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Decomposition products of cylindrospermopsin – a cyanotoxin produced by *Raphidiopsis raciborskii* (Woloszynska)

by

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Abstract

Toxins produced by cyanobacteria (cyanotoxins) and released into water have become a serious problem worldwide due to the increasing morbidity and mortality of living organisms they have caused. The ability to synthesize the cytotoxic alkaloid cylindrospermopsin (CYN) has been demonstrated in several freshwater species of cyanobacteria. CYN is highly chemically stable under environmental factors and decomposes only under alkaline conditions, where it forms derivatives. The toxicity potential of the decomposition products formed at pH 10 combined with high temperature (100°C) or UV-B irradiation (36 µmol m^{-2} s^{-1}) has been research based on the crustacean *Thamnocephalus platyurus* (Thamnotoxkit FTM) and bacteria *Vibrio fischeri* (Deltatox® II) bioassays. This paper is a continuation and completion of our previous experiments and the obtained results showed that the applied conditions contributed to the decomposition of the CYN molecule to non-toxic products and its structural modifications by separating the uracil ring or/and the sulfate group from the tricyclic guanidine moiety, leading to a reduction in its toxicity. To the best of our knowledge, this is the first report describing the toxicity of CYN decomposition products formed under alkaline conditions combined with boiling temperature or UV-B irradiation.

Key words: cylindrospermopsin, decomposition products, toxicity, toxicity bioassays

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Introduction

Toxins produced by cyanobacteria (cyanotoxins) belong to the most harmful substances in natural aquatic environments (Neumann et al. 2007; Everson et al. 2009; Weirich & Miller 2014; Kubickova et al. 2019). One of the most common cyanotoxins is the alkaloid cylindrospermopsin (CYN; Fig. 1), which is produced by several freshwater cyanobacteria, such as *Raphidiopsis raciborskii*, *Anabaena lapponica* or *Umezakia natans* (Ohtani & Moore 1992; Harada et al. 1994; Spoof et al. 2006). In animal cells, CYN shows cytotoxic properties and may negatively affect numerous metabolic pathways in many organs (for a review, see De la Cruz et al. 2013; Adamski et al. 2014). It has been demonstrated that the toxin inhibits translation, most likely through its interaction with proteins (Harada et al. 1994; Froscio et al. 2008; López-Alonso et al. 2013). CYN also impairs the activity of glutathione, causing oxidative stress (Runnegar et al. 1995). Moreover, this compound is able to react with nucleic acids, which leads to the adduct formation and contributes to carcinogenesis or mitosis inhibition (Falconer & Humpage 2001; Humpage et al. 2005). To date, two cases of human poisoning by CYN have been documented (Bourke et al. 1983; Carmichael 2001). Among them, the most publicized incident is the one from 1978, called the Palm Island Mystery Disease, which took place in Queensland, Australia. One hundred forty-eight people, mainly children, were hospitalized after drinking water from the Solomon Dam reservoir covered with a bloom of *C. raciborskii* (Bourke et al. 1983). Numerous studies indicate that the CYN molecule is stable under the influence of various abiotic factors present in the natural aquatic environment. This creates a serious problem and may contribute to the disruption of ecological interactions (Chiswell et al. 1999; Wörmer et al. 2010; Adamski et al. 2016a; Adamski et al. 2016b). The fate of CYN in natural water bodies is still unknown. However, it has been demonstrated that the toxin can accumulate in the tissues of invertebrates (Saker & Eaglesham 1999; Saker et al. 2004; Freitas et al. 2016). The number of publications describing the decomposition of CYN under abiotic factors, microbial activities or catalytic reactions and potential methods of its removal from water has increased significantly in recent years (Yan et al. 2016; El-Sheik et al. 2017; An et al. 2019). Nevertheless, information on the toxicity of its decomposition products is limited.

