Quality Assurance and Quality control methods

Field sampling, microscopic analysis and biovolume measurements.

During each sampling, general physical parameters (temperature, pH) was measured via a YSI 556 MPS probe. Replicate 1 L sterile bottles filled with water from 50 cm below the surface of the lake and were fast transferred to the laboratory within 1 h. Each replicate bottle was treated as independent sample from which individual replicates for analysis were obtained.

Duplicate samples for the enumeration of phytoplankton cells were preserved in glutaraldehyde (1%) and as well as in Lugol's iodine (5%) solutions and stored at 4^0 C.

Cell densities of phytoplankton populations were determined in two ways. The density of cyanobacteria colonies within replicated samples was determined using a Palmer or Sedgewick-Rafter counting chamber. Colonial cyanobacteria were dominated almost exclusively by Microcystis sp., Anabaena sp., and Aphanizomenon sp. Enumeration of Anabaena and Microcystis cells per colony was determined in at least 10 colonies per genera per sample (some samples had <10 colonies per chamber) and used to calculate cells mL^{-1}. Lugol's iodine preserved plankton samples were also settled in counting chambers and enumerated by inverted light microscope. Replicated sample analyses using these methods yielded relative standard deviations of <20% for the major groups quantified. Cell volumes of the three most numerous groups of phytoplankton (Microcystis sp., Anabaena sp., and Aphanizomenon sp.,) were estimated by measuring the length and width of>20 cells from each category on every date and using volumetric equations corresponding to the geometric shape each organism most resembled [1].

Chlorophyll measurements and chemical analysis

In the lab, nutrient samples were filtered onto glass fiber filters (GFF; for dissolved nutrients) and stored frozen. Samples for chlorophyll a analysis were gently filtered onto glass fiber filters (GFF) and stored frozen. Nutrient samples were analyzed spectrophotometrically for nitrate, phosphate, ammonia [2,3] . Duplicate samples were measured also by the General State Chemical Laboratory (Division of Ioannina, Greece). In all cases measurements were made according to Standard Methods for the Examination of Water and Wastewater (21rst edition 2005, A.P.H.A., A.W.W.A and W.P.C.F.).

Chlorophyll a was determined fluorometrically using the method described by Welschmeyer[4]. For all of our samples measurements were made in triplicates, by two different people.

Microcystin analysis and ELISA measurement

Cyanobacterial pellets lyophilized within 1 h of collection. Microcystin extraction was performed as previously described [5] with minor modifications as follows. Two to 15 milligrams of lyophilized cyanobacterial pellets were homogenized with 10 ml of 75% methanol in water for 3 min through grinding. Samples were sonicated for 15 min, and then incubated at room temperature (RT) for 20 min with vigorous shaking. The above step repeated three times. At the end of each incubation, the samples centrifuged at 8500 rpm for 10 min. Supernatants were transferred to new tubes and stored at 4^0 C. Pellets were resuspended in 10 ml 75% methanol and incubated overnight (O/N) with vigorous shaking. The entire process was repeated twice the following day and all supernatants were pooled. Previous work[6] has shown that this extraction protocol gives >90% recovery of anatoxin-a and microcystin-LR. Following extraction, the methanolic extract was stored at -20 ^{0}C until analysis.

We used commercially available ELISA kits (Abraxis, Warminster, PA, USA) to measure microcystin loads according to the manufacturer's instructions. The Abraxis ELISA kit quantifies most types of microcystins (microcystin-LR, microcystin-RR, microcystin-YR microcystin-LF,microcystin-LW, demethylated microcystin-RR, demethylated microcystin-LR, etc.) and expresses the total amount of microcystins in microcystin-LR equivalents. For ELISA measurement, appropriate volumes of each sample were evaporated to dryness by speed vac at low temperatures and the residues were then dissolved in 50 µl of water. All the samples were measured in duplicates.

