

**Stability of cyanotoxins,
microcystin-LR,
microcystin-RR and
nodularin in seawater
and BG-11 medium
of different salinity**

OCEANOLOGIA, 43 (3), 2001.
pp. 329–339.

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Oceanology PAS.

KEYWORDS
Cyanobacteria
Hepatotoxins
Microcystin
Nodularin

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Manuscript received 15 March 2001, reviewed 7 June 2001, accepted 11 July 2001.

Abstract

Microcystins and nodularin are potent hepatotoxins produced by fresh and seawater cyanobacteria. The persistence of three hepatotoxins – microcystin-LR, microcystin-RR and nodularin – was investigated in sterile BG-11 medium of different salinity and in water collected from the Gulf of Gdańsk. After 21 days of incubation at $17 \pm 1^\circ \text{C}$ and constant illumination of about $40 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ the concentration of toxins decreased by about 30–37%. No significant changes in toxin concentration in the BG-11 media of different salinity were observed. When toxins were incubated in non-sterile seawater, their concentrations decreased markedly. It is likely that some strains of bacteria are responsible for the breakdown of the toxins. Nodularin turned out to be more resistant to biodegradation than the two microcystins. The influence of certain components of cyanobacteria cells on the accelerated rate of toxin degradation was also considered.

1. Introduction

Microcystins are a group of monocyclic heptapeptide hepatotoxins produced by various freshwater cyanobacteria such as *Microcystis*, *Anabaena*, *Nostoc* and *Oscillatoria* species (Carmichael 1992). The general structure of microcystins is cyclo[-D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha-]. Apart from two variable L-amino acids, X and Z, microcystins consist of three D-amino acids: alanine (Ala), methylaspartic acid (MeAsp) and glutamic acid (Glu), and two unusual amino acids: *N*-methyldehydroalanine

(Mdha) and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda). The Adda amino acid is responsible for the biological activity of the toxins. Microcystins differ primarily in the two L-amino acids, X and Z, which give its name to the molecule. There are more than 60 microcystin variants, and toxic strains usually produce a mixture of different microcystins (Sivonen & Jones 1999). Microcystin-LR, with leucin L and arginin R residues, is the most common one. The structurally similar pentapeptide hepatotoxin, cyclo[-D-MeAsp-L-Arg-Adda-D-Glu-Mdhb-], where Mdhb stands for 2-(methyl-amino)-2-dehydrobutyric acid, was found in *Nodularia spumigena* and named nodularin after its producer. Owing to the production of these hepatotoxins the occurrence of aquatic blooms of cyanobacteria is of worldwide concern. Hepatotoxins, microcystins and nodularin, act as inhibitors of protein phosphatase 1 (PP 1) and 2A (PP 2A) and are potent liver tumour promoters in rats (MacKintosh et al. 1990, Fujiki et al. 1995). Nodularin has proved to be not only a tumour promoter but a tumour initiator as well (Ohta et al. 1994). Potable water supplies and recreational waters with a possible content of toxic cyanobacteria or their metabolites pose a potential threat to those in contact with them. The incidents of wild and domestic animal poisoning and human health problems attributed to exposure to cyanobacterial toxins have been well documented (Nehring 1993, Bell & Codd 1994, Harding et al. 1995, Mez et al. 1997, Pliński & Codd 1997, Pouria et al. 1998, Codd et al. 1999).

Hepatotoxins are synthesized and retained in cyanobacteria cells but during bloom senescence and cell lysis they are released into the surrounding water. Furthermore, some water treatment processes cause cyanobacteria cell disruption and, in consequence, raise the toxin concentration in the reservoir. Investigations into the stability of these toxins are indispensable to determine how long after the cyanobacterial bloom the water constitutes a threat to all potential users. Microcystins and nodularin are chemically stable (Harada 1995, Harada et al. 1996) and, if not diluted, can persist in water for several days or weeks after the bloom (Lahti et al. 1997). Jones & Orr (1994) observed that after algicide treatment of a heavy bloom of *Microcystis aeruginosa*, the released microcystins persisted in the water for 9 days and then rapidly disappeared. The possible routes of microcystin detoxification were discussed by Harada (1995), who suggested that the microcystin concentration in water may decrease as a result of such processes as dilution by uncontaminated water masses, adsorption on particulate material, temperature and pH-dependent decomposition, photolysis and biological degradation. The laboratory experiments of Cousins et al. (1996) demonstrated that primary degradation of microcystin-LR in reservoir water occurred in less than one week. On the other hand, microcystin-LR

