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Research Article

Morphological, Molecular and Toxicologial Characterization of *Nodularia spumigena* Mertens in Jungens (1822) from Brackishwater Lake Bafa (Turkey)

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Abstract

Nodularia spumigena, a blooming cyanobacteria, produce hepatotoxic peptide nodularin. In this study, morphological, molecular and chemical methods were applied to characterize toxic and blooming *N. spumigena* collected from Lake Bafa on June 2010, Aydın, Turkey. Phylogenetic analysis of *N. spumigena* was performed by sequencing 16S ribosomal DNA or the phycocyanin intergenic spacer (IGS) region, and the nodularin synthetase gene cluster (nda). Nodularin produced by *N. spumigena* was determined by using enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC) and liquid chromatography/mass spectrometry (LC/MS). This is the first report on toxic *N.spumigena* in Lake Bafa, Turkey.

Keywords: Lake Bafa, nodularin, ELISA, HPLC, Cyanobacteria

Bafa Gölü'ndeki *Nodularia spumigena* Mertens in Jungens (1822)'in Morfolojik, Moleküler ve Toksikolojik Karakterizasyonu

Öz

Aşırı çoğalan *Nodularia spumigena*, hepatotoksik peptid nodularini üretir. Bu çalışmada Haziran ayında Bafa Gölünden izole edilen *N.spumigena*'nın karakterizasyonu için gerçekleştirilen morfolojik, moleküler ve kimyasal analizler uygulanmıştır. Filogenetik analizler 16S rDNA, fikosiyanin intergenik spacer (IGS) bölgesi ve nodularin sentez gen kümesi (nda) çoğaltılarak gerçekleştirilmiştir. *N.spumigena*'nın ürettiği nodularinin tayini Enzim Bağlı İmmunosorbent Assay (enzyme-linked immunosorbent assay-ELISA), Yüksek Performanslı Sıvı Kromotografisi (high performance liquid chromatography-HPLC) ve liquid chromatography/mass spectrometry (LC/MS) yöntemleriyle araştırılmıştır. Bu çalışma ile, Bafa Gölü'nde toksik *N. spumigena* ilk defa rapor edilmiştir.

Anahtar Kelimeler: Bafa Gölü, nodularin, ELISA, HPLC, siyanobakteri

Introduction

Toxic cyanobacterial and microalgal blooms cause considerable effects on human health and the habitat and give rise to ecological problems all over the world. Anthropogenic activities, such as agriculture and human waste, increase nutrient availability in lakes, rivers and the coastal areas resulting in increased proliferation and dominancy of cyanobacteria. The main toxin producing cyanobacteria species are; *Microcystis*, *Anabaena*, *Oscillatoria*, *Aphanizomenon*, *Cylindrospermopsis* and *Nodularia*. *Nodularia* is a filamentous, nitrogen-fixing cyanobacterium generally dominant in brackishwater [1, 2]. Maximum toxicity occurs when the *Nodularia* grows at

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salinities from 12 to 15 parts per 1000 of salt [3, 4]. The first world record on toxic bloom of *Nodularia* was reported in Lake Alexandria, Australia in 1878 [5]. At that time the lake had estuarine salinity and after the construction of dams to control the flow, salinity decreased, however, it did not prevent *Nodularia spumigena* Mertens in Jungens (1822) to be exist [6]. There are several reports on *Nodularia* blooms in Autstralian and New Zealand estuaries [1] and brackish lakes [7, 8], Mexican saline lakes and lagoons [9], Uruguay [10], USA [11, 12], and the brackish Baltic Sea [13, 14, 15, 16]. Mortality of domestic animals after contamination of water with *N. spumigena* has been reported from Australia [5], South Africa [17], the Baltic Sea and German North Sea coast [18].

The classical method for identification and assessment of cyanobacterial communities includes microscopic examination [19]. This assessment, however, has been criticized on the grounds that morphology can vary considerably in response to fluctuating environmental conditions [20]. In addition, the parts of cyanobacteria such as hormogonia, akinetes and heterocysts may be difficult to characterize by microscopy and thus the actual diversity can be underestimated [21]. Therefore, cyanobacterial diversity analysis should be investigated by microscopic observation extended with a molecular taxonomy. Studies using phylogenetic markers such as small subunit ribosomal RNA (16S rRNA), internal transcribed spacers (ITS) and phycocyanin genes are very common to assess the diversity and evolutionary relationships of cyanobacteria [22, 23].

In this paper, morphological and molecular techniques were applied to characterize *N.spumigena* and the presence of nodularin was determined by LC/MS and ELISA analysis. This is the first report on toxic *N. spumigena* in Lake Bafa, Turkey.

Materials and Methods

Characteristics of the Sampling Site Lake Bafa is one of the largest coastal lakes in Turkey, located about 10 km away from Miletus, Mugla, in western Turkey (37° 30′ 3′′N 27°31′19′′ E) (Fig. 1 [24]).