In previous studies, we described the chemical structure of the CYN decomposition products formed under alkaline conditions combined with boiling temperature or UV-B irradiation (Adamski et al. 2016a; Adamski et al. 2016b). The objective of this research was to assess their potential toxicity based on tests with *Thamnocephalus platyurus* and *Vibrio fischeri*, which are commonly used and are particularly sensitive to cyanotoxins (for a review, see De la Cruz et al. 2013; Sieroslawska 2013; Bober & Bialczyk 2017). Due to the impossibility of proper separation and purification of previously described CYN degradation products (similar retention time, very small concentrations, using a destructive technique) on UPLC-MS/MS, we decided to verify the toxicity of the mixture of all produced compounds. According to the literature, this approach is correct, because of the potential synergistic effects of reactive chemical compounds, which in this case should result in increased toxicity.

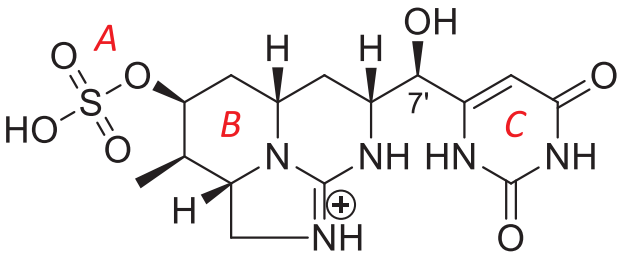


Figure 1

Chemical structure of CYN: A – sulfate group, B – tricyclic guanidine moiety, C – uracil ring

Materials and methods

Cyanobacterial growth conditions and procedure of CYN extraction

Raphidiopsis raciborskii (Woloszynska) Aguilera, Berrendero Gómez, Kastovsky, Echenique & Salerno strain CS-505/7 was cultivated in BG11 medium (Stanier et al. 1971) in a phytotron at 20 ± 1°C. The culture was irradiated with 50 µmol m^{-2} s^{-1} of photosynthetic active radiation (PAR, 400–700 nm) during a 12 h light/12 h dark photoperiod and filtered using GF/C glass fiber filters after 1 month (Whatman, UK). The cellular material was immediately frozen at –20°C and then cyclically thawed and frozen to destroy cell membranes and walls. The cells were further lyophilized, treated with 100% methanol and shaken in darkness until discoloration of the filters. The supernatant was collected and evaporated until a dry residue was obtained. The cyanobacterial extract was

reconstituted on ice with Milli-Q water and centrifuged at 10 000 × g for 5 min. In the next step, the supernatant was filtered using syringe filters (0.22 µm, Merck, Germany) and injected into HPLC. Extraction and purification of CYN from cyanobacterial material were performed as described by Meriluoto and Codd (2005).

Determination of CYN decomposition products

The decomposition of CYN under alkaline conditions combined simultaneously with high temperature or UV-B irradiation was performed as described by Adamski et al. (2016a, b). In short, CYN samples dissolved in buffer at pH 10 were exposed to boiling (100°C ± 1°C) or UV-B irradiation (36 µmol m^{-2} s^{-1}) until complete decomposition of the toxin. The progressive loss of CYN was monitored every half hour using high-pressure liquid chromatography (HPLC) according to the method described by Adamski et al. (2016a, b). In brief, a Waters Atlantis® dC18 column (3.9 × 100 mm, 3 µm) maintained at 35°C was used, and the following gradient elution was applied: from 99 to 88% of water/trifluoroacetic acid (0.05% v/v) for 24 min at a flow rate of 1.0 ml min^{-1}. Eluent B was acetonitrile/trifluoroacetic acid (0.05%, v/v). CYN was quantified using its absorbance at λ = 262 nm. The CYN decomposition products were identified using an ultra-performance liquid chromatography tandem-mass spectrometer (UPLC-MS/MS) coupled with a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole) according to the method developed by Adamski et al. (2016a, b). Chromatographic separation was achieved using an Acquity UPLC BEH (bridged ethyl hybrid) C18 column (2.1 × 100 mm, 1.7 µm) with an Acquity UPLC BEH C18 VanGuard pre-column (2.1 × 5 mm, 1.7 µm) maintained at 40°C under the following conditions: 100% of eluent A for 2 min and a gradient elution from 100 to 30% of eluent A for 10 min at a flow rate of 0.3 ml min^{-1}. Eluent A was water and eluent B was acetonitrile. Both were acidified with formic acid (0.1% v/v). The MS detection settings of the Waters TQD mass spectrometer were as follows: a source temperature of 150°C, a desolvation temperature of 350°C, a desolvation gas flow rate of 600 l h^{-1}, a cone gas flow rate of 100 l h^{-1}, a capillary potential of 3.00 kV, and a cone potential of 20 V. Collision activated dissociation (CAD) analyses were conducted with an energy of 30 eV, and all fragmentations were in the source. The ion spectra were obtained by scanning from 30 to 500 m/z. The concentrations of mixtures of the CYN decomposition products were expressed as the equivalent of the CYN initial concentrations.

