APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1989, p. 1990–1995
0099-2240/89/081990-06$02.00/0

Vol. 55, No. 8

Occurrence of the Hepatotoxic Cyanobacterium *Nodularia spumigena* in the Baltic Sea and Structure of the Toxin

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Received 17 January 1989/Accepted 23 May 1989

Water blooms formed by potentially toxic species of cyanobacteria are a common phenomenon in the Baltic Sea in late summer. Twenty-five cyanobacterial bloom samples were collected from open and coastal waters of the Baltic Sea during 1985 to 1987, and their toxicity was determined by mouse bioassay. All of 5 bloom samples from the southern Baltic Sea, 6 of 6 from the open northern Baltic Sea (Gulf of Finland), and 7 of 14 Finnish coastal samples were found to contain hepatotoxic cyanobacteria. *Nodularia spumigena* and *Aphanizomenon flos-aquae* occurred together in high amounts in blooms from the open-sea areas. In addition, coastal samples contained the species *Anabaena lemmermannii*, *Microcystis aeruginosa*, and *Oscillatoria agardhii*. Eighteen hepatotoxic *N. spumigena* cultures were isolated from water bloom and open-sea water samples. High-pressure liquid chromatographic analysis of both hepatotoxic bloom samples and *Nodularia* strains showed a single toxic fraction. The toxin concentrations of the blooms were ≤2.4 mg/g of freeze-dried material, and those of laboratory-grown cultures were 2.5 to 8.0 mg/g of freeze-dried cells. A single toxin was isolated from three *N. spumigena*-containing bloom samples and three *N. spumigena* laboratory isolates. Amino acid analysis and low- and high-resolution fast-atom bombardment mass spectroscopy indicated that the toxin from all of the sources was a cyclic pentapeptide (molecular weight, 824) containing glutamic acid, β-methylaspartic acid, arginine, *N*-methyldehydrobutyrine, and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid. This indicates that the Baltic Sea toxin is nodularin, the cyclic pentapeptide produced by field and laboratory-cultured *N. spumigena* previously reported from New Zealand brackish water lakes. The signs of poisoning by the pentapeptide were similar to those reported for the heptapeptide toxins from other cyanobacteria.

Mass occurrences of cyanobacteria in the Baltic Sea are a common phenomenon in late summer. Many cases of animal poisoning along the Baltic coast are suspected to have been caused by these *Nodularia spumigena*-dominated blooms (5, 9, 13, 15). In 1878, *N. spumigena* from Australia was the first cyanobacterium to be reported in the scientific literature to cause animal poisonings (8). However, it took 100 years before toxic isolates of this species were available for structural identification of the toxin. A hepatotoxin produced by *N. spumigena* called nodularin was recently isolated from New Zealand bloom material by Rinehart and co-workers (16). The toxin is a cyclic pentapeptide (molecular weight, 824) containing D-glutamic acid, D-erythro-β-methylaspartic acid, L-arginine, *N*-methyl-*Z*-dehydrobutyrine, and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid. Partial structure studies of a New Zealand laboratory culture of *N. spumigena* (4), an Australia laboratory culture (17), and a Baltic Sea bloom sample (7) indicated a structure similar to that of nodularin.

This study investigated the occurrence and toxicity of *N. spumigena* in the Baltic Sea. The structure of the toxin from three different natural bloom samples and three laboratory-grown *Nodularia* cultures was studied by determining amino acid composition and using low- and high-resolution fast-atom bombardment-mass spectrometry (FAB-MS).

