Degradation of the cyanobacterial hepatotoxin, nodularin, under light and dark conditions

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Abstract

The stability of the cyanobacterial hepatotoxin, nodularin, was determined during the incubation of purified toxin, and in nodularin-containing cell-free extracts and whole filaments of the nodularin-producer, Nodularia spumigena in sunlight and darkness. Levels of purified nodularin in aqueous solution remained approximately constant throughout the 9-day trials under all conditions, but decreased in cell-free extracts and whole filaments when incubated under all conditions, with losses being greatest in full sunlight, intermediate in sunlight minus ultraviolet wavelengths and lowest in continuous darkness. Photodegradation and detoxification in Artemia salina bioassays occurred when purified nodularin was irradiated with ultraviolet wavelengths using a laboratory lamp.

Keywords: Cyanobacteria; Nodularia; Nodularin; Toxin; Hepatotoxin

1. Introduction

Toxin production is a common characteristic of cyanobacterial blooms in fresh-, brackish- and marine waters [1]. The toxins, including the nodularin and microcystin hepatotoxins, present short- and long-term hazards to human and animal health [2,3]. Understanding of the regulation of toxin production and persistence is necessary for the protection and safe human use of waterbodies which are prone to cyanobacterial bloom development [1,4,5]. The deaths of farm animals and domestic dogs from hepatotoxicosis after ingesting Nodularia spumigena have been reported for many years (e.g. [6,7]) and can be attributed to nodularin, a potent hepatotoxin which is produced by this filamentous cyanobacterium [8,9].

Nodularin is a pentapeptide (mlz 824 Da) with the structure cyclo (d-erythro-β-methylaspartic acid-L-arginine-Adda-N-glutamic acid-N-methyldehydrobutyryne) [8]. Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.

Surface scums of buoyant N. spumigena filaments and colonies can be produced in lakes and estuaries under calm weather conditions [10] and can be concentrated along leeward shorelines by gentle wind action. A consequence of scum formation is to concentrate the toxigenic cyanobacteria many-fold, so that a lethal oral dose of toxin can be presented to
an animal in a volume below its daily water requirement [11]. Cyanobacterial scums also present substantial hazards to human water contact activities [12]. However, scum formation also exposes cyanobacterial cells and their contents to conditions, namely high light and oxygen levels and low inorganic carbon concentrations, which favour photoinhibition and photooxidative damage [13]. Cyanobacterial scum breakdown and cell lysis eventually occur when scums are stranded on shorelines. In order to investigate the persistence of nodularin under such conditions, we have exposed the toxin in purified form in water, and in intact and broken suspensions of the nodularin-producer *N. spumigena* cells to full sunlight. Further exposures to sunlight minus the UV component, and to UV light from a laboratory source are also described.

2. Materials and methods

2.1. Organism and growth conditions

*Nodularia spumigena* strain UD15, isolated from the brackish water Barrow Ski Club Lake, Lincolnshire, England [14], was grown at 20 ± 4°C in 8 l of modified Z8 medium [15] at 7%o (w/v) salinity (NaCl). Vessels were sparged with filter-sterilized air at about 6 l min⁻¹ under white fluorescent light at an irradiance incident on the growth vessels of about 50 µmol m⁻² s⁻¹. Cells were harvested from 6 l of late growth phase to early stationary phase of batch culture, by filtration through two layers of muslin, and resuspended for exposure trials in 3 l of Milli-Ro quality water containing 10% (v/v) Z8 medium at 7%o salinity (NaCl).

2.2. Preparation of broken cell suspensions

Cells from 6 l, harvested as in Section 2.1, were lyophilized. 3 g dry wt were resuspended in 100 ml water and broken by ultrasonication at full power using an MSE Soniprep (10 × 30 s pulses, with intermittent cooling in ice). Remaining intact filaments and detached cells were removed by bench centrifugation at 1500 rpm for 30 min and the supernatant diluted to 3 l with water.