Toxicity tests

Evaluation and comparison of the toxicity of CYN and its decomposition products were performed using a commercially available Thamnotoxkit F™ toxicity test and a Deltatox® II analyzer according to the respective standard operational procedures. The Thamnotoxkit F™ assay is based on the determination of the percentage mortality of *Thamnocephalus platyurus* larvae (10 larvae in each test well, 3 replicates) after 24 h of exposure to the studied solutions. Percentage changes in the bioluminescence of the bacterium *Vibrio fischeri* were measured at λ = 490 nm on a portable, self-calibrating Deltatox® II photometer. Liquid-dried bacteria were rehydrated using the Microtox reagent and incubated at 15°C for 15 min. After that time, the initial bioluminescence was measured. In the next step, 10 µl of CYN or its decomposition products were added and the bioluminescence intensity was measured after 15 min of reincubation. The quantitative importance of the toxic effects was calculated as the concentration causing the death of 50% of crustacean larvae (LC_{50}) or 50% inhibition of the bacterial bioluminescence (IC_{50}).

Samples of CYN and its decomposition products were adjusted to neutral pH by 0.1 M HCl and prepared at five concentrations, 0.2, 0.4, 0.75, 1.5 and 3 µg ml^{-1}, by dilution in the standard medium (Thamnotoxkit F™) or Milli-Q water (Deltatox® II). The standard medium and Milli-Q water were used as controls in the Thamnotoxkit F™ and the Deltatox® II, respectively.

Chemicals

The Thamnotoxkit F™ test was purchased from MicroBioTests, Inc. (Gent, Belgium). The Deltatox® II test came from Modern Water, Inc. (New Castle, DE, USA). The commercial standard of CYN was obtained from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water came from Millipore (Bedford, MA, USA). All other reagents were of MS/MS, HPLC or analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Results and discussion

Data obtained from the chromatographic studies (not presented here) and mass spectrometry analyses revealed the presence of two and four decomposition products of CYN formed after the exposure of samples to pH 10 combined with boiling (Fig. 2) or UV-B irradiation (Fig. 3), respectively. In Figure 2, the compound with a retention time (R_t) of 1.90 min represents one of the two diastereoisomers indicated