was stable in de-ionized water for over 27 days and in sterilized reservoir water for over 12 days. These results indicated the possibility of microcystin biodegradation in natural waters which, according to the authors, may proceed via the disruption of the conjugated diene system in the Adda side chain. Since Adda is essential to the toxicity of microcystins, the biodegradation product appeared to be less toxic compared to the intact compound. Takenaka & Watanabe (1997) tested several species of bacteria isolated from a Japanese lake and found that, through the activity of alkaline protease, *Pseudomonas aeruginosa* was able to degrade microcystin-LR. Microcystin-LR was also found susceptible to breakdown by *Sphingomonas*, which initiated ring-opening and the production of a linear compound some 200 times less toxic (Bourne et al. 1996). Although these bacteria did not decompose nodularin, biodegradation of nodularin by other strains of bacteria cannot be ruled out.

Microcystins remain stable on irradiation by sunlight; however, the presence of pigments in cyanobacteria cells led to photosensitization and decomposition as a result of the isomerization of a double bond in the Adda chain (Tsuiji et al. 1994). Investigations into the stability of microcystins under UV radiation were carried out; they showed that the toxins were readily decomposed by UV light at the wavelength corresponding to the absorption maxima of the toxins (Tsuiji et al. 1995). The decomposition proceeded by isomerization, and the several photolysis products obtained were not toxic. The influence of humic substances on indirect photochemical degradation of microcystins in natural sunlight has also been investigated (Welker & Steinberg 1999).

Twist & Codd (1997) examined the rate of nodularin degradation under different light conditions. The experiments revealed that in aqueous solution, regardless of the light conditions (darkness, full sunlight, sunlight minus UV wavelengths), nodularin was stable over the 9-day experiment. However, when *N. spumigena* extract was added, the concentration of nodularin decreased significantly. It was thought that this might be due to the presence of some enzymes capable of metabolizing the toxin. The rate of nodularin decomposition, higher in sunlight than in darkness, indicated that the mechanism of the process was photo-oxidative.

The aim of this paper was to assess whether the salinity of the medium could have any influence on the stability of microcystin-LR, microcystin-RR and nodularin. The effect of sterilizing the medium as well as the presence of compounds extracted from cyanobacteria along with the toxins are also reported in this study.

2. Material and methods

Microcystin-LR, microcystin-RR and nodularin standards were purchased from Calbiochem – Novabiochem (La Jolla, CA, USA). As an alternative, microcystin-LR and nodularin were obtained from *M. aeruginosa* PCC 7820 and *Nodularia* sp. PCC 7804 respectively. To isolate the toxins, cyanobacteria cells were harvested during the stationary phase by centrifugation. The dry cells were extracted in 75% methanol by sonication in an ultrasonic bath and continuous stirring for 2 hours at room temperature. The extract was centrifuged, after which methanol was removed from the supernatant by rotary evaporation to the water residue at 35° C. The liquid concentrate was filtered through a Whatman GF/C filter and purified by solid phase extraction (SPE) on Sep-Pak C18 cartridges according to the procedure described below (see: Solid Phase Extraction).

The experiments were conducted in 250 cm³ conical flasks stoppered with bacteriological plugs. 20 µg of toxin standard or the toxin containing extract was added to 100 cm³ of sterilized BG-11 medium of different salinity (0, 4, 8, 16 and 24 PSU), or to water collected from the Gulf of Gdańsk and filtered through a GF/C filter. The required salinity was obtained by adding sodium chloride to the BG-11 medium and then checked with a ProfiLine conductometer (WTW GmbH, Weilheim, Germany). The flasks were maintained for 21 days at 17 ± 1° C under constant illumination of about 40 µmol photon m⁻² s⁻¹. The concentration of toxin in a sample was determined on the 1st, 7th, 14th and 21st day of the experiment. For each analysis 20 cm³ of the media was taken and subjected to solid phase extraction.

