Hungarian Waters: Environmental Risk Assessment

In Hungary, *C. raciborskii* appeared in the 1970s, then in a decade became abundant in Hungarian waters, including Lake Balaton (the largest shallow lake in the Western part of Hungary); then from the early 1980s several cyanobacterial blooms were following each other [reviewed by Padisak (1997)]. Although the trophic level of this lake decreased in

the last decades due to human intervention and the cyanobacterial blooms also seem to be disappeared recently, the *C. raciborskii* species is still spreading in our region, often resulting mass production in several other locations, mostly smaller ponds in Hungary (Vasas et al., 2010a).

When blooming, *C. raciborskii* may produce almost the entire (by up to 95%) total algal biomass (Padisak, 1997; Vasas et al., 2010a), reaching: 60 mg/L in Balaton (1982); 127 mg/L in the Kis-Balaton Water Protection System (2009), and as high as 870 mg/L in a small fishpond, East Hungary not far from the Fancsika pond (1992), as already reported (Padisak et al., 1984; Borics et al., 2000; Horváth et al., 2013). The peak values of *C. raciborskii* blooming, therefore, may represent about the same cyanobacterial concentrations in the water as were established as half maximal effective (EC_{50}) values in our pharmacological or toxicological studies of *C. raciborskii* samples (Fancsika: 397 mg/L, and 971 mg/L, ACT 9505: 734 mg/L, Thamnotox mortality: 574 mg/L; Torokne, 1999); again, highlighting the environmental risk when potentially toxic strains of *C. raciborskii* are blooming.

The hepatotoxic (cytotoxic) cylindrospermopsin (CYN) is the most often detected toxic metabolite of cyanobacteria, including *C. raciborskii* [reviewed by Dittmann et al. (2012) and Moreira et al. (2013)], while some *C. raciborskii* strains are known as producers of PSP neurotoxins (saxitoxin analogs; Molica et al., 2002). Although CYN producing strains only were reported from Australia, Asia, and New Zealand and strains producing saxitoxins are only found in South America, the increasing number of reports of *C. raciborskii* in temperate regions justifies the toxicological studies of European strains.

As mentioned before, no CYN or PSP was detected either in cultures of *C. raciborskii* strains isolated from Hungary, or in the *C. raciborskii* dominated field samples (Torokne et al., 2007; Vasas et al., 2010a; Ács et al., 2013). This result is consistent with other data which reported lack of CYN of this bloom-forming species throughout Europe [reviewed by Poniedzialek et al. (2012)].

Our presented results demonstrated the neurotoxic effects of both the environmental sample (FBS) and the laboratory isolate of *C. raciborskii* (FLI), collected from the Fancsika pond during cyanobacterial blooming. It is very likely, therefore, that toxic (though unidentified) cyanobacterial metabolites made a substantial contribution in the mass destruction in the pond observed in November, 2012. Although lowered oxygen concentration in water resulted by blooming conditions may also cause fish kills (*Sander lucioperca* for example, which is sensitive to hypoxia), in the Fancsika pond; however, the fish mortality did not suggested such species selectivity, therefore, hypoxia was not the only factor resulting the fish kill observed. In addition, cats were also found dead near the pond, also suggesting a kind of intoxication, probably caused by secondary metabolites of the blooming *C. raciborskii*.

Based on our presented pharmacological data, therefore, we conclude that the cholinergic inhibitory effects of Fancsika samples suggest the presence of some yet unidentified anatoxin-a like neurotoxins produced by the *C. raciborskii* strain blooming in the Fancsika pond. This suggestion corresponds with previous pharmacological data regarding other *C. raciborskii* samples isolated in 1995 from Lake Balaton (Kiss et al., 2002; Vehovszky et al., 2012), therefore we cannot exclude that this chemotype of *C. raciborskii* which produce cholinergic neurotoxic metabolites is more widely distributed in this region.

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