DGGE, cloning and sequencing

Initially, PCR amplified products were loaded onto 8% (w/v) polyacrylamide gels 1mm thick, in 1XTAE (20mM Tris acetate (pH 7.4), 10mM acetate, 0.5 mM EDTA) with a denaturing gradient containing 30-80% denaturant (100% denaturant corresponded to 7M urea and 40% (v/v) formamide). Electrophoresis was performed for 16h at 75V and the temperature was set at 60 ^{0} C. Ethidium bromide staining of DGGE gels revealed that even lower ITS bands do not migrate in the gradient containing more than 50% denaturant. For this reason and to achieve higher resolution analysis of our PCR amplified ITS sequences we used a gradient of 35-55% denaturant in our study.

A small block of gel from the middle of all bands detected after ethidium bromide staining was excised and incubated in 50 µl sterile MilliQ water O/N at 4^0 C. The eluents were reamplified by using the original primer set and run on a new DGGE gel to confirm their identity. After identity's confirmation the PCR products subjected to dA addition by incubating for 30 min in the presence of Taq polymerase. This step is crucial, since proofreading polymerases such as Expand high fidelity DNA polymerase (Roche) which was used in our study didn't add dA overhangs at the ends of their PCR products.

PCR products with dA overhangs were purified using a Macherey- Nagel DNA clean-up kit (Nucleospin Extract), and subsequently they were cloned using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Three colonies from each transformation reaction were picked, cultured and plasmid DNA was extracted by using the plasmid kit (Macherey-Nagel). We picked three colonies from each transformation reaction since although the identity of the PCR products had been checked, we could not exclude the possibility that different bands with the same size and GC content migrate together on a DGGE gel. Inserts from all of our plasmids were fully determined by sequencing both strands. Sequencing was performed by Macrogen (Macrogen Inc., Seoul, Korea).

References

1. T.J. Smayda, From phytoplankters to biomass. In: Sournia, A. (Ed.), Phytoplantkon Manual. UNESCO, Paris, 1978, pp. 273–279.

2. T.R. Parsons, Y. Maita, C.M. Lalli, A Manual of Chemical and biological Methods for Seawater Analysis. 1984. Pergamon Press, Oxford.

3. M.N. Jones, Water Res. 1984, 18, 643–646.

4. N.A. Welschmeyer, Limnol. Oceanogr. 1994, 39, 1985–1992.

5. K.M. Karlsson, L.E. Spoof, J.A. Meriluoto, Environ. Toxicol. 2005, 20, 381–389.

6. A. Hotto, M. Satchwell, G.L. Boyer, Environ. Tox. 2005, 20, 243–248.

Table 1. Seasonal variation of: Temperature (T), pH, NH_4, NO_3, NO_2, TP (Total Phosphorous), volumetric microcystin concentrations, Chlorophyll α concentrations and counted cell numbers. (bdl: below detection limit).