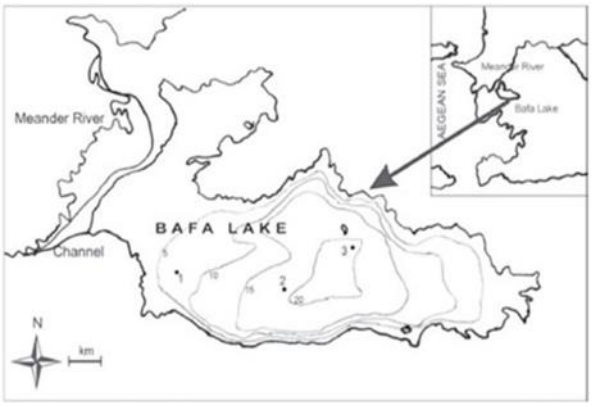

Fig 1. *N. spumigena bloom on the surface water of Lake Bafa.*

Lake Bafa was a marine embayment of Aegean Sea. The lake was formed during late Pleistocene - early Holocene sea-level rise. The palaeogeographical evolution of the region and the subsequent shift in the shoreline has been reconstructed by the in-depth study of numerous geological corings in the alluvial and delta plain of the Great Menderes River [25-28]. The maximum depth is ca. 20 m and the surface cover is approximately 7,000 ha. The main water sources of Lake Bafa are over flow of Great Menderes River and underground water from the nearby mountains [30, 31]. The climate of Lake Bafa is typical mediterranean climate, 5-6 months without rain. The vegetation around the lake belongs to the eu-Mediterranean zone (0–800 m) where it consists of evergreen taxa. The lake provides winter habitat for many bird species that would otherwise be in danger of extinction. Approximately 300,000 birds (mainly pelicans, dwarf cormorants and sea eagles) nest on the coast and isles of the lake [32]. Overall there are 237 genera, 325 species, 22 subspecies and 7 varieties of 80 animal and plant familia and also 16 endemic species determined in the region of Lake Bafa [33]. In order to protect historical and natural values, 12281 ha of this area were designated as Natural Park in 1994.

Sample Collection

The strain of *N. spumigena* used in this study was collected on June, from a *Nodularia* bloom in Lake Bafa (Fig. 2).

Fig 2. *Map of Lake Bafa [54]*

Samples were collected from the surface water, where the colour was conspicuous due to high phytoplankton biomass. Three different sampling points at least 100 m apart were used in the study. Samples were collected from the surface of blooming area with 3L bucket and equally divided into 3 different 1L dark plastic bottles. Formaldehyde solution (40%) was added into the first bottle for microscopic analysis. The second sample bottle was stored in fridge for molecular analysis. The third bottle was also stored in fridge for toxin analyses. This procedure was repeated for each sampling points.

Morphological Characterization

Generally, *Nodularia* species are classified on the basis of their morphology and ecology, such as the presence or absence of gas vesicles, size and shape of trichomes, dimensions and shapes of vegetative cells, akinetes, and heterocytes [34].

N.spumigena collected from Lake Bafa was identified under the microscope (Fig. 3 and Fig. 4) (Leica DM 3000) with a digital camera based on the morphological characteristics from cyanobacterial identification keys [35-37].

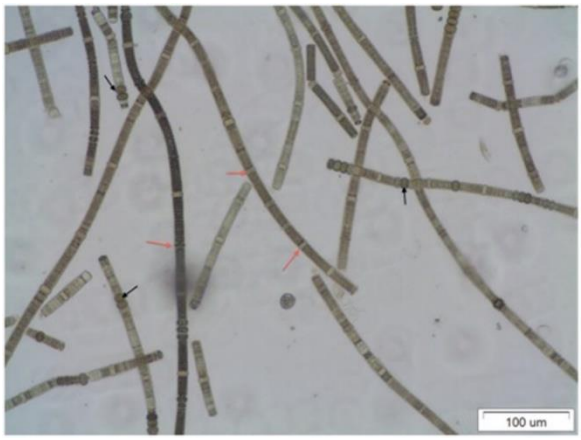

Fig 3. Microscobic photograph of N. spumigena from Lake Bafa, black arrows represent akinetes, red arrows represent heterocytes. Magnification: 100

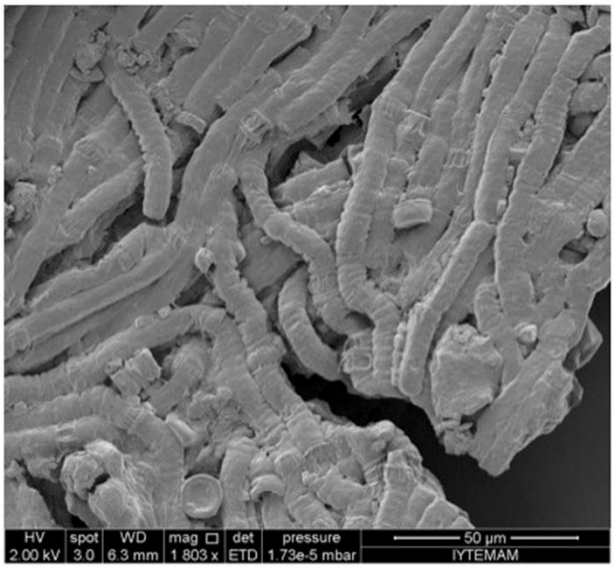

Fig 4. *SEM photograph of N. spumigena from Lake Bafa Magnification: 50 µm*

For morphological examination, bloom sample was examined under the light microscope. Morphology of trichomes, heterocystis, and vegetative cells were examined. 3 different slides were prepared for quantification. In each slide 20 filaments have been measured.

Water quality parameters

The temperature, dissolved oxygen, conductivity and pH of studied area in Lake Bafa were measured using a multi parameter probe (CyberScan 600, EUTECH Instruments) for all sampling points. Probes were calibrated before the process.