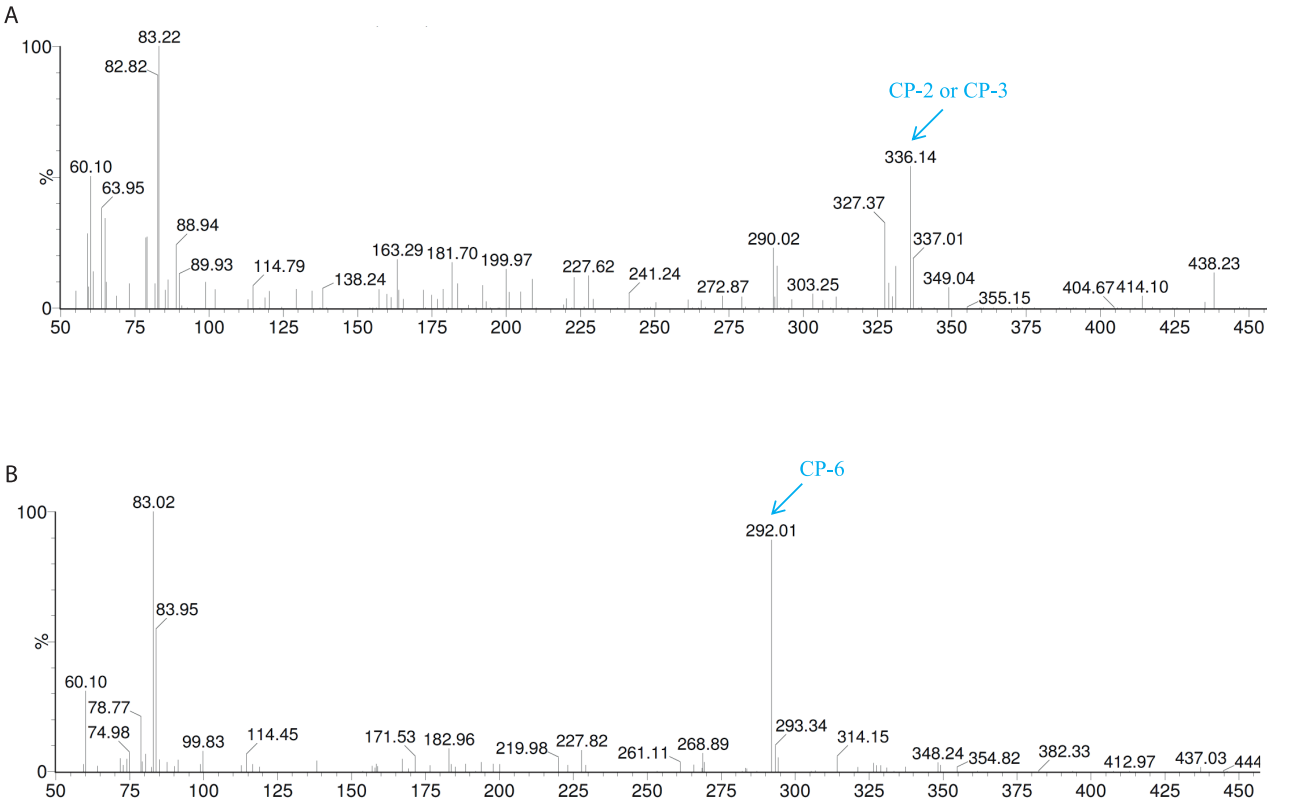


Figure 2

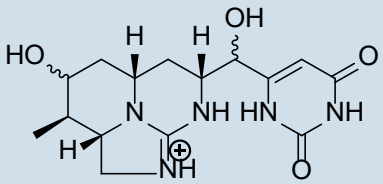
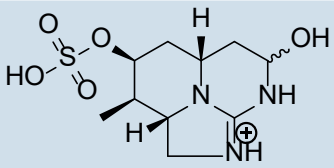
Mass spectra of CYN decomposition products formed under alkaline conditions (pH 10) combined with boiling temperature: A – CP-2 or CP-3, B – CP-6

previously as CP-2 or CP-3, and the substance with an R_t of 3.85 min was identified as CP-6 (Table 1; Adamski et al. 2016a). In Figure 3, in turn, CYN derivatives with an R_t of 1.88 min, 3.87 min, 4.46 min and 4.55 min were labeled as CPI-1, CPI-2, CPI-4 and CPI-5, respectively (Table 2; Adamski et al. 2016b).

The total decomposition of the CYN molecule under both tested conditions resulted in relatively similar compounds. These are the products of separation of the uracil ring (CP-6, CPI-1, CPI-2), the sulfate group (CP-2/CP-3, CPI-4) or both (CPI-5) from the tricyclic guanidine moiety. Recent studies also confirmed the presence of similar CYN decomposition derivatives in uncooked or cooked/steamed muscles of tilapia fish (*Oreochromis niloticus*) (Guzmán-Guillén et al. 2017).

Table 1

Products of total CYN decomposition formed under alkaline conditions (pH 10) combined with boiling temperature

Decomposition products	Retention time [min]	$[M + H]^+$	Fragmentation ions	Proposed structure
CP-2 or CP-3	1.90	336.14	194.1, 176.1	
CP-6	3.85	292.01	212.1, 194.1, 176.1	