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MATERIALS AND METHODS

Sampling. Water bloom samples from the open sea were taken during expeditions in 1985 to 1987 by the research vessels R/V *Aranda* (Finland) and R/V *Arnold Veimer* (USSR). Coastal samples were collected by the Municipal City Laboratory of Helsinki and by the Water and Environmental District Laboratories. The locations of the sampling area and the sampling stations are shown in Fig. 1. Samples of bloom material were taken by plankton net (WP-2; 200-μm mesh size), concentrated by filtering (nylon cloth; 25-μm pore size), and immediately frozen on board or on shore within 12 h of collection. Samples were lyophilized and stored at 4°C. Open-sea water surface samples were collected by water sampler. Identification of the plankton species of the open-sea samples was made in accordance with the Guidelines for Baltic Sea Monitoring (1) published by the Finnish Institute of Marine Research, and the coastal samples were identified as described by Edler et al. (6).

Isolation and cultivation of strains. Isolates of *N. spumigena* were made from subsamples of bloom and water samples as detailed earlier (K. Sivonen et al., Hydrobiologia, in press) by using Z8 agar medium minus nitrogen (10, 11). The salt concentration in the medium was adjusted to about 7.5‰, which corresponds to the salinity of the southern Baltic proper (between approximately 54 and 58°N latitude). On board, cultures were maintained at 18°C under continuous illumination with cool white fluorescent tubes (Philips, Eindhoven, The Netherlands). Cultures removed to the laboratory of the Department of Microbiology, University of Helsinki, were incubated at 26°C with continuous

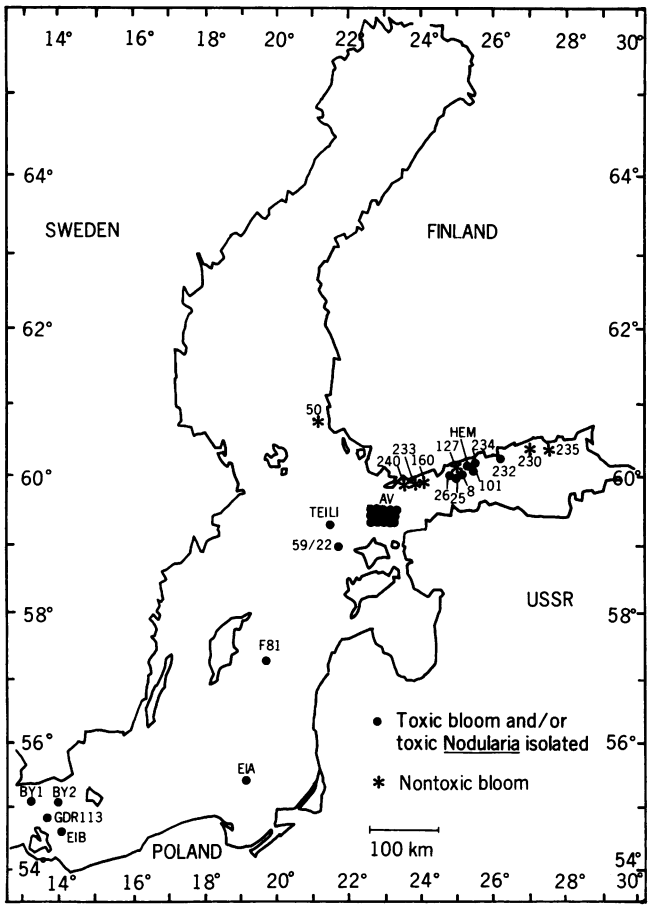

FIG. 1. Outline of the area in the Baltic Sea where samples were taken.

illumination at 500 to 900 lx (cool white fluorescent tubes [Daylight Deluxe; Airam, Helsinki, Finland]). Batch cultivation of isolated *Nodularia* strains took place in 5-liter Erlenmeyer flasks each containing 3 liters of liquid Z8 medium and a 10% inoculum. The culture flasks were aerated with filter-sterilized air (GSWP 02500 [pore size, 0.22 μm]; Millipore Corp., Bedford, Mass.). Cells were harvested by being filtered through nylon cloth at the stationary growth phase after 12 to 14 days of incubation, frozen, lyophilized, and stored at 4°C. Freeze-dried cells were used for toxicity testing and toxin isolation. Toxic strain L575 from New Zealand, grown in BG-11 medium plus 1% NaCl (W. Carmichael, Department of Biological Sciences, Wright State University, Dayton, Ohio), was used as the reference strain (4).

