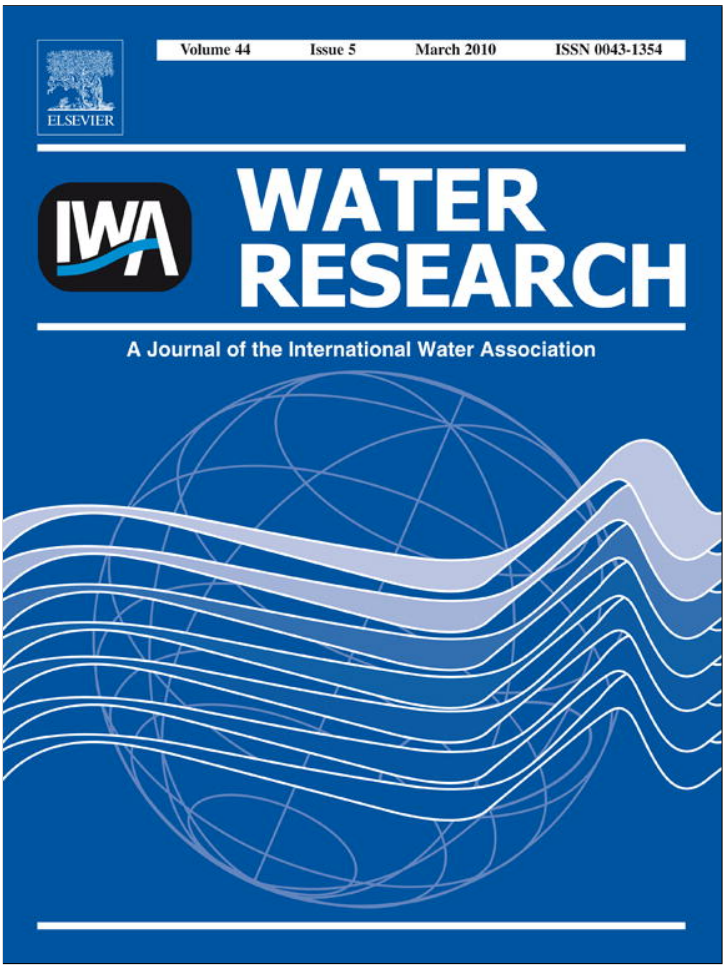

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Health risk evaluation associated to *Planktothrix rubescens*: An integrated approach to design tailored monitoring programs for human exposure to cyanotoxins

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ABSTRACT

Increasing concern for human health related to cyanotoxin exposure imposes the identification of pattern and level of exposure; however, current monitoring programs, based on cyanobacteria cell counts, could be inadequate. An integrated approach has been applied to a small lake in Italy, affected by *Planktothrix rubescens* blooms, to provide a scientific basis for appropriate monitoring program design. The cyanobacterium dynamic, the lake physico-chemical and trophic status, expressed as nutrients concentration and recycling rates due to bacterial activity, the identification/quantification of toxic genotype and cyanotoxin concentration have been studied. Our results indicate that low levels of nutrients are not a marker for low risk of *P. rubescens* proliferation and confirm that cyanobacterial density solely is not a reliable parameter to assess human exposure. The ratio between toxic/non-toxic cells, and toxin concentrations, which can be better explained by toxic population dynamic, are much more diagnostic, although varying with time and environmental conditions. The toxic fraction within *P. rubescens* population is generally high (30-100%) and increases with water depth. The ratio toxic/non-toxic cells is lowest during the bloom, suggesting a competitive advantage for non-toxic cells. Therefore, when *P. rubescens* is the dominant species, it is important to analyze samples below the thermocline, and quantitatively estimate toxic genotype abundance. In addition, the identification of cyanotoxin content and congeners profile, with different toxic potential, are crucial for risk assessment.

1. Introduction

Cyanobacteria blooms are becoming a quite frequent phenomenon worldwide. Several species produce different toxins that have been associated with acute human intoxications after exposure through drinking and bathing waters (Funari and Testai, 2008). The population can be exposed to cyanotoxins by using water for drinking and irrigation purposes, during recreational and professional activities and also through consumption of contaminated

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freshwater organisms and dietary supplements (Funari and Testai, 2008). Due to the health concerns related to cyanotoxin exposure for humans and livestock (Funari and Testai, 2008; WHO, 2004), an increasing number of monitoring programs are being carried out in many countries, to control the possible risk associated with cyanobacteria proliferation. However, most of them are currently based only on cell counts, a parameter which can be inadequate to identify patterns and levels of exposure, crucial in risk assessment and for the adoption of adequate protection measures.