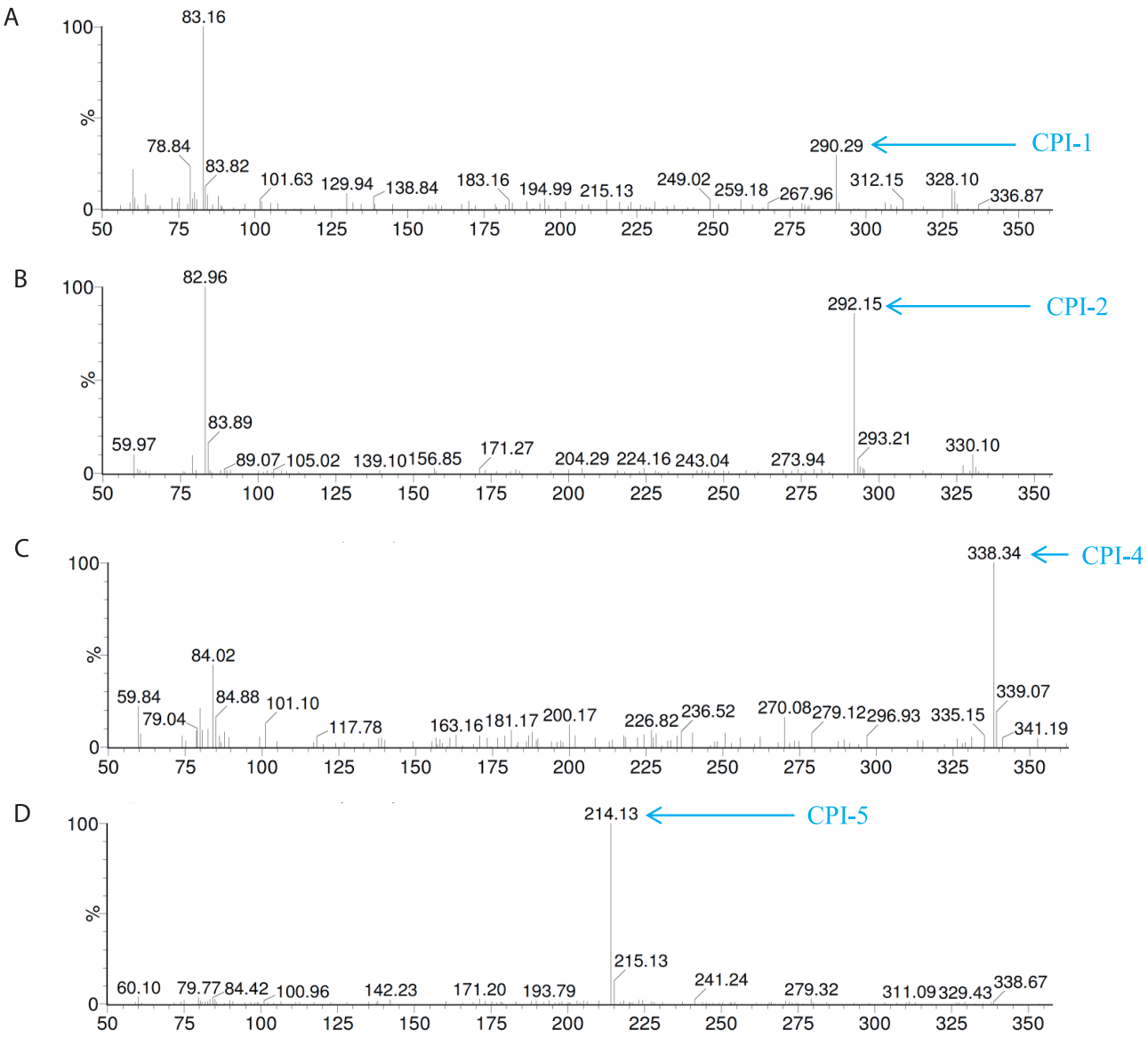


Figure 3

Mass spectra of CYN decomposition products formed under alkaline conditions (pH 10) during UV-B irradiation: A – CPI-1, B – CPI-2, C – CPI-4, D – CPI-5