DNA isolation and PCR analysis

Total genomic DNA from the natural samples was isolated with the ZR Fungal/Bacterial DNA MiniPrep (ZymoResearch) based on manufacturer's instructions and stored at -20ºC. Following DNA isolation DNA was electrophoresed (Elite 300, Wealtech) on 0.8% agarose gel at 5 V $cm^{1.}$

PCR analysis was performed in Bio-Rad MyCycler (thermal cycler), with Helix Amp™ Hypersense DNA polymerase (Nannohelix) with 4 different primer pairs (Table 1) based on manufacturer's instructions.

Table 1. *Targets regions, sequences and references of the primer pairs used for PCR analysis*

Designation	Target	Sequence	Reference
pcβF	*Phycocyanin*	5'-GGCTGCTTGTTTACGCGACA-3'	[50]
pcαR		5'-CCAGTACCACCAGCAACTAA-3'	
27F	16S rDNA	5′-AGAGTTTGATTTACGCGACA-3′	[51]
809R		5′-GCTTCGGCACGGCTCGGGTCGATA-3′	
ndaA-LP	Nodularin	5′-GGA GTT TGG GCG ATG AGT TA -3′	[52]
ndaA-RP		5′-GCC CAA CTA ACT GCA ATG GT-3′	
Cya359F	16S rDNA	5′-GGGGAATYTTCC. GCAATGGG-3′	[53]
Cya781Ra		5′-GAC TACTGG GGTATCTAATCCC**A**TT-3′	
Cya781Rb		5′-GACTACAGGGGTATCTAATCCC**T**TT-3′	

The phycocyanin gene was amplified using pcβF and pcαR primers with 30 cycles of 95 °C for 20 s, 60 °C for 40 s and 72 °C for 40 s. 16S rDNA amplification was performed in 50 µL reaction buffer using 27F and 809R primers, with an initial denaturation step at 95 °C for 2 min followed by 30 cycles of 95 °C for 30 s, 58 °C for 40 s and 72 °C for 40 s and a final extension step at 72 °C for 5 min. The amplification of nodularin gene with ndaA-LP and ndaA-RP primer pair was started with an initial denaturation step at 95 °C for 2 min was followed by 30 cycles of DNA denaturation at 95 °C for 30 s, primer annealing for 40 s at the corresponding annealing temperature, and DNA strand extension at 72 °C for the appropriate extension time and a final extension step at 72 °C for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis in Tris-Boric acid-EDTA (TBE) buffer 1×, at 5V cm^{-1} and stained with SYBR safe and visualized under UV.

Sequence Analysis

Sequence analysis of 5 different PCR amplicons of *N.spumigena* was conducted on ABI 3130XL Genetic Analyzer (Applied Biosystems) at Izmir Institute of Technology, Biotechnology and Bioengineering Central Research Laboratories with universal primers (27F-809R, Cya359F-Cya781Ra, Cya359F-Cya781Rb), phycocyanin gene regions (pcβF-pcαR) and toxin gene regions (nodularin; ndaA-LP and ndaA-RP).

Phylogenetic Analysis

16S rRNA, phycocyanin gene and nodularin gene sequences were aligned with reference sequences retrieved from GenBank using the ClustalW program version 4.0 in MEGA [38]. Phylogenetic trees were constructed with Neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) algorithms using MEGA 5.

Toxin Analysis

Preperation for ELISA:

Collected sample was dried with freeze-drier (Christ Alpha 1-2 LD). For ELISA analysis the sample (0.01 g) was extracted within 10 mL of 75% MeOH. Sample was sonicated for 1 min at 30% power with 3 cycle-probe sonication (Bandelin, Sonoplus HD 2070) in ultrasonic bath (Ultrasonic LC30). Centrifugation (Hettich Rotina 35) was performed at 10,000 g for 10 min at 4 ºC. Supernatant was transferred to a new vial and evaporated by SpeedVac Concentrator (Thermo Scientific Savant SPD121P). 1:100 and 1:1000 dilutions were prepared using milli-Q water and the solutions were filtered through 0.22 µL Millex-HV PVDF filters (Millipore, Bedford, MA, USA).

ELISA:

The extract was analyzed using the competitive ELISA microcystin microplate kits (EP022, EnviroLogix Inc) validated for nodularin and microcystins LR, LA, YR and RR.

Cyanobacterium extracts collected from filters were diluted with MilliQ water for 400-10.000 times in order to give final concentrations within the standard range of 0.16 – 2.5 ng mL^{-1} for ELISA. The ELISA plate was read on microplate reader (SpectraMax 190) at 450 nm. This kit is a competitive ELISA immunoassay with a detection limit of 0.25 ng mL^{-1} for nodularin.

Preparation for LC/MS analysis:

Freeze-dried sample (2 mg) was extracted within 1 mL of 75% MeOH. Sample was sonicated for 1 min with 30% power-probe sonication (Bandelin, Sonoplus HD) in ultrasonic bath (Bandelin RK 103 H). Centrifugation (Sigma 3K30) was performed at 10.000 g, for 10 min at 4 ºC. Supernatant was transferred to a new vial and evaporated under nitrogen stream at 35 °C. The residue was dissolved in 30% MeOH and filtered through 0.22 µL filters (Grace 13 mm HPLC SYR. Filt).

HPLC:

Quantitative determination of nodularin was performed with HPLC Agilent 1200 Capillary, Germany. *N. spumigena* cell extract was separated on Zorbax SB C18 column (150 mm × 0.5 mm, 5 µm) from Agilent. Mobile phase consisted of 0.1% Formic Acid in water (solvent A), and acetonitrile (solvent B). The gradient elution mode was; 30% B at 0 min; 70% B at 5 min; 70% B at 10 min; 25% B at 15 min, injection volume 0.5 µL, flow rate 20 µL min^{-1} and column temperature 30 °C.