Toxicity testing. The acute lethal toxicity (minimum lethal dose) of the freeze-dried bloom and culture materials was tested by an intraperitoneal mouse bioassay using female NMRI mice (n = 3; 20 to 25 g [body weight]; University of Helsinki). The toxicity during the purification steps and that of the purified toxin were determined by a mouse bioassay using male ICR Swiss mice (18 to 25 g [body weight]; Wright State University). A survival time of 1 to 4 h with signs and necrospsy findings typical of hepatotoxic peptide toxins were the criteria used to establish the toxicity of the samples (3).

Quantification, isolation, and characterization of toxin. Quantitative analysis of the toxin was done on a Hewlett-Packard 1090M high-performance liquid chromatograph (HPLC) with a diode array UV-visible detector using a Regis Pinkerton GFF-S5-80 HPLC column (4.6 by 150 mm) plus a Guard Cartridge (Regis Chemical Co.). Samples were prepared as described by Meriluoto and Eriksson (14) and run by using a 1-ml/min mobile phase of 18% acetonitrile and 0.1 M KH_2PO_4 in water (pH 4.5) with detection at 238 nm. Toxin recoveries were 100 to 109% (18 μg of toxin added to the sample before extraction) and 90 to 135% (2 μg of toxin added). The calibration curve was linear from 0.45 to 90.0 μg/ml (n = 6; $r > 0.999$).

TABLE 1. Bloom and water samples collected during 1986 to 1987 from the open Baltic Sea

Open-sea sample type	Sample code	Sampling date	Toxicity of bloom	Dominant cyanobacterial species
Bloom	BY1	13 Aug. 1986	+	*N. spumigena*,[a] *A. flos-aquae*
Bloom	BY2	13 Aug. 1986	+	*N. spumigena*,[a] *A. flos-aquae*
Bloom	GDR113	14 Aug. 1986	+	*N. spumigena*, *A. flos-aquae*
Water	F81	31 July 1987	ND[b]	*A. flos-aquae*, *N. spumigena*[a]
Water	Teili	4 Aug. 1987	ND	*A. flos-aquae*, *N. spumigena*[a]
Water	AV1	8 Aug. 1987	ND	*A. flos-aquae*, *N. spumigena*[a]
Water	AV2	8 Aug. 1987	ND	*A. flos-aquae*, *N. spumigena*[a]
Water	AV3	10 Aug. 1987	ND	*A. flos-aquae*, *N. spumigena*[a]
Bloom	AV33	10 Aug. 1987	+	*A. flos-aquae*, *N. spumigena*[a]
Bloom	AV39	11 Aug. 1987	+	*A. flos-aquae*, *N. spumigena*[a]
Bloom	AV40	11 Aug. 1987	+	*A. flos-aquae*, *N. spumigena*
Bloom	AV45	12 Aug. 1987	+	*A. flos-aquae*, *N. spumigena*[a]
Bloom	AV63	13 Aug. 1987	+	*A. flos-aquae*, *N. spumigena*[a]
Water	AV68	13 Aug. 1987	ND	*A. flos-aquae*, *N. spumigena*[a]
Bloom	AV71	14 Aug. 1987	+	*A. flos-aquae*, *N. spumigena*[a]
Water	AV79	15 Aug. 1987	ND	*A. flos-aquae*, *N. spumigena*[a]
Water	AV91	16 Aug. 1987	ND	*A. flos-aquae*, *N. spumigena*[a]
Water	59/22	8 Aug. 1987	ND	*A. flos-aquae*, *N. spumigena*[a]
Bloom	EIA	1 Sept. 1987	+	*A. flos-aquae*, *N. spumigena*
Bloom	EIB	3 Sept. 1987	+	*A. flos-aquae*, *N. spumigena*[a]

[a] Toxic *N. spumigena* strain isolated from the sample.
[b] ND, Not determined because these samples were not from areas with a dense water bloom concentration.

