Health Risk Associated with Microcystin Presence in the Environment: the Case of an Italian Lake (Lake Vico, Central Italy)

Milena Bruno°[1], Pasquale Gallo*[2], Valentina Messineo°[3], Serena Melchiorre°[4]

°Department of Environment and Primary Prevention, National Institute of Health, Viale Regina Elena, 299-00161, Rome, Italy
[1]mbruno@iss.it
[3]vmessineo@hotmail.com
[4]serena.es@libero.it
*Experimental Zooprophylaxys Institute of Mezzogiorno
Via Salute 2, 80055 Portici (NA), Italy
[2]pasquale.gallo@cert.izsmportici.it

Abstract— **Cyanobacterial toxins are considered a worldwide cause of human poisoning and an important health hazard for human beings. Their presence is generally a direct consequence of a poor human use of surface waters, with increase of nutrient levels due to sewage discharges or to agricultural run-off. At present research groups make efforts to plan effective risk management strategies, and to elaborate risk assessment evaluations taking into account all the possible routes of exposure for the resident human populations. This study shows the case of Lake Vico in Central Italy, hosting annual toxic blooms of *Planktothrix rubescens* with microcystin contamination in lake water, fish species, groundwater and agricultural products, together with arsenic presence in groundwater and lake water. Investigations during 2008-2009 detected microcystin contamination in five fish species (from 0.21 to 411.5 ng/g body weight), in lake water (from 0.42μg/L to 350μg/L) and in treated drinking water plans (from 0.17 to 0.50μg/L). The content of microcystins in some fish samples was confirmed also by liquid chromatography coupled to ion trap mass spectrometry. The calculated EDI according to WHO proposed guidelines went from 5.1 to 123.4 g microcystins, 2-51.4 times higher than the WHO TDI value. In 2010 analyses performed on *Corylus avellana* fruits from cultured trees growing near the lake shore detected microcystin contamination. This evidence, and the presence in the lake, in its groundwater and in local drinking water plans of As levels exceeding the WHO limits for water uses, lead to consider the need to evaluate all the possible environmental variables influencing the assessment of the health risk for local human populations exposed to microcystin contamination.**

Keywords— **Microcystins; Lake Vico; Accumulation; Fish; Water; Risk Assessment**

I. INTRODUCTION

Occurrences of cyanobacterial blooms have been increasingly detected in lakes and reservoirs throughout the world (Falconer and Humpage, 2005), due to anthropogenic eutrophication, catchment modification and climate change (Paerl and Huismann, 2008; Conley et al., 2009). Because toxic cyanobacteria are not able to colonize and grow in human or animal hosts to cause disease, they are not listed among waterborne pathogens in the water industry, leading to possible underestimation of health risk.

75% of monitored water samples contaminated by cyanobacteria also contain toxic metabolites (Sivonen and Jones, 1999) that are a recognized cause of livestock and human poisoning (Falconer, 2005; Bowling, 1992; Stein, 1945, Jochimsen et al., 1998; Carmichael et al., 2001; Behm, 2003).

In some countries (i. e. South Africa) toxic cyanobacteria represent an increasing and serious environmental hazard for livestock and wild animals while human hazard could result from chronic exposure via contaminated water supplies (Oberholster et al., 2005).

Furthermore some toxins, like microcystins, can be transferred along food chain (Smith and Haney, 2006), representing a potential threat to human consumers not only for their presence in raw fish, but also for their stability and concentration during cooking (Bruno et al., 2009) and increase of free microcystins in fish tissue during specific cooking, like boiling (Zhang et al., 2010).

Microcystins are considered among the most dangerous toxic groups, endowed with serious acute and chronic poisoning. They are synthesized non-ribosomally by a large peptide synthetase and polyketide synthase enzyme complex; the responsible gene cluster has been sequenced (Nishizawa et al., 2000; Tillet et al., 2000) and its toxin production seems to be switched on and off in an intermittent manner (Wood et al., 2010). Microcystin – LR, the most studied variant of this group, is a very effective tumor promoter (classified as 2B in the IARC cancerogenic scale, IARC, 2006) which action, mediated by the inhibition of protein phosphatases PP1/PP2A, induces oxidative DNA damage (Zegura et al., 2003), the activation of proto-oncogenes c-jun, c-fos, c-myc (Li et al., 2009) and of the nuclear factor Nrf2 (Nuclear factor erythroid 2-related factor 2; Gan et al., 2010).

Recent studies on polluted lakes pointed out the risk of human exposure to microcystins through both direct (drinking water, recreational activities) and indirect (fish consumption) routes (Zhang et al., 2009; Chen et al., 2009).