LC/MS-MS:

LC/MSn system was used to confirm the presence of nodularin in *N. spumigena* extract. The Bruker HCT Ultra Ion Trap instrument with electrospray ionization ESI operated in the positive mode. The ionization parameters were: capillary voltage 3.8 kV; dry gas temperature 300 °C; dry gas flow 5 L min^{-1}; nebulizer gas pressure 15 psi. In multiple reaction monitoring mode (MRM) ions at m/z 825.4 and 811.4 were monitored to detect nodularin and its demethylated analogue, respectively.

Results

Morphological and molecular identification of *N. spumigena*

In the lake, the filaments were in large mucilaginous masses up to 3 µm in diameter. The mean length of the filaments was 2461 ± 2367 µm (Table2). The trichoms were all straight. There were no curved trichomes observed. In our sample, the length of the trichomes were very long (Max. 6396 µm length). Only a few trichomes were very short (Min. 54 µm length). Cells and heterocysts were discoid and heterocysts were mostly intercalary spaced. The average length and the width of the vegetative cells were 2.7 ± 0.53 and 9.59 ± 0.52, respectively. There were many akinetes at the end of the trichomes and between the vegetative cells. The color of the cell wall was brown. Morphological characterisations of *Nodularia* isolated from Lake Bafa exhibited compatible features with *N. spumigena*.

Water quality analysis

Eight different parameters (temperature, pH, ORP, conductivity, TDS, NaCl, resistivity and oxygen) were considered during the measurement of water quality in Lake Bafa. The parameters were measured with a multi parameter probe (CyberScan 600, EUTECH Instruments) from 3 different sampling points. There were no significant differences ($p>0.05$) between the sampling points thus we used the average values of the measurements (Table 3). The mean temperature was 31.6 ºC and the pH was 8.3. Salinity was measured as 23.8‰. The oxygen was found 3.8 mgL^{-1}, resistivity was 26.6 Ω and, chlorophyll a was 13.6 µg L^{-1}, the oxidation-reduction potential was measured as -98.7 mV.

Table 2. *Morphometric measurements of N. spumigena from Lake Bafa*

	Morphology		**Mean±S.D.**	**Min**	**Max**
Trichomes	Straight	Length (µm)	2461±2367	54	6396
Vegetative cells	Discoid	Length (µm)	2.7±0.53	1.56	3.46
		Width (µm)	9.59±0.52	8.9	10.78
Heterocysts	Discoid	Length (µm)	5.03±0.67	5.74	4.21
		Width (µm)	10.83±0.49	11.37	9.93

Table 3. *The mean of water quality parameters of Lake Bafa during bloom*

Parameters	**Means**
Temperature (°C)	31.6
pH	8.3
ORP (Oxidation-Reduction Potential) (mV)	-98.7
Conductivity (ms)	20.3
TDS (Total Dissolved Solids) (ppt)	18.8
NaCl ‰	23.8
Resistivity (Ω)	26.6
Oxygen (mg/lt)	3.8
Chlorophyll a (µg/lt)	13.6

DNA isolation and PCR analysis

There was only one DNA band on the agarose gel indicating that there were no other contaminants in the sample.

All primers used in the PCR analysis worked for our samples. Cya359F-Cya781Ra, Cya359F-Cya781Rb and, 27F-809R primers were cyanobacteria specific primers. As expected 27F-809R primers gave PCR band at 800 bp, Cya359F- Cya781Ra at 400 bp, Cya359F- Cya781Rb primers at 400 bp.pcβF-pcαR primers specific for phycocyanin gene are generally used for cyanobacterial classifications. Our sample gave result at 650 bp with phycocyanin primers (Fig. 5). ndaA gene region amplified with ndaA-LR and ndaA-RP primer set and we obtained a clear single band at 650 bp. ndaA, nodularin synthetase subunit A gene, is only determined among nodularin producers. Obtaining a PCR band for ndaA supports that our sample is a toxic strain.

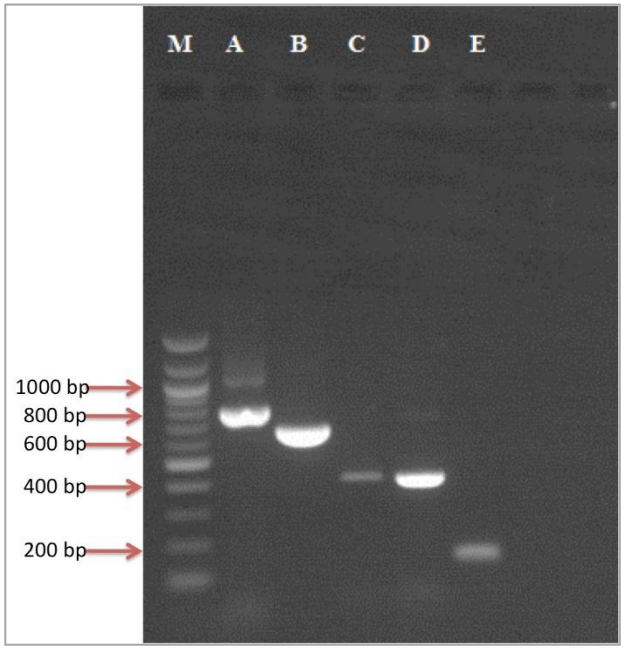

Fig 5. M: 100 bp DNA ladder, A: 16S rDNA (primers 27F-809R), B: cpcB & cpcA (primers: pcβF- pcαR), C: 16S rDNA (primers: 359a-781R), D: 16S rDNA (primers: 359b-781R).