Planktothrix is one of the most important genera in temperate climates amongst the widespread toxin-producing cyanobacteria: it is indeed distributed in middle European and southern sub-alpine mesotrophic and oligotrophic lakes, as well as in North American lakes (Chorus and Bartram, 1999). It is well adapted to low light conditions; during summer stratification, it is usually located within the metalimnion (Reynolds, 1997), due to the availability of nutrients. *P. rubescens* produces microcystins (MCs), the group of cyanotoxins most frequently reported as freshwater contaminants (Sivonen and Jones, 1999), characterized by their potent acute hepatotoxicity. For one of the most acutely toxic MC congeners, namely MC-LR, several studies strongly support a plausible tumour promoter mechanism, on the basis of which IARC classified MC-LR into group 2B (possibly carcinogenic to humans). Due to lack of adequate data *Microcystis* extracts are not classifiable as to their carcinogenicity to humans (group 3) (IARC, 2006). The human health effects associated to MC exposure have been extensively reviewed and reference values have been proposed, based on the analysis of available epidemiological and toxicological data (Funari and Testai, 2008; WHO, 2004; Chorus and Bartram, 1999; Codd et al., 2005).

MC production by a cyanobacterial population depends on its growth rate, with the highest amount produced during the late logarithmic phase, and by other factors such as temperature, light, culture age and nutrient availability (Sivonen and Jones, 1999). The role of these factors has been only partially clarified for *Microcystis* spp. and even less for *Planktothrix* spp. In addition, within a cyanobacteria population, different genotypes can occur and only some of them possess the genes for MC production (Kurmayer et al., 2002). Hence, the toxicity of a given bloom is strongly influenced by its strain composition (Kurmayer and Kutzenberger, 2003), although the factors driving community succession from one genotype to the other are still unknown.

The relationship between cyanobacteria and the rest of the bacterial community within a water body is quite often ignored; however, heterotrophic bacteria may have a role in cyanotoxin production and/or in its modulation (Codd et al., 2005) as well as in generating easily utilizable substrates and nutrients (Hoppe, 1983). Therefore this issue deserves further in depth investigation.

Based on these considerations, the uncertainties on the crucial factors related to cyanobacteria proliferation and toxin production are still numerous. In order to provide a specific scientific basis for the elaboration of tailored monitoring programs aimed to control human exposure, the present study was carried out in Lake Gerosa, a small artificial mountain lake in central Italy. The lake is used for bathing, irrigation, non-commercial fishing and as a potential reservoir for drinking water. Blooms in fall/winter months, attributed to *P. rubescens*, have been reported since 2002. An integrated approach has been applied, consisting of (i) the characterization of the physicochemical and trophic status of the lake and the possible role of the bacterial community in nutrient cycling, (ii) the identification and quantification of the toxic genotype, (iii) the quantification of cyanotoxins and (iv) a tentative risk assessment for the local community.

2. Materials and Methods

2.1. Chemicals

Acetonitrile was obtained from Lab-Scan (Dublin, Ireland), methanol from Panreac (Montcada i Reixac, Spain), glacial acetic acid from Carlo Erba (MI, Italy) and ammonium acetate from Baker (Deventer, Holland). Trifluoroacetic acid (TFA) was from Fluka (Bucks, Switzerland). High-purity water produced with a Milli-Q Milli-ρ system (Millipore, Bedford, MA, USA) was used. Commercially available MC congeners MC-LR, MC-RR, MC-LA, MC-YR and MC-LW were purchased from Alexis (San Diego, CA, USA), whereas Nodularin was from Sigma–Aldrich (St. Louis, MO). Individual standard solutions of the five MCs and Nodularin were prepared by dissolving each compound in methanol (25 ng/μL, final concentrations), and stored at −18 °C in the dark. A composite working standard solution was prepared by mixing congener solutions and diluting with water to obtain concentrations of 1 ng/μL. Nodularin was used as surrogate analyte and added to the water samples at a final concentration of 50 ng/L. Working standard solutions were stored at 4 °C in the dark, and renewed after one working week. SPE cartridges, Supelclean LC-18, 500 mg in 3 mL, were purchased from Supelco (Bellefonte, USA). All reagents used were of high-performance liquid chromatography grade.