Date	T	pH	NH_4 mg/l	NO_3 mg/l	NO_2 mg/l	Total Phosphorus	microcystin µg/l	Chlα µg/l	Total cyanobacterial cell number/l	Microcystis cell number/l	Filamentous cyanobacteria cell number/l	biovolume mm^3/l
11/08/04	27,20	8,40	0,20	0,37	0,0040	0,36	4,80	29,15	4,40E+07	1,20E+07	3,20E+07	4.3
28/09/04	22,70	8,60	0,05	0,43	0,0030	0,40	19,50	50,35	1,77E+08	1,70E+08	7,90E+06	11,987
22/10/04	17,90	8,90	0,38	0,34	0,0050	0,32	12,30	36,00	1,34E+08	1,30E+08	4,00E+06	8,940
11/11/04	12,50	8,30	0,29	0,55	0,0090	0,80	6,10	10,60	2,54E+07	1,80E+07	7,40E+06	2,020
09/12/04	8,20	8,20	0,07	0,23	0,0010	0,85	2,20	5,10	1,22E+07	9,00E+06	3,20E+06	0,932
05/01/05	6,20	8,70	0,20	0,48	0,0030	0,45	0,01	1,30	2,50E+05	2,50E+05	ND	0,016
22/02/05	5,30	8,70	ND	0,39	0,0050	0,60	0,03	1,70	7,50E+05	7,50E+05	ND	0,049
21/03/05	10,00	8,80	0,33	0,55	0,0060	0,50	0,30	8,20	4,50E+05	4,50E+05	ND	0,029
19/04/05	14,30	8,60	0,37	0,45	0,0060	1,20	0,02	9,70	5,10E+05	5,00E+05	1,00E+04	0,034
25/05/05	19,70	8,60	0,15	0,30	0,0020	0,35	0,03	11,30	3,95E+06	7,50E+05	3,20E+06	0,400
21/06/05	24,50	8,50	0,18	0,35	0,0040	0,30	1,70	28,50	3,83E+07	4,30E+06	3,40E+07	4,021
20/07/05	26,20	8,80	0,60	0,25	0,0090	0,16	4,50	80,41	1,22E+08	3,20E+07	9,00E+07	11,990
19/08/05	26,00	8,40	0,10	0,40	0,0060	0,40	3,10	43,22	6,20E+07	1,20E+07	5,00E+07	6,280
13/09/05	22,60	8,70	0,08	0,45	0,0040	0,35	6,10	32,00	8,60E+07	7,00E+07	1,60E+07	6,338
29/09/05	20,50	8,20	0,07	0,18	0,0020	0,55	18,20	38,10	1,48E+08	1,40E+08	8,00E+06	10,036
19/10/05	17,00	8,80	0,45	0,15	0,0060	0,25	14,70	35,20	1,19E+08	1,10E+08	9,00E+06	8,184
24/11/05	13,70	8,30	0,38	0,65	0,0100	0,80	2,30	4,30	9,20E+06	8,00E+06	1,20E+06	0,600
05/12/05	7,40	8,10	0,22	0,43	0,0050	1,01	0,98	3,70	7,40E+06	2,40E+06	5,00E+06	0,656

ND = non detected

Statistical analysis was done with the Instat V3.05 package (GraphPad Software Inc., San Diego, CA) Pearson's test demonstrated a highly significant correlation of microcystin concentrations (µg/l) with Microcystis cell loads (number/l) (*P* value <0,0001) but not with filamentous cyanobacteria (*p*=0,6).