Phylogenetic Analysis

The sequence analysis results of our 16S rRNA, phycocyanin and, nodularin genes were blasted using the BLAST program of NCBI. The obtained sequences have been deposited in the GenBank database under the accession number of JN409386 (16S rRNA), JN377973 (phycocyanin gene), JN562751 (Cya359F, Cya781RA), JN562752 (Cya359F, Cya781RB). Blast results were shown in Table 4 and Table 5.

Phylogenetic analyses were carried out to confirm whether Lake Bafa samples belong to the *Nodularia* genus. cpcB & cpcA gene sequences and 16S rRNA partial sequences of

Lake Bafa isolates were compared with available sequences of *N. spumigena* in GenBank (Table 4, Table 5). Bootstrap analyses were conducted to measure the reliability of observed phylogenetic patterns. For all tree-reconstruction methods, a high bootstrap value (>95%) was obtained, indicating that studied Lake Bafa isolate is *N. spumigena* species (Fig. 6 and Fig. 7).

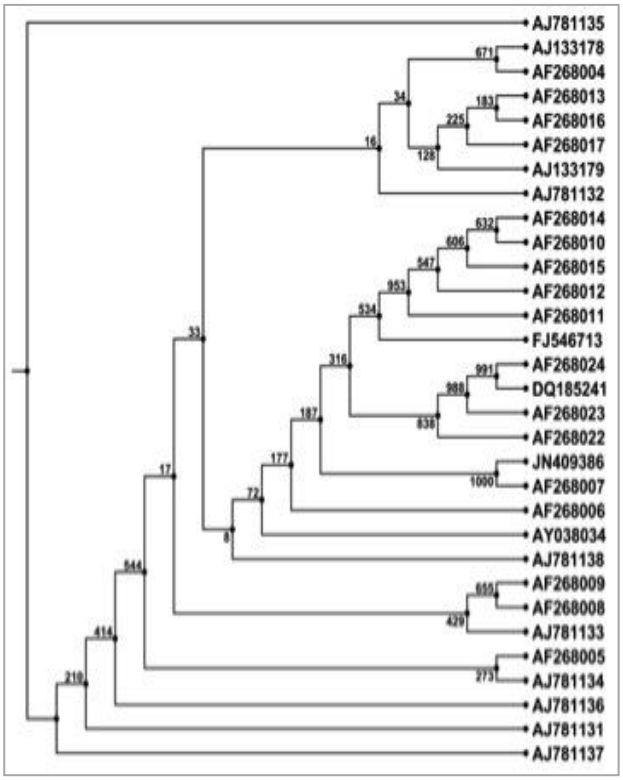

Fig 6. *Maximum likelihood tree of 16S rRNA region of Lake Bafa isolate N. spumigena (JN409386) and 16S rRNA sequences of N. spumigena in GenBank. ZZ82804 (Nostoc sp. 16S rRNA sequence) is used as an outgroup.*

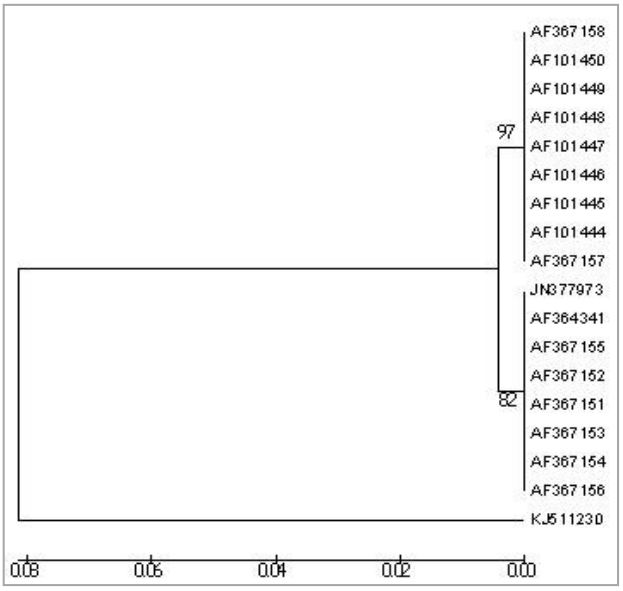

Fig 7*. Maximum Likelihood tree of phycocyanin alpha and beta subunit (cpcβ & cpcα) sequence of Lake Bafa isolate N. spumigena (JN377973) and cpcβ & cpc gene sequences of N. spumigena in GenBank. KJ511230 (Nostoc sp. cpcβ & cpcα gene sequence) is used as an outgroup.*

Toxin analysis

In this study, both ELISA and LC/MS tests indicated that *N. spumigena* isolated from Lake Bafa produced the toxin nodularin. Nodularin was identified by ESI-MS/MS spectra (Fig. 8) and concentration of the nodularin in cyanobacterial material was measured as 312.5 µg g^{-1} d.w. and 405 µg g^{-1} d.w. by ELISA and LC/MS, respectively.