TABLE 2. Bloom samples collected during 1985 to 1987 from Finnish coastal areas of the Baltic Sea

Sample code[a]	Sampling date	Toxicity of bloom	Dominant cyanobacterial species
8	19 Aug. 1985	+	*Nodularia spumigena*, *Aphanizomenon flos-aquae*, *Anabaena lemmermannii*
25	3 Sept. 1985	+	*Nodularia spumigena*, *Aphanizomenon flos-aquae*, (*Dinophysis* spp.)
26	3 Sept. 1985	+	*Nodularia spumigena*, zooplankton
50	9 Oct. 1985	−	*Aphanizomenon flos-aquae*, *Anabaena lemmermannii*, *Nodularia spumigena*
101	31 July 1986	+	*Anabaena lemmermannii*, *Aphanizomenon flos-aquae*, *Nodularia spumigena*
127	14 Aug. 1986	−	*Oscillatoria agardhii*
160	8 Sept. 1986	−	*Nodularia spumigena*, *Aphanizomenon flos-aquae*
HEM	10 Sept. 1987	+	*Aphanizomenon flos-aquae*, *Nodularia spumigena*[b]
230	18 Oct. 1987	−	*Microcystis aeruginosa*; *Nodularia spumigena*, *Aphanizomenon flos-aquae*
232	26 Oct. 1987	+	*Microcystis aeruginosa*; *Aphanizomenon flos-aquae*, *Nodularia spumigena*
233	27 Oct. 1987	−	*Aphanizomenon flos-aquae*
234	29 Oct. 1987	+	*Microcystis aeruginosa*, *Aphanizomenon flos-aquae*, *Nodularia spumigena*
235	31 Oct. 1987	−	*Microcystis aeruginosa*, *Nodularia spumigena*
240	5 Nov. 1987	−	*Aphanizomenon flos-aquae*

[a] All samples were from coastal blooms.
[b] Toxic *N. spumigena* strain isolated from the sample.

Purification of the toxin for structure analysis was based on the method reported by Carmichael et al. (4). Lyophilized cells were extracted with methanol-water (1:1, vol/vol), and the toxin was purified by C-18 Bond Elut cartridges, followed by HPLC using C-18 columns (5 μ-Econosphere [4.6 mm by 25 cm]; Alltech). Toxin was desalted by HPLC (0 to 50% acetonitrile gradient in 5 min) or by using Bond Elut cartridges (toxin eluted with 50% acetonitrile in water). Purified toxin was lyophilized and stored at −80°C.

For amino acid analysis, purified toxins were hydrolyzed in 6 N HCl at 106°C for 24 h before analysis. The amino acids released were precolumn derivatized with phenylisothiocyanate, and the phenylthiocarbamyl amino acids were analyzed by using a Waters Pico Tag HPLC system. The derivatives were loaded onto a C-18 column (15 cm by 4.6 mm) and eluted over 8 min by using a 0 to 60% gradient of acetonitrile in 0.138 M aqueous sodium acetate. The column flow rate was 1.0 ml/min, and the compounds eluted were detected by UV absorption at 254 nm (4, 12).

The intact peptides were analyzed at the University of Illinois by low-resolution FAB-MS (ZAB 10-kV mass spectrometer) by using the Magic Bullet Matrix (dithiothreitol-dithioerythritol at 1:3). The source temperature was 30°C, and the target was bombarded with xenon atoms at 8 kV. This was followed by high-resolution FAB-MS (ZAB-SE) and MS-MS (VG705E4F) to establish the elemental composition.