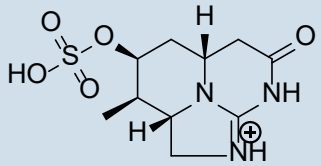
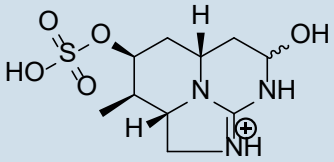
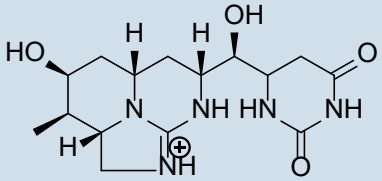
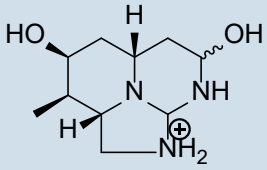
The concentrations of CYN and its decomposition products used in this study reflect the toxin concentrations commonly found in natural water bodies (for a review, see Adamski et al. 2014) as well as its highest concentration ever detected in nature (1.5 µg ml^{-1}; Santos et al. 2015). Moreover, the toxicity of the concentration twice as high as the highest concentration determined for CYN (3 µg ml^{-1}) was analyzed. This allowed a satisfactory comparison of the toxicity of the toxin and the mixture of its derivatives.

The LC_{50} value of CYN for *T. platyurus* larvae was determined to be 0.39 µg ml^{-1}. Sieroslawska (2013) obtained a lower LC_{50} value for the toxin equal to 0.27 µg ml^{-1} in a similar assay. However, none of the examined concentrations of the CYN decomposition products caused larval death (Table 3).

The results obtained by applying the Deltatox® II test, according to the classification described by Hsieh et al. (2004), showed a high sensitivity of *Vibrio fischeri* to the toxin with an IC_{50} dose < 0.2 µg ml^{-1} (Table 3). Inhibition of its bacterial bioluminescence was observed for all tested CYN concentrations (Fig. 4A). On the other hand, exposure of *V. fischeri* to even the highest analyzed concentration of CYN decomposition

Table 2

Products of the total CYN decomposition formed under alkaline conditions (pH 10) during UV-B irradiation

Decomposition products	Retention time [min]	$[M + H]^+$	Fragmentation ions	Proposed structure
CPI-1	1.88	290.29	210.1, 192.1	
CPI-2	3.87	292.15	212.1, 194.1, 176.1	
CPI-4	4.46	338.34	196.1, 178.1, 137.1, 110.1	
CPI-5	4.55	214.13	196.1, 178.1, 137.1, 110.1	

products did not cause any significant changes in the level of bacterial bioluminescence (Figs 4B and C; Table 3).

The presented results confirmed the less toxic character of these derivatives. As a consequence, it can be assumed that they do not pose a threat to the life and health of organisms such as crustaceans and bacteria living in water reservoirs.

Taking into account the less toxic properties of the studied decomposition compounds, our results suggest that the toxicity of CYN is determined by the presence of the tricyclic guanidine moiety, the uracil ring and the sulfate group (Fig. 1), and the structural modification of any of the above-mentioned parts may contribute to the reduction of its toxic properties. In comparison to the CYN decomposition process described in our previous studies (Adamski et al. 2016a,b), its complete breakdown was also associated with the loss of all compounds structurally similar to CYN. The obtained results clearly show that the applied conditions result in the decomposition of CYN to less toxic products. Norris et al. (1999) studied the *in vitro* toxicology of the CYN analogue, 7-deoxy-cylindrospermopsin (7-deoxy-CYN), and found that this compound was not toxic in mouse bioassays. There is no additional hydroxyl group at

Table 3

Toxicity of CYN and its decomposition products assessed with Thamnotoxkit F™ and Deltatox® II tests expressed as the concentration causing death of 50% of crustacean larvae (LC_{50}) or 50% inhibition of the bacterial bioluminescence (IC_{50})

Tested substances	Toxicity tests	
	Thamnotoxkit F™ LC_{50} [µg ml^{-1}]	Deltatox® II IC_{50} [µg ml^{-1}]
CYN	0.39	< 0.2
CYN decomposition products formed under boiling temperature at pH 10	> 3	> 3
CYN decomposition products formed under UV-B irradiation at pH 10	> 3	> 3