Several Italian lakes have been investigated for the presence of cyanobacterial populations causing periodic blooms with toxin production (Messineo et al., 2009) and fish contamination (Bruno et al., 2009). This study was undertaken in order to investigate possible fish, agricultural products and drinking water contamination in the Italian Lake Vico, known to host a microcystin producing population of Planktothrix rubescens (Mazza et al., 2008).

II. MATERIALS AND METHODS

A. Study Site

Lake Vico is an extended volcanic basin inside a crater depression of the Cimino Volcano Complex, 60 km north of Rome, Central Italy. The surface is 12.09 km2, the volume is 260.7 m3 x 106 and the depth reaches 48.5 m (mean depth 21.6 m); the total turnover time is 17 years (Carollo et al., 1974; Dyer, 1995). The lake is part of a Regional wildlife Park, and is listed as a wetland of international importance by Ramsar Convention. The neighbourhoods of Lake Vico host extended hazel woods of an appreciated and famous cultivar (the Gentle Rounded Roman). The lake serves as drinking water reservoir for 10,000 people of two little towns, Ronciglione and Caprarola, and is a recreational area throughout the year.

Progressive eutrophication of the lake has been documented over the past several decades (Barbanti et al., 1971; Gelosi, 1985; Dyer, 1995; Franzoi, 1997; Mazza et al., 2008).

B. Fish, Water and Fruit Sampling

Samplings from January, 2008 to March, 2009 were carried out in the lake while hosting a Planktothrix rubescens population with a long-lasting, extended winter bloom. Twenty-five adult fish catched in different zones of the lake and fourteen water samples, plus nine fish samples previously collected and preserved during years 2006 (March) and 2007 (June), were analyzed for microcystin contents. Two adult trouts (Salmo trutta) from a fish farm were used as negative control.

The analyzed fish species were the zooplanktivorous species Coregonus lavaretus (whitefish, 27 individuals), the zoobenthivorous species Tinca tinca (tench, 3 individuals), the carnivorous species Lepomis gibbosus (pumpkinseed, 2 individuals), Perca fluviatilis (perch, 2 individuals) and Esox lucius (pike, 1 individual). The two perches were analyzed only by ion trap LC/ESI-MS/MS.

Ten water samples (1 L of volume) were collected by filling 1 L Pyrex glass bottles 10-20 cm below the water surface from five stations (Fogliano, Renari, Bella Venere, Fondo Cencio and Riva Azzurra) close to the lake shore. Four water samples from municipal and private water supplies were collected from January 2008 to March 2009. The samples were stored in ice chests and transported to the laboratory. For microscopic observations subsamples were analysed by an inverted microscope (Leitz Labovert FS), according to Utermöhl (1931) and Lund et al. (1958), using 25 ml sedimentation chambers for phytoplankton identification and cell density estimation.

In 2010 another long-lasting bloom of P. rubescens occurred in the lake from winter until May. Ten green fruits were sampled in July 2010 from three hazel trees growing on the bank of the lake. Ten green fruits from hazel trees growing 20 Km far from the lake area were sampled too, and analyzed as blank.

C. Chemicals and Reagents

Microcystin-LR (MC-LR), microcystin-YR (MC-YR) and microcystin-RR (MC-RR) were supplied by DHI Water and Environment (Denmark); microcystin-LF (MC-LF) and microcystin-LW (MC-LW) were supplied by Vinci Biochem (Vinci, Italy) in screw capped vials containing 25 µg standard. All the reference materials were of analytical grade purity.

Trifluoroacetic acid (TFA), HPLC grade acetonitrile, methanol and glacial acetic acid (99%) were from Carlo Erba (Milan, Italy). HPLC grade water was produced using a MilliQ system (Millipore, Bedford, MA, USA). C18 end-capped (EC) solid-phase extraction (SPE) cartridges with 1 g sorbent bed and 6 mL reservoir volume (Isolute, UK) were purchased from Step-Bio (Bologna, Italy).

D. Standard Solutions of MCs

MC-RR, MC-LR and MC-YR reference materials were purchased in standard stock solutions in methanol, at 10 µg/mL, 10 µg/mL and 6.3 µg/mL, respectively. To prepare MC-LF and MC-LW standard stock solutions at 10 µg/mL, the vial content (25 µg) of each compound was dissolved in 2.5 mL methanol. A MCs mix standard solution at 2.5 µg/mL, containing MC-RR, MC-LR, MC-LW and MC-LF, was prepared by mixing equal volumes of each standard solution at 10 µg/mL, while the MC-YR standard stock solution was opportunely diluted in methanol to prepare a working solution at 2.5 µg/mL. All the standard stock solutions were stable for at least six months if stored in the dark at -20°C. The mix working standard solutions of the five MCs at 10, 25, 50, 75, 100 ng/mL were prepared daily from the standard mix solutions at 2.5 µg/mL by diluting with methanol.