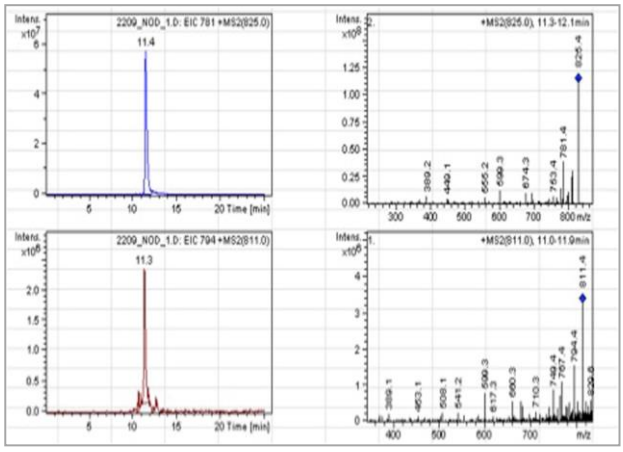

Fig 8. *Mass spectrum of nodularin isolated from N. spumigena strain from Lake Bafa.*

Table 4. *Cyanobacterial strains used in this study to constract the phylogenetic tree by c-phycocyanin beta subunit (cpcB) and c-phycocyanin alpha subunit (cpcA) genes*

Name	**Accession**	**Similarity with JN377973**	**Strain**	**gene**	**Reference**
N.spumigena	AF364341	100 %	kac13-pc	cpcB and cpcA genes, partial cds	Janson,S. and Graneli,E
N.spumigena	AF101450	99 %	nsph03	cpcB and cpcA genes, partial cds	Bolch,C.J.S., Orr,P.T., Jones,G.J., Blackburn,S.I.
N.spumigena	AF101449	99 %	nsor18	cpcB and cpcA genes, partial cds	Bolch,C.J.S., Orr,P.T., Jones,G.J., Blackburn,S.I.
N.spumigena	AF101448	99 %	nsla0	cpcB and cpcA genes, partial cds	Bolch,C.J.S., Orr,P.T., Jones,G.J., Blackburn,S.I.
N.spumigena	AF101447	99 %	nskr07	cpcB and cpcA genes, partial cds	Bolch,C.J.S., Orr,P.T., Jones,G.J., Blackburn,S.I.
N.spumigena	AF101446	99 %	nsgl02	cpcB and cpcA genes, partial cds	Bolch,C.J.S., Orr,P.T., Jones,G.J. Blackburn,S.I.
N.spumigena	AF101445	99 %	nsbr05	cpcB and cpcA genes, partial cds	Bolch,C.J.S., Orr,P.T., Jones,G.J. Blackburn,S.I.
N.spumigena	AF101444	99 %	nsb105	cpcB and cpcA genes, partial cds	Bolch,C.J.S., Orr,P.T., Jones,G.J. Blackburn,S.I.
N.spumigena	AF367156	100 %		cpcB and cpcA genes, partial cds	Laamanen,M.J.,Gugger,M.F., Lehtimaki,J.M., Haukka,K.,Sivonen,K.
N.spumigena	AF367157	99 %		cpcB and cpcA genes, partial cds	Laamanen,M.J.,Gugger,M.F., Lehtimaki,J.M., Haukka,K.,Sivonen,K.
N.spumigena	AF367158	99 %		cpcB and cpcA genes, partial cds	Laamanen,M.J., Gugger,M.F., Lehtimaki,J.M., Haukka,K., Sivonen,K.
Nodularia sp.	AF367155	100 %		cpcB and cpcA genes, partial cds	Laamanen,M.J., Gugger,M.F., Lehtimaki,J.M., Haukka,K., Sivonen,K.
N.spumigena	AF367154	100 %		cpcB and cpcA genes, partial cds	Laamanen,M.J., Gugger,M.F., Lehtimaki,J.M., Haukka,K., Sivonen,K.
Nodularia sp.	AF367153	100 %		cpcB and cpcA genes, partial cds	Laamanen,M.J., Gugger,M.F., Lehtimaki,J.M., Haukka,K., Sivonen,K.
Nodularia sp.	AF367152	100 %		cpcB and cpcA genes, partial cds	Laamanen,M.J., Gugger,M.F., Lehtimaki,J.M., Haukka,K., Sivonen,K.
N.spumigena	AF367151	100 %		cpcB and cpcA genes, partial cds	Laamanen,M.J., Gugger,M.F., Lehtimaki,J.M., Haukka,K., Sivonen,K.
N.spumigena	AF367156	100 %		cpcB and cpcA genes, partial cds	Laamanen,M.J., Gugger,M.F., Lehtimaki,J.M., Haukka,K., Sivonen,K.

Table 5. *Cyanobacterial strains used in this study to constract the phylogenetic tree 16S ribosomal RNA gene*