2.3. Extraction and purification of nodularin

Lyophilized *N. spumigena* UD15 cells (5 g) were added to 100 ml aqueous acetone (50% v/v) and magnetically stirred at room temperature for 1 h. The suspension was centrifuged (4000 rpm for 10 min), the supernatant retained and the pellet re-extracted as before. The pooled supernatants were acidified to 0.1% trifluoracetic acid (TFA, v/v), filtered (Whatman GF/C), and diluted with water to 21. The cyanobacterial extract was then passed through four 1 g Sep-Pak C₁₈ Environmental cartridges (Waters Chromatography Millipore), which had previously been conditioned by passing through 20 ml methanol followed by 40 ml water, at about 2 ml min⁻¹. Nodularin was eluted from the cartridges by applying a 5% incremental gradient of aqueous acetonitrile in 20 ml volumes. The fractions were examined by high performance liquid chromatography (HPLC) [16]. Fractions containing nodularin were pooled and rotary evaporated at 45°C in vacuo to dryness, resuspended in 200 µl methanol (HPLC grade, Rathburn), identified and quantified as nodularin using analytical HPLC with diode array detection (DAD). Equipment consisted of a Waters 600E pump assembly, a 991 DAD set to scan over 200–299 nm and a 717 WISP autosampler. The column was a C₁₈ µ Bondapak (3.9 mm internal diameter × 300 mm), maintained at 40°C. The elution solvents were water plus 0.05% (v/v) TFA and acetonitrile plus 0.05% TFA. Separation of the components was achieved using a 30–35% acetonitrile gradient over 10 min. Quantification of nodularin was by integration of the peak area and comparison with that of a known amount of nodularin [16].

2.4. Outdoor exposure conditions

Exposure experiments were carried out in September 1994 on the laboratory roof, in the type of weather conditions commonly experienced during autumn, when cyanobacterial blooms and scums occur in the UK. Temperatures ranged from 12 to 15°C and the photon flux density (400–700 nm) ranged from 40 (dull) to 180 (sunny) µmol m⁻² s⁻¹. Glass beakers containing 500 ml of aqueous purified nodularin, nodularin plus cell lysate, or whole cell suspensions were weighed and placed in
Fig. 1. Levels of nodularin during incubation at ambient temperature (12-15°C): (a) purified nodularin in aqueous solution; (b) cell-free extracts of *Nodularia spumigena* UD15; (c) whole filaments of *N. spumigena* UD15. Exposure conditions: ■—■, continuous darkness; △—△, full sunlight; ○—○, sunlight minus UV wavelengths (light ≥ 400 nm). Values are the mean of duplicate determinations which did not vary more than 10% from the mean.

open topped glass tanks of dimensions 1 m×30 cm×30 cm. These were situated in the open air, in direct sunlight. During infrequent periods of rain during daylight, and routinely at night, the surfaces of the tanks were covered in plastic sheeting which transmits wavelengths above, but not below 250 nm. The tanks were partially filled with water in order to increase the humidity, reduce evaporation of the open experimental samples, and to even out short-term temperature fluctuations otherwise experienced in the 500 ml samples. Each tank held duplicate test solutions, with three types of exposures being investigated over 9 days: full sunlight; wavelengths above 400 nm (achieved by placing a sheet of glass which screened out UV wavelengths above the tank); and continuous darkness (tank covered by black plastic). Evaporation of water from the test samples was measured by weighing the beakers at 3-day intervals to calculate weight loss not due to sample removal.

For the analysis of purified nodularin in aqueous
solution, 20 ml samples were taken, frozen to $-20^\circ$C and lyophilized directly. For nodularin determination in samples containing cell-free *Nodularia* extracts, 20 ml samples were filtered through a Whatman GF/C filter and the filtrate frozen to $-20^\circ$C and lyophilized. Twenty ml samples of whole cell treatments were GF/C-filtered and the filter discs and filtrates frozen and lyophilized to permit analysis of intracellular and extracellular nodularin pools, respectively. All lyophilized samples were resuspended in 1 ml methanol prior to HPLC analysis with DAD.