E. Water Analysis

The filtered water samples, from raw lake water and from municipal water supplies, were directly analyzed in triplicate with the EnviroGard ELISA kit for microcystin detection. Microcystins dissolved in water were analyzed.

F. Fish Tissue Extraction

Extraction was performed as in Bruno et al., 2009. Briefly, tissues (5.0 ± 0.1 g, liver or muscle or ovary) were homogenized in 10 mL 100% MeOH for 15 min. using a Potter Homogenizer (Polytron), then sonicated 5 min. at 30–40°C in an ultrasonic bath (Elgasonic Swiss made, 25 kHz) to disrupt cells. The sample was then centrifuged for 5 min. at 5000 g and the supernatant decanted and filtered. The extraction was repeated on the pellet, the sample was centrifuged and the supernatant filtered on the same filter previously used. The filter and the funnel were washed three times with little volumes of MeOH; the two supernatants and the washings were collected together, then reduced to a small volume (1-2 mL) by rotavapor at 40°C and brought to a volume of 5 mL with 100% MeOH. One mL of this extract (corresponding to 1.0 g tissue) was then diluted with 1 mL of distilled water and applied to a HLB SPE Waters OASIS cartridge preconditioned with 1 mL of 100% MeOH followed by 1 mL of distilled water. The column was washed with 1 mL of MeOH/distilled water 5%. Microcystins were eluted with 1 mL of 100% MeOH.

The 100% MeOH fraction was then dried by rotavapor at 40°C; the residue, re-suspended in 2 mL distilled water, was stored at -30°C for subsequent microcystin analysis in triplicate with the EnviroGard Elisa kit.

G. Fruit Extraction

The fruits were deprived of the shell, weighted (mean weight 2 g), grinded to powder first with a mortar and pestle then in an electric grinder for 10 min., and finally sonicated in distilled water for 5 min. at 30 – 40 °C, as described for fish tissue extraction.

After the two sonications and centrifugations, the supernatants were pooled and analyzed in triplicate with the EnviroGard kit.

H. Microcystin Analysis by Enzyme-Linked Immunosorbent Assay (ELISA)

The EnviroGard Microcystins Plate Kit (Strategic Diagnostics Inc., Newark, DE, USA) is a direct competitive ELISA for quantitative detection of microcystins and nodularins (limit of quantification, LOQ = 0.1 ppb). It does not differentiate between microcystin-LR and other microcystin variants but detects their presence to differing degrees. The concentrations at 50% inhibition (50% B/Bo absorbance signal) for these compounds (ppb) are: microcystin-LR 0.31, microcystin-RR 0.32, microcystin-YR 0.38.

The final reaction solution absorbances of the kit were measured at 450 nm with an Anthos 2010 microplate spectrophotometer (Anthos – Labtech, Salzburg, Austria).

I. Fish Sample Clean Up

The muscle of two perches from Lake Vico, after removal of skin, was homogenized by ultra-turrax; 5.00 ± 0.01 g were weighed, extracted by shaking for 3 min with 20 mL of the mixture MilliQ water/methanol 1/3 v/v containing 0.1% trifluoroacetic acid (TFA), then centrifuged at 1086 g for 10 min. The upper liquid phase was separated and reduced to about 5 mL in rotovapor at 40°C.

Twenty mL MilliQ water were added, and the sample were loaded by gravity on a SPE C18 end-capped cartridge, previously rinsed with 5 mL methanol and equilibrated with 5 mL MilliQ water. The cartridge was washed with 5 mL of MilliQ water/methanol 95/5 v/v, dried under vacuum for 30 min. The SPE column was eluted with 5 mL TFA 1% in methanol v/v; the sample was dried under a nitrogen stream at 40°C, then dissolved in 1 mL methanol and analysed by ion trap LC/ESI-MS/MS.