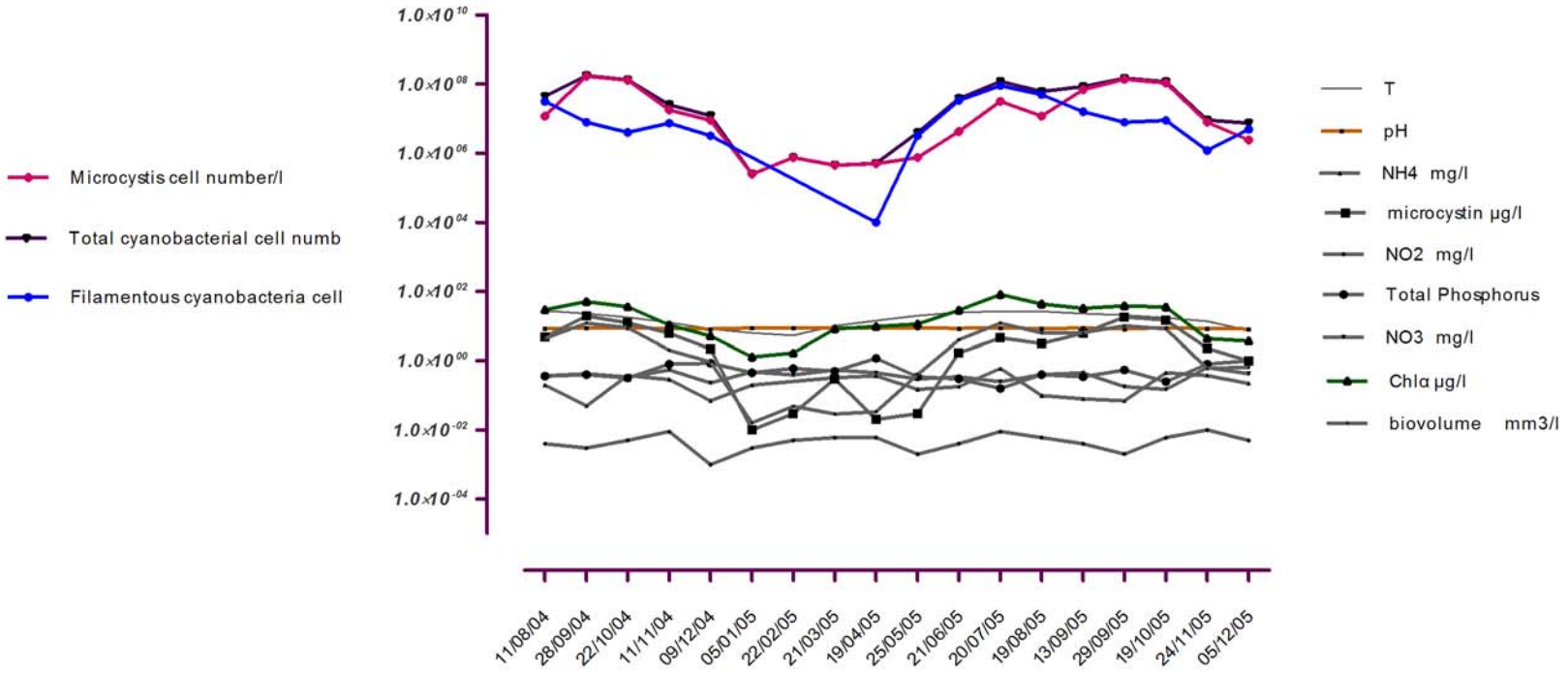

Table 2. Sequence analysis of excised DGGE bands. (*M.sp: Microcystis sp., A.spiroides: Anabaena spiroides, Aph.sp: Aphanizomenon sp.*, UFC: uncultured freshwater cyanobacterium, T: toxic strain, NT: non toxic strain, UNT: strain with unknown toxicity).

DGGE Band. No.	Base pairs sequenced	Identical to excised DGGE band	Closest matching organism	Base pairs compared (main stretch)	Similarity	Base pairs compared (second stretch)	Similarity
1	524		***M.sp K65,* AJ605173.1, (UNT)**	458	100%		
2	441		***A.spiroides,* AJ293215.1**	280	95%		
3	538	6,10,14	***M.sp. K17-m,* AJ605146.1, (NT)**	466	99%		
			***M.sp AICB 34*, AY672727.1 (UNT)**	464	100%		
4	Heterodublex						
5	533	9	***M.sp K17-l,* AJ605144 (NT)**	462	100%		
6	538	3,10,14	***M.sp. K17-m* AJ605146.1 (NT)**	466	99%		
			***M.sp AICB 34,* AY672727.1 (UNT)**	464	100%		
7	538	11,15	***M.sp AICB 34,* AY672727.1 (UNT)**	466	99%		
8	535		***M.sp K39,* AJ605185.1, (T)**	463	99%		
9	533	5	***M.sp K17-l,* AJ605144, (NT)**	462	100%		
10	538	3,6,14	***M.sp. K17-m,* AJ605146.1, (NT)**	466	99%		
			***M.sp AICB 34,* AY672727.1 (UNT)**	464	100%		
11	538	7,15	***M.sp AICB 34,* AY672727.1 (UNT)**	466	99%		
12	534		***M.sp K17-l,* AJ605144, (NT)**	462	99%		
13	448	18	***A.spiroides,* AJ293215.1**	270	94%		
14	538	3,6,10	***M.sp. K-17-m,* AJ605146.1, (NT)**	466	99%		