Primers and probes used for polymerase chain reaction were custom synthesized by MWG Biotech. BioRad (Hercules, CA) supplied iQ Supermix for Taq Nuclease Assay. Platinum® Taq Polymerase, PCR buffer and $MgCl_2$ were purchased from Invitrogen Ltd (Paisley, UK). Lysozyme (100,000 units/mg) was supplied by Sigma–Aldrich (St. Louis, MO). All other chemicals were of the highest purity available from commercial sources.

DAPI (4,6-diamidino-2-phenylindole) and the fluorescent substrates for ectoenzymatic activities (4-methyl-umbelliferone, 4-MUF; 7-amino-4-methyl-coumarin, 7-AMC; L-leucine-7-AMC; 4-MUF-phosphate; 4-MUF-*N*-acetyl-β-D-glucosaminide) were supplied by Sigma–Aldrich (St. Louis, MO).

2.2. Description of the site and sampling

Lake Gerosa (42°53′N, 13°21′E) is an artificial freshwater basin (2.5 km length, 370 m width, 50 m depth, 628 m a.s.l.) located in central Italy with very small influent and effluent.

Water samples were collected monthly (October 2006–June 2007) in the center of the lake (surface, −25 m, −50 m) and at the influent and effluent. Ten liters per site were collected with a polycarbonate Niskin bottle, stored in the dark in acid clean polycarbonate bottles or in dark glass bottles, at *in situ* temperature, until arrive in the lab, within 24 h.

2.3. Physical measurements and nutrients analysis

Temperature and pH were determined by a multiparametric probe (PBI International), oxygen concentration by Winkler titration and transparency by the Secchi disk. Nutrient concentrations (total P and N, P-PO_4, N-NO_3, N-NO_2, N-NH_4) and chlorophyll-*a* were measured according to Italian standard methods (APAT and IRSA-CNR, 2003).

2.4. Planktothrix abundance

Planktothrix abundance was determined by epifluorescence microscopy. Five to 10 mL formaldehyde-fixed samples (final concentration 4%) were filtered onto 0.8 μm black membrane filters (25 mm diameter). The filaments were counted by autofluorescence under blue light (450–490 nm) (Olympus BX51). The number of cells per filament was determined by average, on a minimum of 50 filaments, at each sampling date.

2.5. Microcystins detection

Microcystin total content (intracellular plus dissolved toxin) was measured on the whole water samples, after freeze-thawing twice. For immunochemical detection (ELISA), 5 mL water samples were filtered by membrane filters (0.22 μm, Millex®-GV), and analyzed directly with a commercially available Microcystins Envirologix Plate Kit (Envirologix, Portland, Maine, USA), by measuring the absorbance at 450 nm (Wallac Victor^2 spectrofluorometer, Perkin Elmer Inc, USA). The average variation coefficient was 5.6% and it was never higher than 15%; the limit of quantification (LOQ) was 0.16 μg/L.

For liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis, water samples extraction was performed according to Lawton et al. (1994) by solid phase extraction (SPE). The LC-MS/MS determinations were carried out as previously described (Bogialli et al., 2006).

The amount of MC congeners was quantified by the external standard quantification procedure, referring to a standard calibration curve (correlation coefficient $R^2 = 0.98$–0.99), after exclusion of any matrix effect. Nodularin was used as quality control for the extraction efficiencies. The two demethylated forms of MC-RR and MC-LR were quantified by assigning the same molar response factors of the corresponding fully methylated MCs (Bogialli et al., 2006). The within laboratory variation of the data was not greater than 25%. The limit of detection (LOD) varied between 0.5 ng/L (MC-RR, MC-LA) and 2 ng/L (MC-LW); LOQ was calculated on the basis of a minimal accepted value of the signal-to-noise ratio (S/N) of 10 and resulted between 1 ng/L (MC-RR) and 5 ng/L (MC-LW) . MC-LF, MC-LW, MC-LR were never found above the LOD of the applied method.