L. Ion trap LC/ESI-MS/MS Analysis

Analyses were carried out using a LC/ESI-MS system, equipped with a quaternary pump Surveyor LC pump Plus, a Surveyor plus autosampler and LCQ Advantage ion trap mass spectrometer with an electrospray ion source (ESI) (Thermo Fisher, Milan, Italy). Chromatographic separation was performed injecting 50 µL sample volume on a 4 µm particle 250 × 3.0 mm Max RP 80 Å Synergi stainless steel column (Phenomenex, Torrance, CA, USA), at 0.3 mL/min flow rate, using 0,05% TFA in water as mobile phase A, and 0,05% TFA in acetonitrile (ACN) as mobile phase B. The chromatography was carried out by linear gradient at room temperature, according to the following programme: 2 minutes at 30% B, then from 30% B at time 2 to 100% B in 16 minutes, holding on for 8 minutes, finally to 30% B in 3 minutes; the equilibrium time between runs was 12 min. The mass spectrometer was periodically calibrated with standard solutions of Ultramark, caffeine and Met-Arg-Phe-Ala peptide provided by the manufacturer. During the LC/ESI-MS/MS experiments, mass spectra were acquired in the positive and negative ionisation mode; the spectrometer parameters were optimised by tuning on the [M+H]+ ion of MC-RR, at m/z 1038.5, and on the [M-H]- ion of MC-LF, at m/z 1023.5. Tuning was performed at 0.3 mL/min LC flow rate. The following experimental LC/ESI-MS/MS parameters were set: capillary temperature 300 °C, spray voltage 4.5 kV, microscan number 3, maximum inject time 200 ms, Further experimental conditions (collision energy, isolation width), and the diagnostic ions for MS/MS qualitative analysis, are also reported (Table 1). The LC/ESI-MS/MS analysis was carried out by monitoring the signals of the precursor ion → product ion transitions (selected reaction monitoring, SRM mode) from the LC/ESI-MS/MS dataset of each MC; to improve method specificity and sensitivity for both MC-LW and MC-LF, a MS3 event was introduced, to obtain another diagnostic ion (Table 1). The data were acquired and processed using the XcaliburTM software, version 1.3, from Thermo Fisher.

Both blank samples and samples spiked at 0.5ng/g and 1.0 ng/g with each MC were analysed. The chromatograms obtained extracting the precursor ion→main product ion transition (SRM) signals from the LC/ESI-MS/MS dataset were integrated to calculate the calibration curves. Calibration curves for testing method linearity in solvent were calculated by linear regression, using standard solutions in methanol 10, 25, 50, 75, 100ng/mL. During each working session, a blank reagent, blank and spiked samples were analyzed.

TABLE I.
EXPERIMENTAL CONDITIONS AND SELECTED DIAGNOSTIC IONS FOR ION TRAP LC/ESI-MS/MS ANALYSIS

	MS^2 conditions			**MS^2 and MS^3 conditions**	
	MC-RR	**MC-YR**	**MC-LR**	**MC-LW**	**MC-LF**
Ionization mode	**positive**	**positive**	**positive**	**negative**	**negative**
Maximum injection time (ms)	300	300	300	300	300
Isolation width (m/z)	2	2	2	2	2
Molecular ion (m/z)	1038.5	1045.6	995.7	1023.5	984.5
Selected precursor ion for MS^2 (m/z)	1038.5	1045.6	995.7	1023.5	984.5
Collision energy (%) for MS^2 transition	32	35	35	38	40
Selected diagnostic MS^2 ions (m/z)	1020.6	1027.4	977.7	1005.5	966.5
	909.5	1017.5	967.6		
		916.5			
Selected precursor ion for MS^3 (m/z)				1005.5	966.5
Collision energy for MS^3 transition (%)				37	38
Selected diagnostic MS^3 ions (m/z)				804.2	765.5

III. RESULTS

Lake Vico (Latium Region) hosts populations of P. rubescens blooming every year, from winter to late spring. The populations, resting at – 25 m during summer, slowly ascend in autumn, forming blooms that reach the surface in winter. Phytoplanktic fish feed on the blooms, accumulating the toxins.

During 2008-2009 blooms, P. rubescens represented 95% of the total phytoplankton. Seven out of ten analyzed water samples from five lake stations showed the presence of microcystins from February to May 2008, from a minimum of 0.42 □ g/L (May 2008) to a maximum of 350 µg/L (March 2008, Bella Venere station). Three samples from a public fountain and two private tap waters of Caprarola town showed presence of microcystins from 0.17 µg/L to 0.50 µg/L (March, 2009; table 2). The town assumes water for drinking purposes

from the lake, but its water plan is not provided by adequate filters (i.e. carbon filters) for biotoxins or heavy metals, and fixed quantities of chloride compounds are used throughout the year, even when algal biomasses are too much extended to allow a satisfactory treatment with the available chloride.