			***M.sp AICB 34,* AY672727.1 (UNT)**	464	100%		
15	538	7,11	***M.sp AICB 34,* AY672727.1 (UNT)**	466	99%		
16	527		***M.sp V145,* AY827827.1 (NT)**	529	97%		
17	441		***A.spiroides,* AJ293215.1**	267	94%		
			***Aph.sp W35,* AY827830.1**	246	95%		
18	448		***A.spiroides,* AJ293215.1**	270	94%		
			***Aph.sp W35,* AY827830.1**	247	95%		
19	534		***M.sp K17-l,* AJ605144 (NT)**	443	99%		
20	447		***A.spiroides,* AJ293215.1**	270	94%		
			***Aph.sp W35,* AY827830.1**	246	96%		
21	534		***M.sp K17-l,* AJ605144 (NT)**	462	99%		
22	523		***M.sp B23,* AY827804.1 (NT)**	524	97%		
23	525		***M.sp. B23,* AY827804.1 (NT)**	525	97%		
24	529	29	***M.sp K39,* AJ605185.1 (T)**	463	100%		
25	529		***M.sp K39,* AJ605185.1 (T)**	463	99%		
26	529	35	***M.sp V145,* AY827827.1 (NT)**	527	99%		
27	Heterodublex						
28	507		***UFC***	271	98%		
29	529	24	***M.sp K39,* AJ605185.1 (T)**	463	100%		
30	534		***M.sp. K39,* AJ605185.1 (T)**	463	99%		
31	441		***A.spiroides,* AJ293215.1**	270	95%		
			***Aph.sp T51,* AY827800.1**	244	95%	102	96%
32	447	42	***A.spiroides,* AJ293215.1**	270	95%		

			***Aph.sp T51*, AY827800.1**	246	95%	102	96%
33	447		***A.spiroides*, AJ293215.1**	280	94%		
			***Aph.sp T51*, AY827800.1**	245	95%	102	94%
34	485		***A.spiroides*, AJ293215.1**	280	95%		
			***Aph.sp W35*, AY827830.1**	245	96%	103	93%
35	529	26	***M.sp V145*, AY827827.1 (NT)**	527	99%		
36	449	41	***A.spiroides*, AJ293215.1**	280	96%		
			***Aph.sp T51*, AY827800.1**	244	96%	102	96%
37	490		***A.spiroides*, AJ293215.1**	270	96%		
38	451		***A.spiroides*, AJ293215.1**	280	96%		
			***Aph.sp T51*, AY827800.1**	246	96%	102	96%
39	669	43	***A.sp. BC-Ana-0026*, AJ496732.1**	285	96%		
40	521	44	***M.sp K75-u*, AJ605205.1 (NT)**	438	98%		
41	449	36	***A.spiroides*, AJ293215.1**	280	96%		
			***Aph.sp T51*, AY827800.1**	244	96%	102	96%
42	447	32	***A.spiroides*, AJ293215.1**	270	95%		
			***Aph.sp T51*, AY827800.1**	246	95%	102	96%
43	669	39	***A.sp. BC-Ana-0026*, AJ496732.1**	285	96%		
44	521	40	***M.sp K75-u*, AJ605205.1 (NT)**	438	98%		
45	533		***M.sp. K17-m*, AJ605146.1 (NT)**	428	100%		
46	444		***A.spiroides*, AJ293215.1**	270	96%		
47	443		***A.spiroides*, AJ293215.1**	274	96%		
			***Aph.sp W35*, AY827830.1**	244	96%	103	94%

48	445		***A.spiroides*, AJ293215.1**	280	96%		
49	522	52	***M.sp K75-u*, AJ605205.1 (NT)**	457	99%		
50	532		***M.sp. K17-m*, AJ605146.1 (NT)**	467	97%		
51	451		***A.spiroides*, AJ293215.1**	280	97%		
52	522	49	***M.sp K75-u*, AJ605205.1 (NT)**	457	99%		
53	522		***M.sp K75-u*, AJ605205.1 (NT)**	455	99%		
54	447		***A.spiroides*, AJ293215.1**	270	94%		
			***Aph.sp W35*, AY827830.1**	244	95%	103	93%
55	522	56	***M.sp K75-u*, AJ605205.1 (NT)**	457	99%		
56	522	55	***M.sp K75-u*, AJ605205.1 (NT)**	457	99%		
57	525		***M.sp K9*, AJ605150.1 (T)**	459	99%		
58	443		***A.spiroides*, AJ293215.1**	280	95%		
			***Aph.sp W35*, AY827830.1**	246	96%	124	94%
59	441	63	***A.spiroides*, AJ293215.1**	270	95%		
			***Aph.sp W35*, AY827830.1**	246	95%	103	94%
60	441		***A.spiroides*, AJ293215.1**	274	95%		
61	524		***M.sp K9*, AJ605150.1 (T)**	459	99%		
62	444		***A.spiroides*, AJ293215.1**	270	95%		
			***Aph.sp T51*, AY827800.1**	245	95%	102	96%
63	441	59	***A.spiroides*, AJ293215.1**	270	95%		
			***Aph.sp W35*, AY827830.1**	246	95%	103	94%
64	446		***A.spiroides*, AJ293215.1**	270	95%		
			***Aph.sp T51*, AY827800.1**	245	95%	102	96%