2.5. Laboratory exposure of nodularin to UV

Purified nodularin in Milli-Ro-deionized water (120 µg ml$^{-1}$) was placed in quartz cuvettes (path-length 1 cm) and exposed to UV radiation using a 19 W Hanovia tube (UVλ emission max., 254 nm). The cumulative UV dose incident on the surface of the cuvette (mJ cm$^{-2}$) was calculated from $E \times t$, where $E$ was lamp intensity (1 mV m$^{-2}$) and $t$, the exposure time (seconds). The absorbance of triplicate samples of nodularin was measured after different exposure times at 238 nm, the $\lambda_{\text{max}}$ for aqueous nodularin [8,16] and at 300 nm.

2.6. Brine shrimp toxicity assay

Aqueous samples of UV-exposed and -non-exposed purified nodularin were assessed using the brine shrimp (*Artemia salina*) bioassay [17], concentrations of nodularin solution required to cause 50% mortality ($LC_{50}$) being determined.

3. Results

3.1. Exposure to sunlight

Levels of purified nodularin in aqueous solution did not vary significantly over 9 days of diel exposure to full sunlight, sunlight minus UV ($\geq$400 nm) or constant darkness (Fig. 1a). However, when a cell-free filtrate of ultrasonicated *N. spumigena* cells was included, nodularin levels decreased under all conditions (Fig. 1b). No loss of nodularin occurred within the first 2 days in continuous darkness, although a decline in toxin levels was detected after 72 h. Nodularin levels in the dark after 9 days (216 h) were about 55% of those at zero time and an overall loss rate of about 0.1 µg ml$^{-1}$ day$^{-1}$ occurred.

Higher losses of nodularin occurred when exposed with cell-free filtrate to diel sunlight, no 48-h lag being apparent in contrast to dark-maintained toxin and filtrate (Fig. 1b). The highest losses of nodularin (about 95% over 9 days) occurred in the full sunlight exposures, toxin levels decreasing from 2 to about 1.35 µg ml$^{-1}$ over the initial 2 days. Loss rates in full sunlight thereafter were similar to those in continuous darkness. Exposure of nodularin to sunlight

Fig. 2. Reduction in concentration of purified nodularin in aqueous solution (initial concentration 120 µg ml$^{-1}$) to UV light (Hanovia lamp, emission max 254 nm) indicated by reduction of $\lambda_{\text{max}}$ (238 nm) of nodularin peak measured by HPLC-DAD (–––). ○–○, concomitant increase in absorbance at 300 nm. Values are the means of 3 determinations; standard deviations were $\leq 10\%$ of mean values.)
minus UV (i.e. to light ≥400 nm) resulted in an intermediate overall loss over the 9-day trials, about 30% of the initial toxin level remaining (Fig. 1b). As with the full sunlight exposures, no initial lag was observed in the onset of nodularin loss when exposed to minus UV.

Exposure of whole filaments of \( N\text{. spumigena} \) also resulted in nodularin losses over the 9-day exposures (Fig. 1c). Toxin losses in dark-maintained cultures were not apparent within the first 24 h, although an approximately constant loss rate then occurred resulting in about an overall 55% reduction in toxin, as also occurred with nodularin incubated with dark-maintained cell extracts. No nodularin was detected in the extracellular medium throughout cell incubation in the dark. When cells were exposed to full sunlight, or to sunlight minus UV, a loss of about 75% of the intracellular pool of nodularin was recorded after 24 h (Fig. 1c). This was largely accompanied by a transient detection of nodularin in the extracellular phase (aqueous filtrate), indicating partial release of the toxin from the cells, rather than initial toxin-photodegradation in vivo. Intracellular nodularin levels thereafter remained constant in the light at a residual 25% of initial concentrations (Fig. 1c), whilst the extracellular pool decreased within 24 h in the presence of full sunlight or sunlight minus UV to below detection limits (data not shown; 10 ng on HPLC column, 400 ng sample\(^{-1}\)).