3. Solid phase extraction and HPLC

The SPE procedure involved activation of the 500-mg Sep-Pak tC₁₈ cartridge (Waters, Milford, MA, USA) by passing 10 cm³ of methanol through it, followed by 10 cm³ of MilliQ water from an ultra-pure water system (Millipore, Bedford, MA, USA). The aqueous sample was then passed at a flow rate of about 5 cm³ min⁻¹. The cartridge was washed with 10 cm³ of MilliQ water and the toxins were eluted with 5 cm³ of 100% methanol. The solvent was evaporated to dryness in vacuo at 35° C and the residue dissolved in 1 cm³ of 100% methanol for analysis with the Waters HPLC system. A model 626 pump with a model 600S controller, a Rheodyne 9125 injector with a 20 µl loop and a 996 photodiode-array detector (PDA) operating in the 200–300 nm range were used. All data were collected and processed using Waters Millennium software. Toxins were identified by their retention time and characteristic absorption spectra with a maximum at

238 nm. Separations were performed on a LiChrospher 100 RP-18 column (25 cm × 0.4 cm I.D., 5 μm particle size) and a 100 RP-18e LiChroCart cartridge (Merck, Darmstadt, Germany) with a 30-min linear gradient of 30–60% aqueous acetonitrile containing 0.05% TFA. The flow-rate was maintained at 1 cm³ min⁻¹. All solvents were of HPLC grade. Quantitative analysis of the toxins was carried out using a calibration curve based on peak area measurements for standard solutions.

4. Results and discussion

Many species of cyanobacteria, including those normally living in brackish and fresh waters, are capable of growth and bloom formation over a wide range of salinity (Reed & Stewart 1985), and their expansion and adaptation to new geographical regions is therefore quite probable. Moreover, in the case of some species of cyanobacteria, salinity was found to be one of the most important factors influencing the production of toxins (Blackburn et al. 1996, Hobson et al. 1999). The objective of this work was to determine whether this factor can modify microcystins and nodularin stability in the environment.

Seven BG-11 media of salinity 0, 4, 8, 16 and 24 PSU were used. To examine the rate of toxin decomposition, the peak areas of the toxin on the HPLC chromatogram obtained at the beginning of the experiment and after one, two and three weeks were compared. On a given day of the

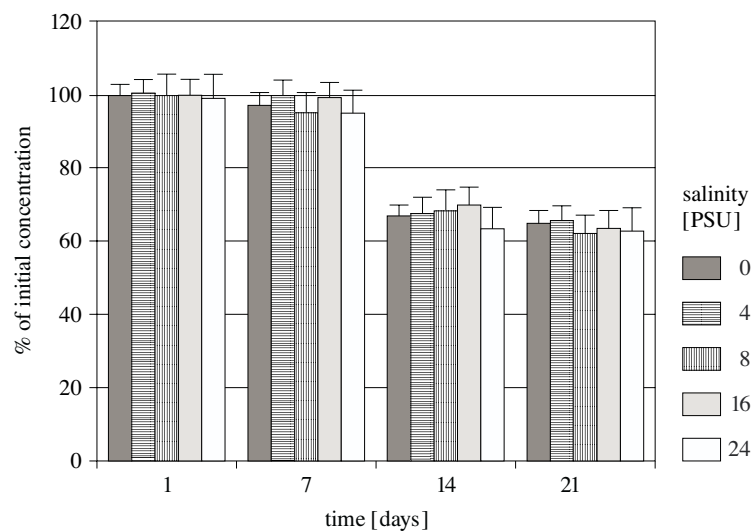


Fig. 1. Changes in microcystin-RR concentration in BG-11 medium of different salinity (0, 4, 8, 16, 24 PSU)

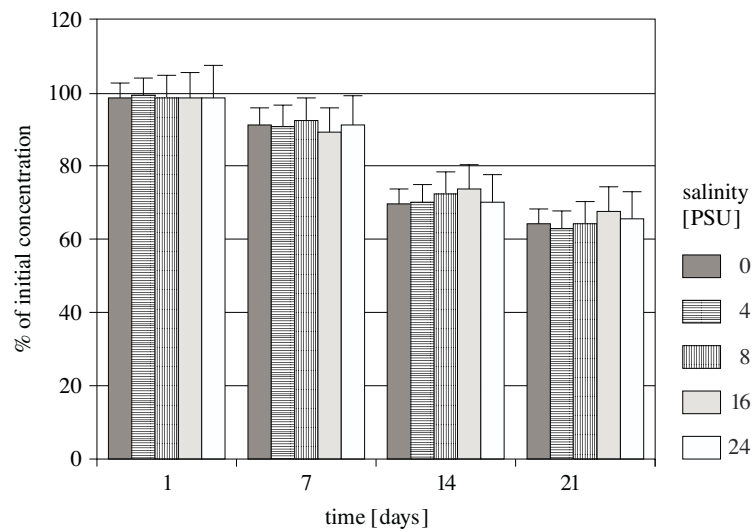


Fig. 2. Changes in microcystin-LR concentration in BG-11 medium of different salinity (0, 4, 8, 16, 24 PSU)