Name	**Accession**	**Similarity with JN409386**	**Strain**	**Nodularin**	**gene**	**Reference**
N.spumigena	AF268008	99 %	NSLA02A4	+	16S rRNA	Moffitt,M.C., Blackburn,S.I. and Neilan,B.A.
N.spumigena	AF268005	99 %	HEM	+	16S rRNA	Moffitt,M.C., Blackburn,S.I. and Neilan,B.A.
N.spumigena	AF268004	99 %	BY1	+	16S rRNA	Moffitt,M.C., Blackburn,S.I. and Neilan,B.A.
N.spumigena	AJ781138	99 %	AV63	+	16S rRNA	Lyra,C., Laamanen,M., Lehtimaki,J.M., Surakka,A. and Sivonen,K.
N.spumigena	AJ781137	99 %	F81	+	16S rRNA	Lyra,C., Laamanen,M., Lehtimaki,J.M., Surakka,A. and Sivonen,K.
N.spumigena	AJ781135	99 %	Huebel 1987/310	+	16S rRNA	Lyra,C., Laamanen,M., Lehtimaki,J.M., Surakka,A. and Sivonen,K.
N.spumigena	AJ781134	99 %	HEM	+	16S rRNA	Lyra,C., Laamanen,M., Lehtimaki,J.M., Surakka,A. and Sivonen,K.
N.spumigena	AJ781133	99 %	Huebel 1987/311	+	16S rRNA	Lyra,C., Laamanen,M., Lehtimaki,J.M., Surakka,A. and Sivonen,K.
N.spumigena	AJ781132	99 %	Huebel 1988/306	+	16S rRNA	Lyra,C., Laamanen,M., Lehtimaki,J.M., Surakka,A. and Sivonen,K.
N.spumigena	AJ781131	99 %	PCC9350	+	16S rRNA	Lyra,C., Laamanen,M., Lehtimaki,J.M., Surakka,A. and Sivonen,K.
N.spumigena	KF360086	99 %	CH307	+	16S rRNA	Fewer,D.P., Jokela,J., Paukku,E., Osterholm,J., Wahlsten,M.,Permi,P., Aitio,O., Rouhiainen,L., Gomez-Saez,G.V. and Sivonen,K.
N.spumigena	KF360088	99 %	AV45	+	16S rRNA	Fewer,D.P., Jokela,J., Paukku,E., Osterholm,J., Wahlsten,M. Permi,P., Aitio,O., Gomez-Saez,G.V. and Sivonen,K.

Table 6. *Blast results of Bafa Lake isolate N. spumigena (BF001) ndaA gene sequence*

Name	Accession	Similarity with BF001	Strain	gene	Reference
N.spumigena	CP007203	99 %	CCY9414	Nodularin	Voss,B., Bolhuis,H., Fewer,D.P., Kopf,M., Moke,F., Haas,F.,El-Shehawy,R., Hayes,P., Bergman,B., Sivonen,K.,Dittmann,E.,Scanlan,D.J., Hagemann,M., Stal,L.J. and Hess,W.R.
N.spumigena	AY210783	99 %		Nodularin	Moffitt,M.C. and Neilan,B.A.
N.spumigena	DQ842505	93 %	AV1	Nodularin	Jonasson,S., Vintila,S. and El-Shehawy,R.

Discussion

There is not any previous report on the occurrence of *N.spumigena* bloom in Lake Bafa and this is the second report in Turkish fresh and brackish waters. The first *N. spumigena* bloom in Turkish freshwater was reported by Akcaalan et al. [39], and it was also the first report in European freshwater lakes. A halotolerant organism, *Nodularia*, has been shown to have a strong growth potential in estuaries; where it benefits from land-derive nutrients [40]. Domestic and industrial wastes that contain high levels of nitrogen and phosphorus, lead to cyanobacterial blooms. The toxicity of strains varies according to environmental conditions such as nutrient levels, water temperature, salinity and the composition of plankton community.

The in situ measurements of water quality parameters from June are given on Table 3. The negative ORP result indicates low amount of dissolved oxygen and high amount of metals and contaminants. It also depicts high quantity of dead and decaying materials in the water. In clean water, the ORP value should be as high as 300 to 500 mV, however, in Lake Bafa it was measured as -98.7 mV. This is an expected result because of the high inputs of the sewage and industrial wastes into the lake. According to Inland Water Quality Parameters (Regulation of Surface Water Quality Management, Republic of Turkey Ministry of Forestry and Water Affairs); Class I brackish lake water parameters should be; pH 6.5-8.5, temperature $\leq$ 25 ºC, conductivity < 400 µS cm^{-1}, dissolved oxygen > 8.0 mg L^{-1}, chlorophyll a 3.5-9.0 µL^{-1}. Our measurements with pH was 8.3, conductivity 20.3 µS cm^{-1}, dissolved oxygen 3.8 µS/cm and chlorophyll concentration 13.6 µL^{-1} indicating that the Lake Bafa is an eutrophic lake in Class IV. All these parameters have impact on bloom formation and toxin concentration. Discharge from irrigated agricultural lands along the Lake Bafa coast may cause eutrophication in the lake. In addition to the domestic wastes of nearby village Kapikiri, farming and livestock breeding related pollution sources around the lake are the other factors that trigger bloom formation.

According to guidelines of WHO and Falconer et al [41, 42] on drinking and bathing waters there are 3 levels of cyanobacterial abundance that affect human health. In first level the chlorophyll a concentration is 10 µg L^{-1} or below. The toxic cyanobacterial toxin is expected to be below or close to the drinking water limits. In the second level, chlorophyll a concentration is 50 µg L^{-1} which represents potential health risks like irritation or allergenic responses. In the third level that the authors recommended prohibition of swimming and to prevent contact with water where the chlorophyll a concentration is higher than 100 µg L^{-1}[43].

Aydın Province Governership published a report on Buyuk Menderes River Basin Management Plan in 21.06.2011 highlighting the eutrophic parameters of Lake Bafa that leads cyanobacterial blooms, fish and bird deaths. The report mentioned about the low amount of dissolved oxygen and the high salinity that endanger the aquatic life dramatically. The population of *Cyprinus carpio* Linnaeus, 1758

and *Silurus glanis* Linnaeus, 1758 became extinct in the lake where the population of *Anguilla anguilla* Linnaeus, 1758, *Diplodus sargus* Linnaeus, 1758 and *Dicentrarchus labrax* Linnaeus, 1758 decreased in last decade.