Of the 34 lake fish, 59 fish tissue samples were analyzed with ELISA kit for microcystin presence: 93.3 % of total fish samples (31 muscle, 27 viscera and 1 ovary) were contaminated, at concentration values ranging from a minimum of 0.21 to a maximum of 411.5ng/g b.w. (wet weight). Microcystins were detected in 97% of viscera samples (ranging from 1.37 to 411.5 ng/g with an average concentration of 29.84 ng/g), in 87% of muscle samples (from 0.21 to 26.56 ng/g with an average concentration of 13.32 ng/g) and in the ovary sample. In January, 2008 microcystin concentration in fish muscle samples (Coregonus lavaretus) reached 26.56ng/g (tab. 2).

Control trouts were negative. The presence of microcystins in two fish (Perca fluviatilis) was confirmed by ion trap LC/ESI-MS/MS analysis. The compounds MC-RR, MC-LR, MC-LF, MC-YR abd MC-LW were completely separated by reversed phase HPLC on a C12 stationary phase, and unambiguously identified by at least 2 product ions produced in the MS2 and MS3 SRM mode on ion trap. The limits of quantification (LOQs) of the method are 0.5 ng/g for MC-RR, MC-LR, MC-YR, and 1.0 ng/g for MC-LW, MC-LF, showing ion trap mass spectrometry is a very sensitive and reliable hyphenated technique for microcystin determination in fish muscle. Mean recoveries ranging from 74.5% (MC-LW) to 90.1% (MC-LR) were measured; method repeatability at 1 ng/g was evaluated by the coefficient of variation (RSD %), ranging between 3.8 – 18.2 %, over six determinations. The contamination levels measured in the two fish samples analyzed are reported in Table 3; only MC-LF was identified in the samples analyzed, collected in 2006.

The difference of concentrations between muscle and viscera (the latter being generally more contaminated) varied noticeably (from two thousand-fold in a whitefish of 2006, March to equality in a whitefish of 2008, June).

To calculate EDI from our data, we used both the WHO TDI values of Chorus and Bartram (1999) and the more recent USEPA TDI values (2006).

In Lake Vico region, fish are used to be consumed as preferred main course from spring to autumn. Fish are usually consumed roasted, and viscera are often not completely removed (liver, in particular).

For an adult human weighing 60 kg and ingesting 300 g serving of fish muscle (Magalhaes et al., 2003; Mohamed et al., 2003), according with the recent USEPA guidelines based on Heinze (1999) and developed for acute and chronic risk (0.006 and 0.003 microcystin g/kg b.w./day, respectively, USEPA, 2006), the microcystin level of 54,8 % of muscle samples from Lake Vico analyzed from March, 2006 to March, 2009 was even 22 -fold the recommended TDI value for acute risk, while the viscera positive for microcystins (27 samples) exceeded even 343-fold this value.

The WHO proposed TDI value (0.04 g/kg body weight/day, Chorus and Bartram, 1999) for an adult of 60 kg b.w. ingesting 300 g of edible fish, was exceeded by one muscle sample (3.3-fold, January, 2008) and by 59,2% of viscera samples (Fig. 1).

Professional freshwater fishing in Lake Vico officially begins not before April, due to the low temperatures and strong winter winds typical of this area.

In our study, in this season of legal fishing a percentage of 57% sampled fish (edible organs) according to WHO provisional TDI, and 54.8% (muscle tissue) and 100% (edible organs) sampled fish according to USEPA provisional TDI, was unsafe for consumers.

The hazelnut sample analyzed with the ELISA method for microcystin contamination gave a value of 1.62ng/g, while the sample taken 20 km far from the lake was negative. These two analyses were repeated using an ELISA kit of different sensitivity (the Abraxis anti-ADDA moiety kit which detects all the MCYST analogues containing the ADDA residue epitope) during other experimental studies. This test exhibited a value of 2.3ng/g for the sample coming from the lake shore, and a negative result for the other sample (unpublished results).