RESULTS

Bloom samples collected from the open sea contained both *N. spumigena* and *Aphanizomenon flos-aquae* (Table 1; Fig. 1), and all were hepatotoxic to mice, causing death within 1 to 3 h. Half of the 14 Finnish coastal samples were hepatotoxic (Table 2; Fig. 1). The cyanobacterial species variation was higher in coastal samples than in open-sea samples. In addition to *Nodularia* and *Aphanizomenon* species, *Oscillatoria*, *Microcystis*, and *Anabaena* species were present (Table 2).

Eighteen *N. spumigena* single-colony isolates were made from bloom and open-water samples (Tables 1 and 2). They were all hepatotoxic in the mouse bioassay. The signs of hepatotoxicity obtained with all samples and isolates were similar to those reported for hepatotoxic peptides of other cyanobacteria (3, 4).

HPLC analysis showed one toxic peak with the same retention times and similar UV spectra in all of the hepatotoxic bloom and laboratory culture samples (Fig. 2). The toxin concentrations of the bloom samples were ≤2.4 mg of toxin per g of freeze-dried bloom material (Table 3). When the toxin content of the bloom samples (measured by HPLC) was high, toxicity (expressed as the minimum lethal intraperitoneal dose was also high (Table 3). The 50% lethal dose (i.p.) of the purified toxin for mice was approximately 70 μg/kg (body weight). Toxin concentration was directly related to the proportion of *N. spumigena* organisms present in the bloom samples, indicating that they were responsible for the toxicity observed. The toxin concentrations of the laboratory-grown culture isolates varied from 2.5 to 8.0 mg/g of freeze-dried cells.

Amino acid analysis showed the presence of equimolar amounts of β-methylaspartic acid, glutamic acid, and arginine in all of the bloom and culture materials analyzed (Table

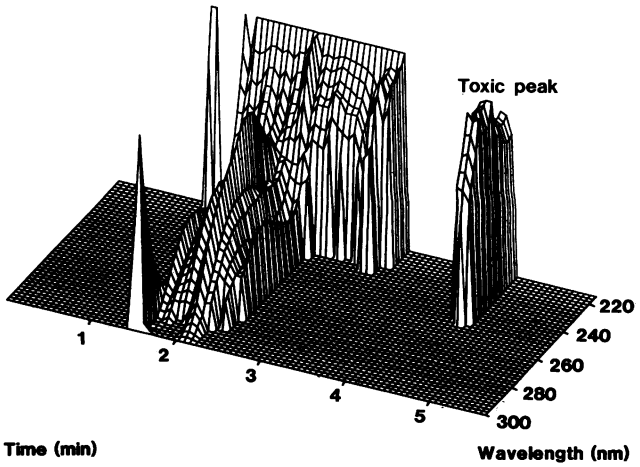

FIG. 2. HPLC diode array chromatograph showing toxic peak from BY2 water bloom of *N. spumigena* collected in the Baltic Sea. The toxin eluted at 4.6 min with a UV maximum at approximately 238 nm.

TABLE 3. Toxicity, toxin concentrations, and relative abundance of *N. spumigena* in Baltic Sea bloom samples

Year and sample code	Toxicity (MLD mg/kg)[a]	Toxin concn (mg/g)	Proportion of *N. spumigena* (%)[b]
1985			
8	250	NT[c]	+
25	1,500	<0.1	+
26	2,000	<0.1	+
1986			
101	1,000	0.2	+
127	—	ND[d]	0
160	—	ND	+
BY1	250	1.0	+
BY2	200	1.5	+
GDR113	650	0.4	+
1987			
AV 33	2,000	0.1	4.1
AV 39	500	0.4	3.9
AV 40	500	0.5	+
AV 45	1,000	0.3	9.4
AV 63	1,000	0.3	7.6
AV 71	500	0.5	26.7
HEM	1,500	<0.1	1.5
EIA	250	1.6	34.6
EIB	125	2.4	43.5
230	—	ND	+
232	2,500	0.1	+
233	—	ND	0
234	1,250	0.2	+
235	—	ND	+
240	—	ND	0

[a] MLD, minimum lethal dose; —, nontoxic sample.
[b] Proportion of *N. spumigena* in the phytoplankton biomass determined from open-sea bloom samples; A plus indicates the presence of *N. spumigena* in the sample when the proportion was not determined.
[c] NT, Not tested.
[d] ND, Toxin not detected.