2.6. Molecular investigation

Each sample (250–500 mL) was filtered on 0.2 μm Supor Membrane Filters (Pall Corporation) and stored at −20 °C. For total genomic DNA extraction, each filter was cut and incubated for 10 min in PBS at 4 °C; cells were pelleted by centrifugation (3400 × g, 10 min) and re-suspended in 500 μL TNE buffer (50 mM Tris–HCl, 5 mM EDTA, 50 mM NaCl, pH 8). DNA extraction was performed according to Burns et al. (2004), except for the lysis step with lysozyme (50 mg/mL) extended overnight (37 °C). Genomic DNA was re-suspended in 100 μL sterile water. DNA concentration and purity (calculated as the A_{260}/A_{280} ratio) were measured with a BioPhotometer (Eppendorf, AG, Hamburg, Germany).

The molecular identification of *P. rubescens* species was carried out by detecting the intergenic spacer region within the phycocyanin operon (PC-IGS region; Kurmayer et al., 2004). Experimental conditions, primers and TaqMan probes for *PC* and *mcyB* (see below) are reported in Table 1. A *Microcystis aeruginosa* culture (CCAP1450/6) was used as negative control. MC producers within *P. rubescens* population were identified by amplification of a sequence within *mcyA* and *mcyE*, according to Kurmayer et al. (2004) and Mbedi et al. (2005), respectively. PCR products were analyzed by electrophoresis on a 2% (w/v) ethidium bromide stained agarose gel in TBE buffer (0.1 M Tris, 0.09 M boric acid, 0.1 mM EDTA).

To quantify the toxic genotypes (*mcyB*) with respect to *Planktothrix* general population (*mcyB/PC* operon ratio), Taq Nuclease Assay was carried out as previously described (Kurmayer and Schober, 2003). The standard curves were obtained with genomic DNA of *P. rubescens* strains CCAP1460/3 and CCAP1460/10. Each sample was analyzed in triplicate. qPCR amplification efficiencies of natural water samples (0.90–0.98) were similar to the qPCR amplification efficiencies of the *Planktothrix* standard strains (0.95–0.99).

2.7. Bacterial abundance and ectoenzyme activities

Bacteria were counted by epifluorescence microscopy. Five milliliters formaldehyde-fixed (4%) and DAPI stained (1 μg/mL final concentration) samples were filtered onto a 0.22 μm black membrane filter. At least 20 frames or 200 cells per sample were counted, from two replicate slides, at 1000× magnification (Olympus BX51), under UV excitation. Ectoenzyme activities (cell-surface-bond and periplasmic), leucine aminopeptidase (Leu), *N*-acetyl-β-D-glucosidase (chitinase, NAGA) and alkaline phosphatase (Pho) were measured at saturating substrate concentrations, using fluorogenic analog substrates (Hoppe, 1983). These consist of a fluorescent fluorophore (4-MUF or 7-AMC) linked to the substrate molecule. The final concentrations used were: 125 μM 4-MUF-phosphate; 100 μM 4-MUF-*N*-acetyl-β-D-glucosaminide and 250 μM L-leucine-7-AMC. Fluorescence was measured at 355/460 nm excitation/emission (Wallac Victor^2 spectrofluorometer, Perkin Elmer INC, USA). Quantification of 4-MUF and 7-AMC was achieved by calibration with standard solutions.

3. Results

P. rubescens was the only potentially toxic cyanobacterium morphologically and genetically identified in Lake Gerosa. It has never been found in the influent; cell density in the effluent was always in the low range of that detected in the lake ($2.3 \times 10^6 \pm 0.6 \times 10^6$ cell/L). Based on these observations, results related to influent and effluent sampling sites were considered irrelevant and not shown.

Table 1 – Oligonucleotide sequences for the PCR amplification of the phycocianin interspacer (PC-IGS) region and of the microcystin biosynthesis (*mcy*) genes.