TABLE II
MICROCYSTIN CONTENT (PPB) IN WATER AND FISH TISSUE EXTRACTS FROM VARIOUS SPECIES OF LAKE VICO

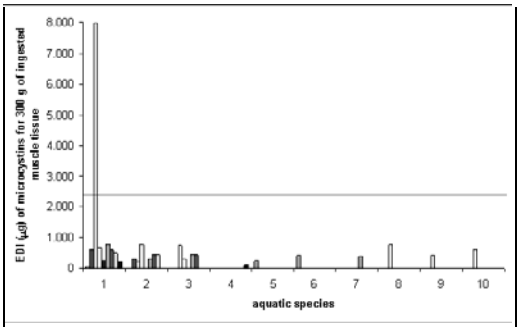
Fish	March 2006	June 2007	January 2008	February	March	April	May	June	November	March 2009
Coregonus lavaretus 1 muscle	0,21	1,99	26,56	2,19	0,85	2,58	1,99	1,68	0,71	
Coregonus lavaretus 1 viscera	411,5	1,37	39,19	28,57 Ovary 2,14		39,53	7,7	7,7		4,3
Coregonus lavaretus 2 muscle	n.d.	1,05	0,76	2,53		1,01	1,45	1,42	n.d.	
Coregonus lavaretus 2 viscera	3,68	19,65	3,16	42,17		6,96	11,03	25,4	2,97	
Coregonus lavaretus 3 muscle		0,80	n.d.	2,43		1,906	0,49	1,35	n.d.	
Coregonus lavaretus 3 viscera		1,84	18,01	43,2		10,46	8,58	5,94	3,33	
Coregonus lavaretus 4 muscle									0,33	
Coregonus lavaretus 4 viscera									2,41	
Lepomis gibbosus 1 muscle	0,83									
Lepomis gibbosus 2 muscle	1,40									

Esox lucius muscle						1,24				
Tinca tinca 1 muscle			2,53							
Tinca tinca 1 viscera			23,47							
Tinca tinca 2 muscle			1,33							
Tinca tinca 2 viscera			7,1							
Tinca tinca 3 muscle			2							
Tinca tinca 3 viscera			26,6							
Water Fogliano station				1,42						
Water Renari station				2,9	1,6					
Water Bella Venere st.				1,6	350		0,42			
Water Fondo Cencio st.					1,38		n.d.			
Water Riva Azzurra st.							n.d.	n.d.		
Public fountain via Belli Caprarola			0,35							
Tap water Poggio dei Cerri Caprarola				0,34						0,17
Tap water Prano Caprarola										0,50

VISCERA = LIVER, HEART, SPLEEN N. D. = NOT DETECTED

IV. DISCUSSION

The aqueduct of Caprarola city, although not provided by adequate filters for microcystins, is planned to mix the waters coming from the lake with the groundwater from two wells placed out of the caldera, quite far from the lake. This can explain the reduced microcystin quantity detected in the tap waters and the public fountain of the city in respect to the levels detected in the contemporary samples of the lake stations (table 2). Assuming that one adult drank 2 L of water/ day, the estimated daily intake (EDI) of microcystins from drinking water by local humans of Caprarola city in the examined period, went from 0.34 to 1μg.



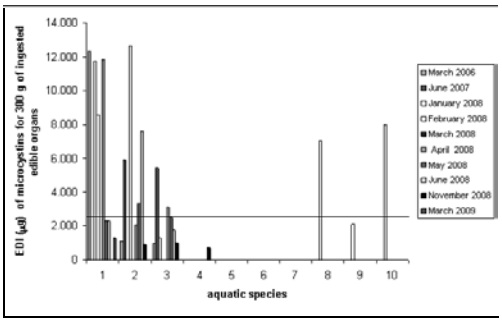


Fig.1 Estimated daily intake (EDI) of microcystins for muscle tissue (left graphic) or edible organs (right graphic) of *Coregonus lavaretus* (1-4), *Lepomis gibbosus* (5-6), *Esox lucius* (7), *Tinca tinca* (8-10). Horizontal line: WHO human (60 kg body weight) TDI for microcystins.

These levels are consistent with the microcystin contamination of drinking water detected in two toxic P. aghardii bloom episodes occurred in Finland (1989, between 0.1 and 0.5μg/L) and in Sweden (1994, 0.82 μg/L), responsible for severe outbreaks of acute illness (Annadotter et al., 2001).

Unfortunately, no epidemiological investigations could be planned in the Caprarola area to verify possible increased frequencies of gastrointestinal syndromes in the toxic bloom season, as suspected in other cases (Bruno, 2010).

Microcystin accumulation could be determined with ELISA kit in most of the fish samples, but during the P. rubescens blooming season, from November to April, the distribution of toxins in fish muscle (particularly in a planktonic species like whitefish) showed an inhomogeneous trend, with samples showing highly contaminated viscera and low concentrations in muscle. The differences among microcystin contents in fish from the same sampling could be explained with different routes of exposition (contaminated food, absorbance through the gills, etc.) for the single individual through feeding and/or resting places in the winter lethargy, due to winter slowing of metabolism and consequent reduced toxin depuration (Nolan et al., 2010).