4; Fig. 3). Low-resolution FAB-MS spectra of the samples showed that the molecular weight of the toxin in all of the samples was (*m/z*) 824 (Table 4; Fig. 4). The molecular formula of the toxin, based on high-resolution FAB-MS elemental analysis, was $C_{41}H_{61}N_8O_{10}$.

DISCUSSION

Hepatotoxic *N. spumigena* was common in Baltic Sea water blooms and open-sea water samples during all 3 years

TABLE 4. Molar ratios (based on arginine) of amino acids and molecular weights of toxins isolated from water blooms and cultures of laboratory isolates of Baltic Sea *N. spumigena*

Sample	β-Methylaspartic acid molar ratio[b]	Mol wt (*m/z* + H)
EIA bloom	1.0	825.4
EIB bloom	1.1	825.8
BY2 bloom	1.1	825.6
BY2 strain	1.2	825.6
BY1 strain	1.0	825.4
AV 45 strain	1.1	825.6

[a] Sample numbers refer to locations shown in Fig. 1.
[b] Partial coelution of glutamic acid and β-methylaspartic acid in the Pico Tag HPLC system was corrected for (11). The molar ratios of arginine and glutamic acid were 1 (reference) and 1.2, respectively, in each sample.

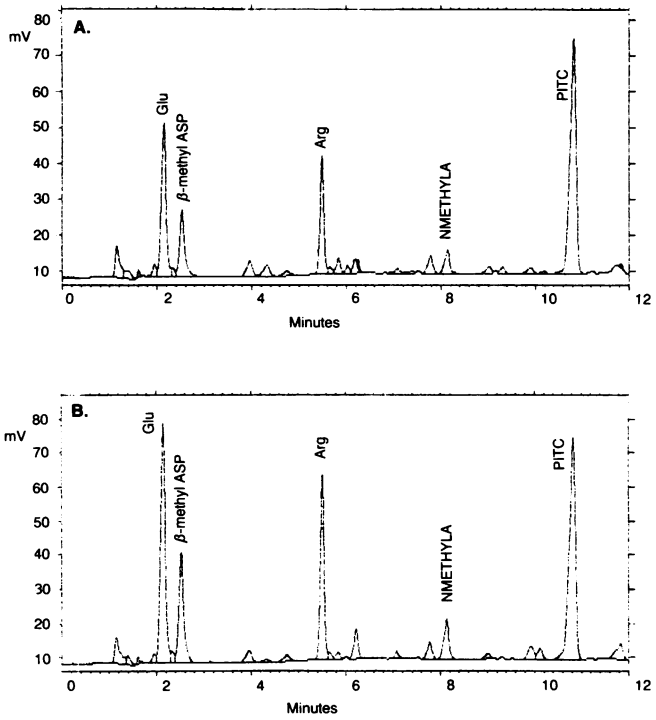

FIG. 3. HPLC and amino acid profile of *N. spumigena* hepatotoxin. (A) Extracted from water bloom material of sample BY2 (5 μg). (B) Extracted from laboratory-cultured strain from sample BY2 (8 μg). Analysis was by Waters Pico Tag precolumn derivatization with phenylisothiocyanate to yield phenylthiocarbamyl amino acids. For molar ratios, see Table 4. PITC, unidentified phenylisothiocyanate derivative produced during sample derivatization; NMETHYLA, *n*-methylamine, a hydrolysis product of *n*-methyldehydrobutyric acid; β-methyl ASP, β-methylaspartic acid.