Target gene	Designation	Sequences 5′ to 3′	Amplicon size (bp)	Reference
PC-IGS[a]	PC_F	TGCTGTCGCCTAATTTTTCA	271	Kurmayer et al., 2004
	PC_R	CCACTGATCAGGCTGTCAGA		
mcyA[a]	mcyA_F	ATCAAACAGATGTACTGAC AGGT	174	Kurmayer et al., 2004
	mcyA_R	AGGCCAGACTATCCCGTT		
mcyE[b]	mcyE_F	TTACCTAATTATCCCTTTCA AAG	589	Mbedi et al., 2005
	mcyE_R	CAATGGGTAAGGTTTGCTT		
PC-IGS (real-time PCR)[c]	PlPc_F	GCAGGAATTACTCCTGGAG ATTGT	71	Kurmayer and Schober, 2003
	PlPc_R	GCCGCAGCGAGATCAAAG		
	PlPc_probe	FAM-CGCTCTGGCTTCTGAA GTCGCCG-TAMRA		
mcyB (real-time PCR)[c]	Pl-mcyB_F	ATTGCCGTTATCTCAAGCGAG	76	Kurmayer and Schober, 2003
	Pl-mcyB_R	TGCTGAAAAAACTGCTGCAT TAA		
	mcyB_probe	FAM-TCAGAGGAAAGAGCTT CACCTCCACAAAAA-TAMRA		

a DNA (1 μL) was amplified in 20 μL (final volume) containing Invitrogen Platinum® Taq Polymerase (1 U), PCR buffer (20 mM Tris–HCl, 50 mM KCl, pH 8.4), 1.5 mM $MgCl_2$, 300 μM dNTPs and 0.5 μM each primer. Thermal profile: initial denaturation (94 °C, 3 min), 40 melting cycles (94 °C, 30 s), annealing (52 °C, 30 s) and elongation (72 °C, 30 s).

b DNA (1 μL) was amplified in 20 μL (final volume), containing Invitrogen Platinum® Taq Polymerase (1 U), PCR buffer (20 mM Tris–HCl, 50 mM KCl, pH 8.4), 1.9 mM $MgCl_2$, 200 μM dNTPs and 0.5 μM of each primer. Thermal profile: initial denaturation (96 °C, 10 min), 35 denaturation cycles (96 °C, 10 s), annealing (50 °C, 10 s) and extension (72 °C, 30 s) and final elongation (72 °C, 10 min).

c Five microliters of DNA sample from a standard strain or a field sample were amplified in a final volume of 25 μL containing 12.5 μL of 2× iQ Supermix, 300 nM *PC* primers, 900 nM *mcyB* primers, 100 nM TaqMan probe for *PC*, 250 nM TaqMan probe for *mcyB*. Amplification was carried out using an iQ5 Real-Time PCR Detection System (BioRad, Hercules, CA). Thermal profile: initial denaturation (95 °C, 10 min), 45 melting step cycles (95 °C, 15 s), annealing-extension step (60 °C, 1 min). The threshold cycle was converted into starting target copy number. Series of 10-fold dilutions of DNA for *PC* and *mcyB* genes from the standard strains (range 1×10^2–1×10^7) were amplified and DNA content was related to the cell number equivalents.

3.1. *Physicochemical parameters*

Water temperature ranged from a minimum of 6.4 °C, in winter during the mixing condition, to a maximum of 22 °C in surface water, during summer. The onset of column stratification in March maintained the temperature at −50 m always below 10 °C. Transparency was minimum in late autumn, corresponding to the peaks of *P. rubescens* and heavy precipitation; then it increased until the maximum in May–June 2007.

Prolonged hypoxic conditions and a slightly lower pH characterized the bottom layer, except from February to March, when water was oxygenated by mixing processes (Table S1). Thus, although the thermocline was at ~10–20 m, major differences were observed between the bottom layer and the rest of the water column. Low levels of total phosphorus (P-tot) were measured (2–24 μg/L), during the entire sampling period with a bottom-to-surface concentration gradient (Fig. S1A). Phosphate was always below the detection limit, except in October–November on the bottom (~4 μg/L). Higher nitrogen (N-tot) levels were detected on the bottom, where ammonia ($N\text{-}NH_4$) accounted for nearly all N-tot in autumn (annual range of N-tot and ammonia: <25–850 μg/L and <5–170 μg/L respectively; Fig. S1B,C). Consistently, in the same period, nitrate ($N\text{-}NO_3$, 2–225 μg/L) concentration was negligible at the three depths (Fig. S1D), and nitrite was always <LOQ (1 μg/L, data not shown). Therefore, during the blooming season nitrogen at surface and at −25 m was mainly in its organic form. In summer, the surface layer was nitrogen-depleted and nitrate was the major form at the bottom.