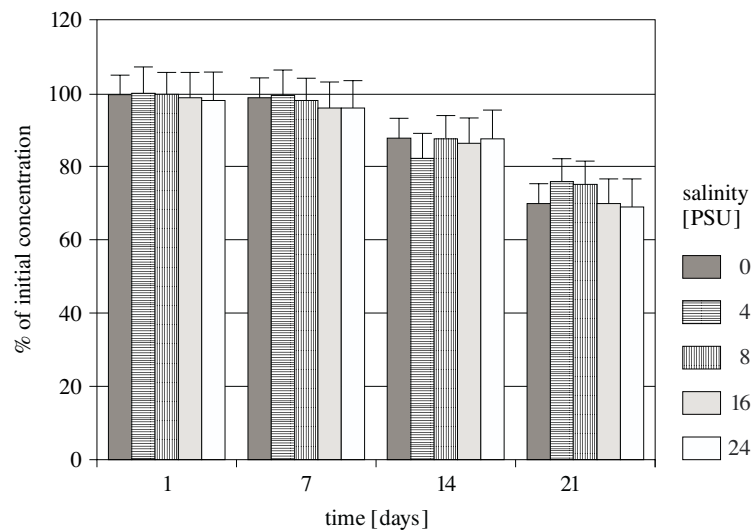


Fig. 3. Changes in nodularin concentration in BG-11 medium of different salinity (0, 4, 8, 16, 24 PSU)

experiment, the discrepancies in toxin recoveries from media of different salinities did not exceed 5.5% and were within the range of relative standard deviation of the results in replicate measurements (3.3–7.5%). Therefore, it can be concluded that the stability of the three toxins does not depend on

the salinity of the media. At all salinities, over the first 7 days of exposure to light intensity of about $40 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ and temperature of $17 \pm 1^\circ \text{C}$, the concentrations of toxins did not change significantly (Figs. 1, 2 and 3). During the next two weeks they decomposed slowly to over 60% of the initial concentration in the case of microcystin-LR and -RR, and to over 70% in the case of nodularin. These results support the previous findings of Jones & Orr (1994), Tsuji et al. (1995), Cousins et al. (1996) and Twist & Codd (1997), who showed that microcystins and nodularin are chemically stable. In sterilized medium they can persist for several weeks or months without any change in their chemical structure and toxicity.

In another experiment, we examined the degradation rate of the three toxins diluted in seawater taken from the Gulf of Gdańsk. The water was passed only through a GF/C filter and used unsterilized. The results (Fig. 4) indicate a toxin degradation that is rapid when compared with the sterile BG-11 medium. During the course of the 21-day experiment, the concentration of microcystin-LR, microcystin-RR and nodularin was reduced to 18.7, 9.5 and 31.2% of the initial concentration respectively. Additionally, discrepancies in the rate of degradation between individual toxins were observed. While on day 7 the concentration of microcystin-LR decreased to 42.8% of the initial value, the concentrations of microcystin-RR and nodularin decreased to 80.2 and 88.6% respectively. The lesser persistence of cyanotoxins in the unsterilized medium can be

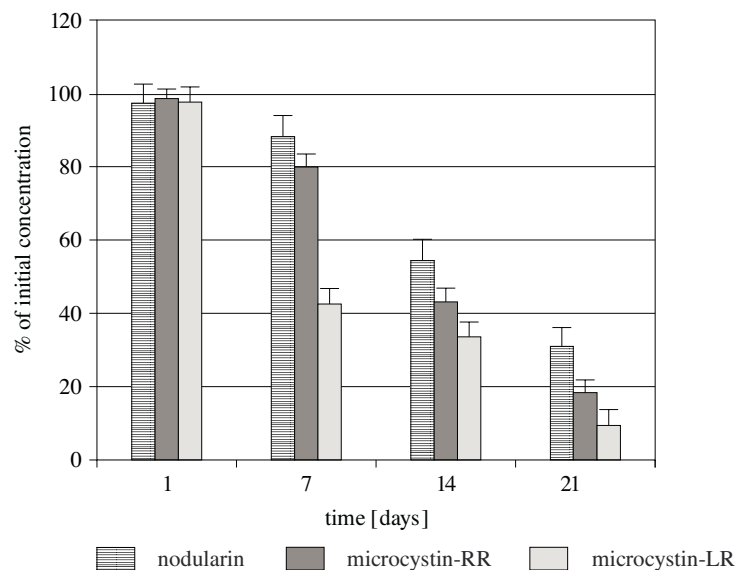


Fig. 4. Decrease in the content of cyanotoxins in the water taken from the Gulf of Gdańsk (non-sterile medium)