### 3.2. Laboratory exposure to UV

A dose-dependent decrease in purified nodularin concentrations in aqueous solution occurred in response to exposure to UV wavelengths, as indicated by a decrease in absorbance at 238 nm (nodularin \( \lambda_{\text{max}} \)) (Fig. 2) and reduction in nodularin peak height and area in HPLC. This was accompanied by an increase in absorbance at 300 nm (Fig. 2), and of additional peaks on HPLC with retention times of 12.13 and 14.26 min (retention time for nodularin: 13.43 min). The UV-dependent decrease in nodularin levels also resulted in a decrease in acute toxicity in the 16-h brine shrimp bioassay, i.e. from a \( \text{LC}_{50} \) value of 3.3 µg ml\(^{-1}\) for nodularin standard to no detectable toxicity in the samples exposed to a UV dose of 2100 mJ cm\(^{-2}\).

### 4. Discussion

Microcystin-LR, which is subject to biodegradation by aquatic bacteria is a stable molecule, toxin levels being unchanged after storage in sterilized aqueous solution for more than one year [18,19]. The long-term stability of nodularin has not been reported, although toxin levels were approximately constant in the dark at ambient temperatures throughout the present 9-day studies. These results indicate that the persistence of nodularin in aqueous systems is influenced by other components of the toxin-producer cells and by light. In the 9-day outdoor exposure trials used here, levels of purified nodularin in water did not fall significantly unless \( N\text{. spumigena} \) cell-free extract was present. In the latter case, the decrease in toxin levels in continuous darkness (Fig. 1b) may be due to the activity of nodularin-metabolising or -degrading enzymes. Microcystin-LR degradation by mixed and single strains of bacteria has been found when toxin samples are enriched with environmental material containing mixed microbial populations [18,19], although the bacterial strains tested for their ability to degrade nodularin, did not do so [19]. The possibility that bacterial degradation was involved in the loss of nodularin reported here is not excluded since the exposures were carried out in open systems, but this is thought to be unlikely without a prior enrichment programme. A second, and more likely possibility is that the cell-free extracts of \( N\text{. spumigena} \) may contain (a) nodularin-metabolizing/degrading enzymes(s).

In addition to the possibility of an enzymic/metabolic mechanism of nodularin breakdown, the increased losses of nodularin in sunlight, versus continuous darkness (Fig. 1b, c), indicate a contribution of photodynamic and/or photooxidative processes. Rates of decomposition and isomerisation of microcystin-LR in the light are accelerated by the addition of chlorophyll \( a \) and \( \beta \text{-carotene} \) [20], indicating photosensitized reactions in the breakdown of this toxin. Nodularin losses in vitro were constantly greater in full sunlight (UV plus visible wavelengths) than in sunlight from which UV was excluded (Fig. 1b). This indicates that the photodegradation of nodularin was proceeding via pigment-photosensitized reactions driven by visible light [13] and that UV was
also involved. Although nodularin absorbs UV (Amax 238 nm) and irradiation of the pure toxin with a UV lamp caused nodularin losses (Fig. 2), no reduction in nodularin level was attributable to UV when the pure toxin was incubated in full sunlight minus added photosensitizers, or when whole *N. spumigena* filaments were exposed to full sunlight. This may be due to insufficient levels of UV available during the exposure period and/or the presence of UV-protective compounds in *N. spumigena*.

Cyanobacterial toxin-producing blooms are increasing globally in occurrence, population density and extent. For example, *N. spumigena* blooms, dominant in the Baltic Sea, are intruding into new areas [21]. The phenomena of cyanobacterial toxin biodegradation, metabolism and photodegradation are an area where increased knowledge may find applications. Understanding the lifespan of cyanobacterial toxins, their susceptibility to degradation procedures and the toxicology of their metabolic and breakdown products has implications for the nature conservation, water supply and recreation sectors, where waterbody management and water treatment are necessary for conservation and the protection of animal and human health.

References