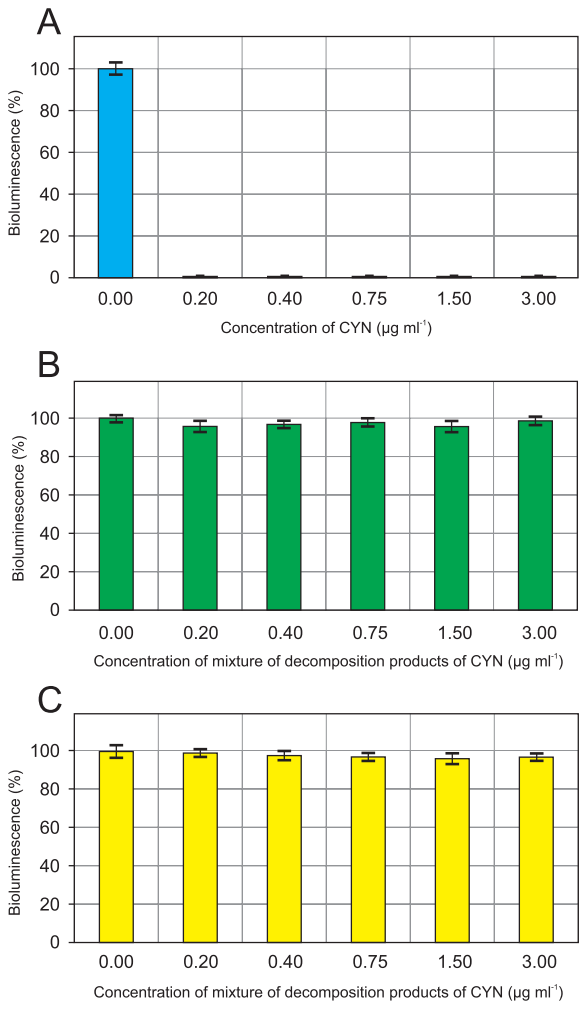


Figure 4

Decrease in bioluminescence observed in the Deltatox® II test: A – pure CYN (aqueous solution), B – mixture of CYN decomposition products formed under alkaline conditions (pH 10) at 100°C, C – mixture of CYN decomposition products formed under alkaline conditions (pH 10) during UV-B irradiation

the C-7 position in 7-Deoxy-CYN, confirming that even small modifications in the chemical structure of CYN significantly change its properties. Information on the toxicity of CYN and the degradation of its molecule in the natural aquatic environment is still limited. Metcalf et al. (2002) indicated that the CYN concentration of 20 µg ml^{-1} did not result in 100% mortality of the brine shrimp *Artemia salina* within 24 h. However, prolonged exposure to 40 h caused the death of all tested organisms. The LC_{50} values ranged from 8.1 to 0.71 µg ml^{-1} after 24 and 72 h of incubation, respectively. Moreover, the toxin was less toxic than the extract from the cells of *C. raciborskii* containing CYN (Metcalf et al. 2002). Chiswell et al. (1999) described that the toxin in cyanobacterial extract is less stable. These results suggest that the toxicity of CYN is complicated and many factors in the natural aquatic environment, such as the presence of photosynthetic pigments and enzymes, could influence its properties. The synergistic interaction of substances, such as other toxins or their decomposition products, also appears to be important (De la Cruz et al. 2013; Pinheiro et al. 2016). In nature, abiotic factors probably only slightly contribute to the decomposition of CYN, because of its high chemical stability. Most likely, the activity of microbial communities may contribute to the significant degradation of CYN in the environment. However, potential products of these reactions are still unknown (Fabbro et al. 2001; Klitzke et al. 2010; Klitzke & Fastner 2012; Mohamed & Alamri 2012).

To date, only some limited information has been reported on the removal of CYN from drinking water in wastewater treatment plants (De la Cruz et al. 2013). The results presented in this study advance the knowledge about the toxicity of CYN decomposition products and may be useful in the design and implementation of water treatment systems.

In many cases, extensive and persistent cyanobacterial blooms contribute to the alkalization of water (Gao et al. 2012). The combination of alkaline pH and UV irradiation affects the amount of CYN in the natural environment. Further studies should focus on the process of CYN degradation in nature and the toxicity of its decomposition products formed under such conditions.

Conclusions

During the complete decomposition of CYN under alkaline conditions (pH 10), combined with high temperature (100°C ± 1°C) or UV-B irradiation (36 µmol m^{-2} s^{-1}), less toxic decomposition products were formed. The obtained results indicate that the modifications of the chemical structure of CYN, such as the separation of the uracil ring or the sulfate group and changes in the guanidine moiety, decrease its toxic properties. Given the limited information available on the fate and toxicology of CYN in the natural aquatic environment, further studies should focus on these issues.

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