attributed to the presence of bacteria thought to be responsible for the biodegradation of the compounds (Jones & Orr 1994, Cousins et al. 1996). We did not identify the bacteria responsible for the breakdown of the toxins, but strains capable of decomposing both microcystins and nodularin were probably present in the seawater used in our experiment. However, the results indicated that under the experimental conditions nodularin was more resistant to biodegradation than microcystins. Cousins et al. (1996) reported that microcystin-LR degradation in reservoir water occurred after 13 days. When bed sediments were added to reservoir water, toxin degradation reached completion after only 6 days. According to these authors, the higher rate of toxin degradation was related to the increase in microbial population in the sample. It is to be expected that in laboratory experiments as well as in natural waters the rate of toxin degradation will depend on the type and size of microbial population occurring there. If this is the case, the results of studies on the persistence of cyanotoxins in various aquatic systems may be difficult to compare.

In the last experiment, extracts of *M. aeruginosa* PCC 7820 and *Nodularia* sp. PCC 7804, which contained microcystin-LR and nodularin respectively, were used instead of the commercial reagents. As can be seen from Fig. 5, the concentration of both toxins gradually decreased in BG-11 medium and during 21 days of the experiment fell below the detection limit of the analytical method. This accelerated rate of degradation

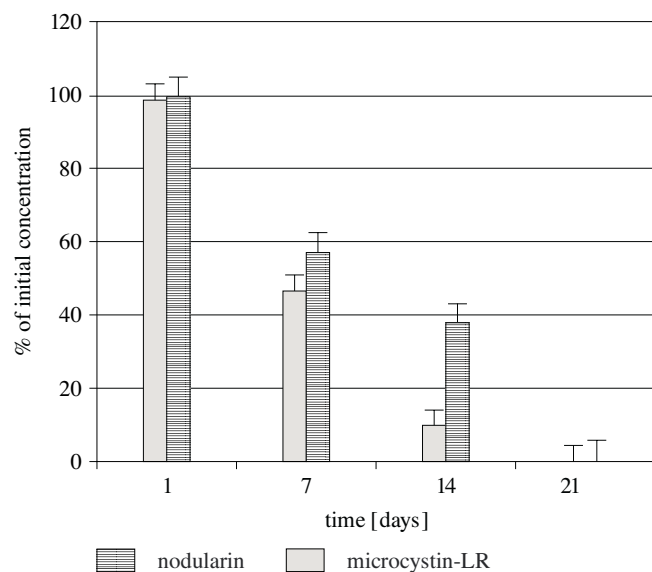


Fig. 5. Decrease in the concentration of microcystin-LR and nodularin isolated from cyanobacteria cells in the BG-11 medium

may be related to the presence of some cell components extracted from cyanobacteria together with the toxins. Twist & Codd (1997) reported a light-dependent decrease in concentration of the nodularin extracted from *N. spumigena* cells. This effect was attributed to the presence of some nodularin metabolizing or degrading enzymes. According to Tsuji et al. (1994), microcystin-LR underwent photochemical breakdown in full sunlight to non-toxic derivatives. In the presence of cell pigments the process was accelerated and in two weeks, more than 90% of the toxin decomposed. It was also postulated by other authors (Feitz et al. 1999, Welker & Steinberg 1999) that such components of the cyanobacteria cell extract as chlorophyll *a*, β -carotene and xanthophylls contribute to the photosensitized degradation of microcystins, which proceeds by isomerization to a compound with a much weaker tumour-promoting activity.

So far, most of the work on the degradation of cyanobacterial hepatotoxins has been done on microcystin-LR and only a few studies on other microcystins and nodularin are available. The processes of toxin degradation, either by breakdown of one of the peptide bonds and ring opening, or by isomerization of the Adda side chain, result in the detoxification of water bodies. To be able to assess properly the health risk associated with the use of contaminated waters, our knowledge about the persistence of the hepatotoxins in aquatic systems should be more extensive.

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