65	445		***A.spiroides*, AJ293215.1**	270	96%		
			***Aph.sp T51*, AY827800.1**	246	96%	102	95%
66	444		***A.spiroides*, AJ293215.1**	280	95%		
			***Aph.sp T51*, AY827800.1**	245	95%	102	94%
67	446		***A.spiroides*, AJ293215.1**	280	96%		
			***Aph.sp W35*, AY827830.1**	244	96%	103	94%
68	445		***A.spiroides*, AJ293215.1**	270	94%		
			***Aph.sp T51*, AY827800.1**	245	95%	102	95%
69	528		***M.sp V145*, AY827827.1 (NT)**	527	99%		
70	525		***M.sp. B23*, AY827804.1 (NT)**	525	98%		
71	524		***M.sp. B23*, AY827804.1 (NT)**	524	98%		
72	524		***M.sp K9*, AJ605150.1 (T)**	459	98%		

Table 3. Sample number, collection date, microcystin concentrations in lake water expressed as microcystin-LR equivalents, species, tissue, microcystin concentrations in tissues expressed as microcystin-LR equivalents in ng g^{-1} wet weight (MC-LR eq. ng g^{-1} ww) and the critical amount of edible muscle necessary to ingest to reach a tolerable daily intake (TDI) of microcystin (0.04μg kg^{-1} BW, or 2μg for an adult weighing 50 Kg BW) determined by WHO. (*Anodonta cygnea*: Swan mussel, *Carassius gibelio*: Prussian carp, *Barbus albanicus*: Albanian barbell, *Cyprinus carpio*: Common carp, *Rutilus ylikiensis:* an endemic roach of Greek freshwaters).

Sample No	Collection Date	microcystin in lake water μg/l	Organism	Tissue	MC-LR eq. ngr/gr. ww	Critical Amount for TDI (gr)
1	26/10/2004	10,80	*Anodonta cygnea*	muscle	69,60	28,70
2			*Anodonta cygnea*	muscle	87,40	22,80
3			*Carassius gibelio*	muscle	3,16	63,90
3				liver	44,20	-
4			*Barbus albanicus*	muscle	3,04	657,80
4				liver	53,80	-
5			*Cyprinus carpio*	muscle	3,14	636,00
5				liver	48,40	-
6	19/3/2005	0,20	*Anodonta cygnea*	muscle	62,40	32,00
7			*Carassius gibelio*	muscle	2,24	892,00
7				liver	21,00	-
8			*Rutilus yliciencis*	muscle	4,71	424,00
8				liver	26,20	-
9	12/6/2005	0,80	*Anodonta cygnea*	muscle	87,60	22,80
10			*Anodonta cygnea*	muscle	49,80	40,16
11			*Carassius gibelio*	muscle	2,06	1000,00
11				liver	17,60	-
12			*Carassius gibelio*	muscle	2,48	806,00
12				liver	20,40	-
13			*Cyprinus carpio*	muscle	3,04	657,00
13				liver	46,00	-
14	30/9/2005	17,80	*Anodonta cygnea*	muscle	102,20	19,60
15			*Anodonta cygnea*	muscle	72,80	27,40
16			*Carassius gibelio*	muscle	2,46	813,00
16				liver	14,20	-
17			*Carassius gibelio*	muscle	3,30	606,00
17				liver	41,60	-
18			*Barbus albanicus*	muscle	2,94	680,00
18				liver	56,20	-
19			*Cyprinus carpio*	muscle	2,56	781,00
19				liver	42,20	-