In Yabanli et al [44]'s results measured on October 2006 in Lake Bafa the pH was 7.7, dissolved oxygen 5.5 μScm^{-1}, salinity was measured as 0.16 ‰, and the temperature was 23 ºC. Because of low temperature and salinity, there was no cyanobacterial bloom in the lake. The main differences between two studies are temperature and salinity that supports the *N. spumigena* formation conditions. In 1987 the salinity of Lake Bafa was 4‰ and from that year on the salinity started to increase to; 7‰ in 1989, 12‰ in 1999, 16‰ in 2006 and 23‰ in 2011. The reason why *N. spumigena* was not blooming in Lake Bafa before 2011 might be the lower salinity levels of the lake water. The high temperature and long daylight triggered the bloom formation. The requirement for irrigation water during the spring and the summer period considerably decreases the freshwater turnover in the lake and this is considered as the main reason of high salinity and low oxygen levels. It also diminishes the natural circulation in the lake.

The phytoplankton composition of Lake Bafa in October 2006 consisted of *Anabaena* and *Oscillatoria* but not *N. spumigena* [44]. In our study on June 2011, *N.spumigena* was the only phytoplankton species in blooming part of the lake.

Bolch et al. [45] found a close genetic identity between *N. spumigena* taxons from Baltic Sea and Australia, despite two regions had salinity and temporal differences. To make an accurate identification, it is important to compare the genetic variation of *N. spumigena* strains from different locations that have different water quality around the world. In our study, PCR, LC/Mand ELISA analyses confirmed that it was the nodularin producing *N. spumignea* that was present in Lake Bafa at the time of our sampling. Identification based on morphological features of Lake Bafa isolate also confirms that it is *N. spumigena*. 16S rRNA (Accession Number: JN409386) and phycocyanin (cpcB & cpcA) (Accession Number: JN377973) and nodularin gene sequences analyses confirmed the morphological identification with high bootstrap value (>99%), demonstrating that the sample is *N. spumigena* (Table 4, 5 and 6). Based on different phylogenetic tree constructions; NJ (Neighbour Joining), MP (Maximum Parsinomy) and ML (Maximum Likelihood) methods used in our analysis for 16S rRNA, phycocyanin genes have similar strain cluster. According to the ML tree of 16S rRNA region, Lake Bafa isolate appeared to be a sister taxon (with 100% bootstrap value) with toxic *N. spumigena* isolated from Australia (AF268007). Lake Bafa isolates are found in the same cluster with other Australian and Baltic Sea isolates. The NJ tree constracted for phycocyanin gene regions represented high similarity (>99%) between Lake Bafa isolate and other isolates found in the GenBank. The percentage of similarity and genetic distance support that the *N. spumigena* strain analysed in this study is a toxic species. Moffit et al [46] and Lyra et al. [47] observed 16S rRNA gene sequence similarities within the *N. spumigena* phylogenetic cluster, which was consistent with our results. According to Lyra et al [44], planktonic *Nodularia sp*. with gas vesicles produces nodularin. This group studied 27 *Nodularia sp*. isolates from Gulf of Finland; all *N. spumigena* were isolated from plankton and characterized based on their gas vacuoles. Strains that did not produce nodularin, lacked gas vacuoles and were mainly isolated from different types of benthic habitats. Our samples were isolated from plankton and had gas vesicles, and this finding also supports it that was a toxic *Nodularia* species.

Nodularin extracted from *N. spumigena* culture was used as a standard for ELISA and LC/MS analysis. Nodularin concentration in d. w. of the cyanobacterial material was 405 $\mu g\ g^{-1}$ d.w. by LC/MS and 312.5 $\mu g\ g^{-1}$ d.w by ELISA. This amount is lower than Iznik Lake *N. spumigena* concentration revealed by Akcaalan et al [48] which was 444.6 $mg\ g^{-1}$ d.w. by ELISA and 578 $\mu g\ g^{-1}$ d.w by HPLC, it is, however, higher than permitted health limits. According to the World Health Organization (WHO) [41] guideline, the microcystin and nodularin value in drinking water is less than 1 $ng\ mL^{-1}$.

According to our predictions, the temperature of the lake water will increase on the surface as well as down to 3 meters depth. Evaporation because of the high temperature will most likely increase the lake salinity. This can have severe effects since *N. spumigena* may benefit from such an increase. Further, penetration of light in the lake would decrease as a consequence of dense blooms, shadowing macrophytes and potential increase in nutrition and recruitment of fish. Thus, climate change in Lake Bafa may provide suitable habitat for cyanoabcteria such as *N. spumigena* and thus have severe effect on whole food chain.

There is no available data on Turkish cyanobacterial blooms and molecular identification of cyanobacteria in literature. *M. aeroginosa* (Kützing) Kützing blooms reported in Küçükçekmece Lagoon [49], Golden Horn [50] and Eğirdir Lake [51]. Akcaalan et al [52]. identified *N. spumigena* bloom and they also identified *Planktothrix rubescens* bloom in Iznik Lake. However, all samples were identified morphologically in these studies. Our study is the first data on molecular identification of Turkish toxic blooming cyanobacteria.

In conclusion, this is the first report of toxic *N. spumigena* bloom in Lake Bafa and second report in Turkey and European freshwaters. Occurance of toxic *N. spumigena* bloom in Lake Bafa may threaten the wildlife in the lake and damage the environment. The cyanobacterial toxin analysis should also be taken into consideration for human and animal health in the area.

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