Different whitefish populations in the same lake could be in lethargy or in a reduced activity. These differences could require an appropriate sampling frequency and in general a food safety testing specific to fish species, season and lake: i. e., one sample showed in January the highest levels of microcystin in muscle (tab. 2); more homogeneous values could be seen in spring-summer samples, even if an extracellular microcystin value of 350μg/L in March, 2008 (Bella Venere station) possibly produced an high value (39.53 ng/g) in viscera tissues of one fish sampled at the beginning of April in the same station.

TABLE 3
CONCENTRATION OF MICROCYSTINS DETERMINED BY ION TRAP LC/ESI-MS/MS IN SOME FISH SAMPLES FROM LAKE VICO.

Sample	MC-RR	MC-LR	MC-YR	MC-LF	MC-LW
Perca fluviatilis 1	nd	nd	nd	14	nd
Perca fluviatilis 2	nd	nd	nd	21	nd

ND: NOT DETECTED (< LOQ)

The zooplanktivorous Coregonus lavaretus is the fish species of main commercial value in the lake. The species population of Lake Vico is annually exposed to long-lasting blooms of P. rubescens, which microcystin production causes toxic accumulation in fish tissues.

German studies on sub-chronic and chronic exposure to P. rubescens in whitefish showed gill pathology, alterations in liver, kidney, gastrointestinal tract and in fitness, even after exposure to low cell densities (1.5 x 106/day for up to 28 days). The maximum microcystin concentration in whitefish tank was 11μg/L (MC-LReq, mean value). Stress and organ damage have been considered causal for weight and fitness reduction of coregonids in Bavarian lakes (Ernst et al., 2006; 2007). Data provided by our study related to 2008 microcystin levels in lake water,giving a peak value of 350μg/L, and data from the 2007 study (Mazza et al., 2008), giving a peak of 55.3μg/L, show the same periodic annual exposure for Lake Vico whitefish population, formerly observed in Bavarian lakes. Specific studies on fish pathologies have not yet planned, but reports from local professional fishermen refer to weight loss in whitefish individuals and smaller stocks in the fish populations, without any fishing increase.

Microcystin accumulation could be determined in most of the fish samples, indicating that an important percentage of the harvested aquatic species in the legal fishing season is unsafe for consumers. The EDI values, calculated in 2008-2009 for commercial fish according to WHO proposed TDI value, went from 4.8 to 122.4μg (2-51 times higher).

Totally, during the peak of the cyanobacterial bloom in 2008-2009 a daily intake of microcystins by local humans of Caprarola from fish and drinking water could be 5.1-123.4μg MC-LReq., 2-51.4 times higher than the unique TDI value proposed by WHO for acute and chronic effects, less restrictive than USEPA limits.

Microcystins accumulate in aquatic and crop plants through diffusion in eutrophized water bodies (Pflugmacher et al., 1999; Romanowska-Duda and Tarczynska, 2002) or through irrigation with water from contaminated plans (Codd et al., 1999; Mohamed and Al Shehri, 2009). The biochemical target of microcystin molecules makes every living organism vulnerable to their toxic effect. They represent a threat for quality and yield of crop plants and a sanitary hazard due to accumulation in edible vegetables (Ming-Yong and Hong-Tu, 1994; Takeda et al., 1994; Kurki-Elasmo and Meriluoto, 1998; Codd et al., 1999; McElhiney et al., 2001). Lake hazelnuts grow near the lakeshore and are sustained by the lake groundwater, which shows microcystin contamination for the year period corresponding to the P. rubescens blooms (Mazza et al., 2008).

Hazelnut production is the main agricultural activity in the Viterbo district and the Cimino Complex. Hazelnuts are not only exported, but also used to produce ice creams, cakes, cookies and chocolate creams, and are largely consumed by local populations. A prudential estimation of human daily consumption of hazelnuts and their derived products during the year in the Cimino population can be evaluated ranging from 30 to 150 g/day. The level of microcystin contamination, measured in the fruits grown near the lake in 2010, gives an estimated daily intake (EDI) ranging from 48 ng (70 ng according to the anti-ADDA ELISA) to 240 ng (340 ng according to the anti-ADDA ELISA) for the population of the two little towns (Ronciglione and Caprarola) in the volcanic caldera hosting Lake Vico.

The calculated EDI have not to be considered comprehensive, lacking the analysis of hazelnuts in 2008-2009, and the analysis of lake blooms and fish contamination in 2010.