of the study and in all of the areas sampled. It was even isolated from water samples during times when blooms were not detected. *N. spumigena* was considered to be responsible for toxicity in all of the samples, because (i) it was present in all of the toxic samples, (ii) toxicity was always hepatic, (iii) all of the isolates of *N. spumigena* were hepatotoxic, and (iv) the toxicity was correlated with the proportion of *N. spumigena* organisms in the blooms. *A. flos-aquae*, which codominated with *N. spumigena* in the open-sea samples, has not been reported to produce hepatotoxins. Bloom samples in which *A. flos-aquae* did dominate were nontoxic. *Anabaena*, *Oscillatoria*, and *Microcystis* isolates from freshwater sources are known to produce hepatotoxins, but the toxins produced are heptapeptides (3). The one coastal sample dominated by *Oscillatoria agardhii* was nontoxic. Most of the *Microcystis aeruginosa*-dominated blooms from the late autumn of 1987 were nontoxic or slightly toxic, indicating that *M. aeruginosa* was not the toxin-producing organism in these samples. Although we did not isolate nontoxic *N. spumigena* strains from the Baltic Sea during this study, they are known to exist (Sivonen et al., in press). The occurrence of nontoxic *N. spumigena* was also implied by the dominance of *N. spumigena* in nontoxic sample 160 (Table 2). In other nontoxic samples in which *N. spumigena* was present (samples 50, 230, and 235 [Table 2]), its amount may have been too small to cause detectable toxicity.

The highest toxin concentrations in the samples collected were found in blooms that originated in the southern Baltic

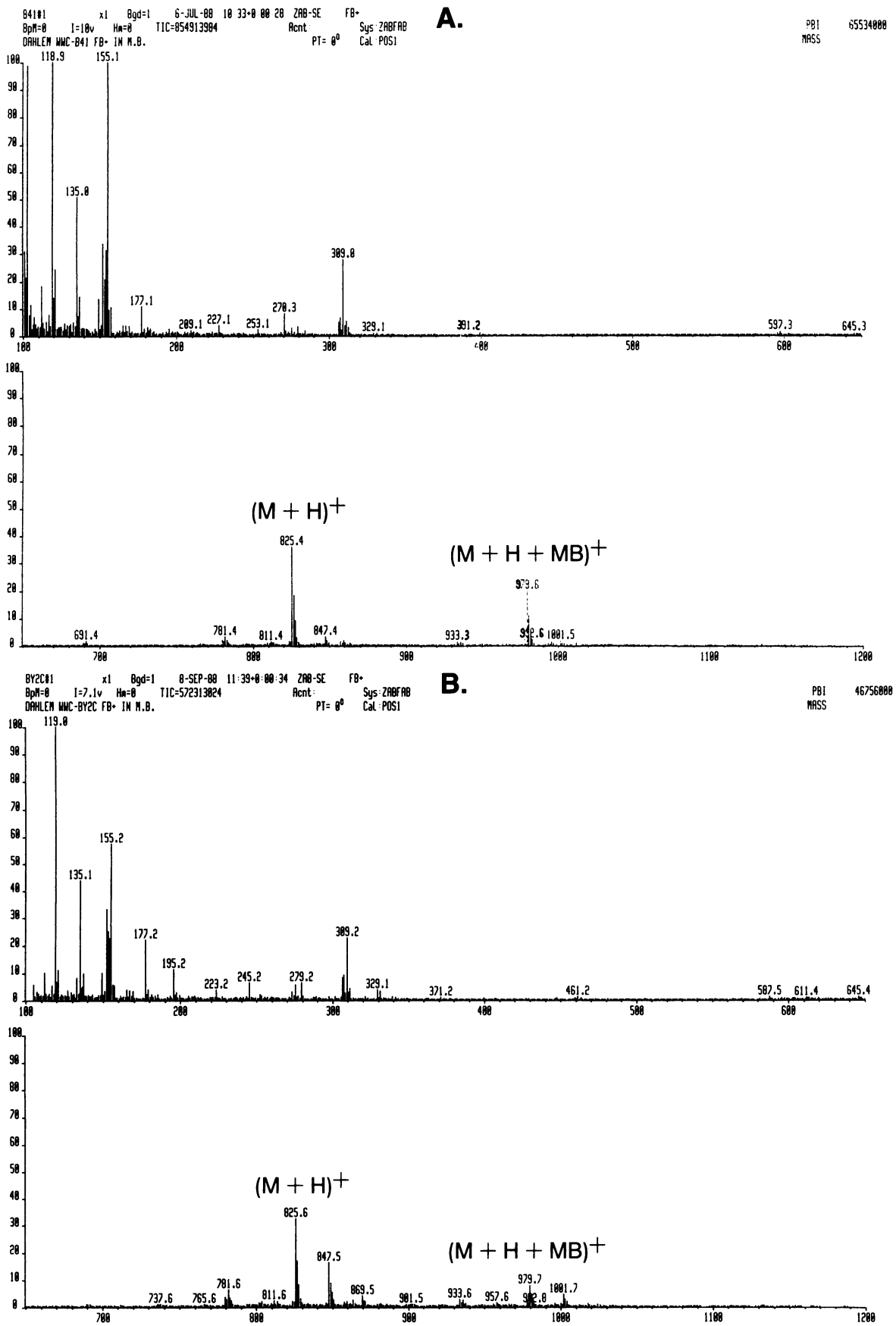

FIG. 4. FAB-MS spectrum of *N. spumigena* hepatotoxin in Magic Bullet (MB) matrix. (A) Extracted from water bloom material of sample BY2. (B) Extracted from laboratory-cultured strain from sample BY2.

Sea. Laboratory-grown *Nodularia* cultures were found to be more toxic than water bloom samples. Lower toxicity in the natural samples was due partly to the diluting effect of nontoxic cyanobacteria and/or zooplankton. Some of the cultures isolated produced high amounts of toxins. Carmichael et al. (4) have found toxin concentrations of 1.96 to 2.99 mg/g of freeze-dried cells in the *Nodularia* culture isolated in New Zealand. In that study, a different culture medium for *Nodularia* sp. and a different technique to analyze the toxin concentrations were used.

We found that the toxin isolated from Baltic Sea natural bloom materials and laboratory cultures of *N. spumigena* appeared identical and similar to the toxin from New Zealand *N. spumigena* (4, 16). The Baltic Sea toxin was also similar to that reported from other studies with *N. spumigena* (7, 16, 17). The studies reported here indicate that one toxin accounts for most if not all of the toxicity found in the samples or isolated strains. This is in contrast to other cyanobacterial species, i.e., *Microcystis* and *Oscillatoria* spp. and water blooms from South Africa (2), Japan (18), Finland (Sivonen et al., unpublished data), and elsewhere (3), which indicate that several similar toxins can be produced by a single strain.

ACKNOWLEDGMENTS

The technical assistance of Riitta Saastamoinen, Jaana Lehtimäki, and Else Peuranen and amino acid analysis by William Evans are gratefully appreciated.

This study was supported by grants from the Academy of Finland, the University of Helsinki, the Maj and Tor Nessling Foundation, and the Finnish Cultural Foundation. The toxin purification work was supported in part by U.S.A. Medical Research Defense Command contract DAMD17-87-C-7019 awarded to W. W. Carmichael. FAB-MS spectra were obtained in the Mass Spectrometry Laboratory in the School of Chemical Sciences, University of Illinois, supported in part by a grant from the National Institute of General Medical Sciences (GM 27029). The ZAB-SE mass spectrometer was purchased in part with a Public Health Service grant from the Division of Research Services, National Institutes of Health (RR01575); a grant from the National Science Foundation (PCM-8121494); and a Public Health Service grant from the National Institute of General Medical Sciences (GM 27029).

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