The frequency of cyanobacterial blooms is an average of 74% for several Mediterranean Countries (Oberholster et al., 2005). In Italy, 46% of lakes and reservoirs was interested by this phenomenon in 2003 (Codd et al., 2005). Future nutrient loading coupled with climatic warming may promote increase in toxic cyanobacterial blooms (Davis et al., 2009).

Microcystin toxins are unlikely to have impact in Western Countries as acute poisoning cause. More serious could be the risk of hepatic or gastrointestinal cancer occurrence through chronic exposure to subacute doses in drinking water (Humpage et al., 2000; Zegura et al., 2003; Herfindal and Selheim, 2006). Moreover, recent studies suggest the possibility that damage could occur at lower levels than the provisional WHO TDI (Herfindal and Selheim, 2006; Chen et al., 2009), and remark that the toxin level has more value than cell number in water control (Conti et al., 2005; Messineo et al., 2006).

In fact preliminary epidemiological studies pointed out worldwide evidence of increased frequency for primary liver and colon tumors in areas with drinking water reservoirs affected by microcystin contamination (Ueno et al., 1996; Zhou et al., 2002; Fleming et al., 2002; Svircev et al., 2009).

Given the possible overlapping of acute human poisoning symptoms with a range of gastrointestinal illnesses, pathogens and agricultural or industrial pollutants are firstly suspected during outbreaks of enteric diseases, while microcystin toxins are considered as last chance (Texeira et al., 1993; Bruno, 2010).

Microcystins can be associated with the presence of other compounds like the neurotoxin BMAA (-N-methylamino-L-alanine), which generalized production in cyanobacterial species represents a new potential long-term human health hazard (Brand et al., 2010; Jonasson et al., 2010). The cyanobacterium P. rubescens also produces anabaenopeptins and aeruginosins (Ferranti et al., 2011), cyanopeptides characterized by minor toxicity compared with microcystins, which effect still has to be added to the total toxicity of this species. The presence of these toxins is generally not investigated when microcystins are detected, and a complete toxic risk assessment is not estimable.

Arsenic monthly detections in Vico lake water during a 2007 study (personal communication) gave values from 18 to 42μg/L (WHO limit for human consumption: 10μg/L). Arsenic was detected also in drinking water from the municipal water supplies of the two towns (from 19 to 54μg/L) and in groundwater wells nearby the lake (from 49 to 557μg/L). Arsenic is a cancerogenic chemical element classified as 1 in the IARC scale, and responsible for neural, liver, lung, lymphatic, skin tumors and pathologies. Its presence in lake water is quite constant throughout the years, and its pathogenic action could be possibly enhanced by the presence of microcystins, strong tumor promoters (Nishiwaki-Matsushima et al., 1991). These evidences, besides highlighting the need to plan a focused epidemiological study on Lake Vico human populations, show that contemporary presence in lake water of several components as cyanobacterial toxins and chemical toxic contaminants may lead to heavier risk evaluations in respect to a single detected toxin, suggesting also the need to fix lower limits for microcystins in case of carcinogens presence. Generally, the risk assessment estimate for water consumption may not be a simple evaluation.

Previous data related to 2007 P. rubescens blooms refer to total microcystin levels up to 55.3µg/L in lake water with a scum of 59.5 x 10^9 cell/L (January 2007), and up to 123 ng/L in groundwater wells (Mazza et al., 2008).

Apart from the registered maximum cell number, this toxic bloom was not evident at a naked eye; in 2010 the bloom responsible for fruit contamination was clearly evident at people from February to May (the blooms are red-coloured). This marked evidence may have implied a higher toxin production compared to the 2007 and 2008 blooms, with consequent higher microcystin contamination in fish tissue, groundwater and in the town aqueduct. A different, higher human risk level could have been caused in 2010.

Similar evidences in other Italian lakes (Messineo et al., 2006; Bruno et al., 2009) led us to consider that the risks associated with consuming contaminated drinking water, aquatic and agricultural products have to be systematically evaluated in respect to the annual population dynamics of the examined toxic species, but also to variations in total toxin production along multiannual series, as well as to the trophic evolution of the water body. So it might be more correct to think to the cyanobacterial toxin risk assessment as a dynamic variable, to which trend are not unrelated the epidemiologic conditions (i.e. of the age classes) of the exposed populations, too.

V. Conclusions

The contemporary presence of cyanotoxins (microcystins) and chemical risk factors in drinking water supplies, irrigation and recreational lakes like Lake Vico poses a potential hazard to human health and to agricultural and farming quality. The human risk assessment in these particular environmental conditions must be needfully considered into new schemes more comprehensive of other concurrent risk factors, in order to protect the health of the various groups of people at best.

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