3.2. *Seasonal population dynamic of P. rubescens*

P. rubescens bloomed in late autumn, reaching the highest density on the surface and at −25 m in November 2006 (Fig. 1A). In December the bloom was over and the number of cells gradually decreased until summer, when *P. rubescens* disappeared from the surface. At −25 m *P. rubescens* cell density fluctuated between 5.7 and 2.1×10^6 cells/L. At −50 m, *P. rubescens* never bloomed, but it was always present, with a very limited seasonal variation (Fig. 1A).

3.3. *Microcystin content*

The highest total (intracellular plus dissolved) MC levels, as detected by ELISA in the period November 2006–June 2007, were found in December both at surface and −25 m water samples. The toxin levels on the surface progressively decreased with time, up to levels below quantification limit (April–June, 2006). although in deeper waters MCs were detectable during the entire sampling period (Table 2).

In most samples MC total content values, as detected with the LC-MS/MS method, were either similar or more frequently

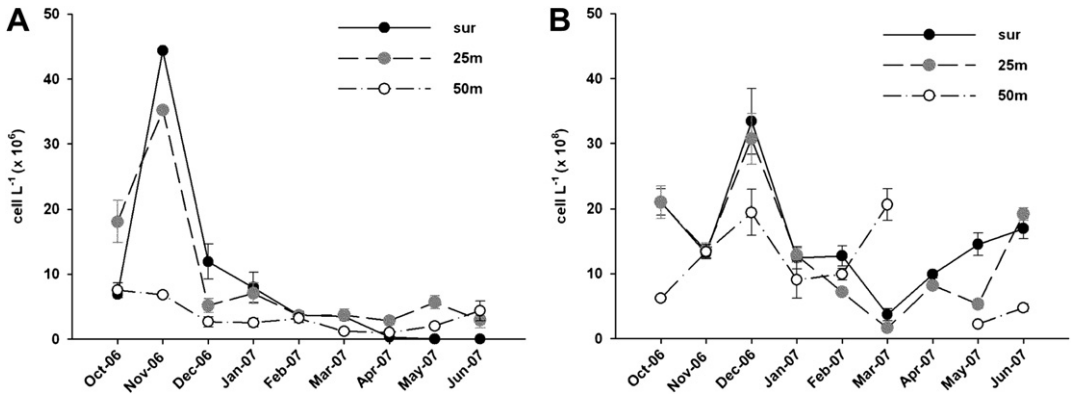

Fig. 1 – Seasonal dynamic of (A) *P. rubescens* and (B) bacteria in the lake, at the three sampling depths. Results are expressed as averages ± SE.

lower than those measured with the ELISA method, although the trend was similar. In surface waters, the total MC concentrations peaked in October and December and then progressively decreased; differences among monthly measurements were larger than two orders of magnitude. At −25 m, MC concentrations showed a limited monthly variability with higher values measured in October, December and April. In May–June MC concentrations were up to 20-fold higher than in superficial waters. At −50 m, MC concentrations were always very low, except in June, when MC level was the highest in the water column: this value corresponded to the highest abundance of *P. rubescens* at −50 m (Fig. 1A).

The MC concentration profiles roughly paralleled *P. rubescens* densities (Fig. 1A), although the highest MC concentrations did not exactly correspond to the *P. rubescens* density peak.

Among the tested MC variants, MC-LF, MC-LW, MC-LR were never found above the LOD of the LC-MS/MS method. The most abundant variants were the demethylated forms of MC-RR and MC-LR (Dem-RR and Dem-LR), with a clear predominance of the two isomers of the Dem-RR, [Dha^7]MC-RR and [D-Asp^3,Dhb^7]MC-RR, whose concentrations are reported as sum (Table 2).

3.4. *Molecular investigation*

The amplification of a 271 bp fragment within the PC-IGS region in all samples, and the absence of products with *Microcystis* specific primers, confirmed *P. rubescens* as the only cyanobacterium present in Lake Gerosa. Results on PC-IGS and *mcy* genes products in standard *P. rubescens* cultures, negative control and samples are shown in Fig. S2. The

Table 2 – Total MC concentrations (LC-MS/MS and ELISA).

		Oct 2006	Nov 2006	Dec 2006	Jan 2007	Feb 2007	Mar 2007	Apr 2007	May 2007	Jun 2007
Surface	Dem-RR[a]	1.02	0.056	0.40	0.032	0.026	0.019	0.12	0.013	0.007
	RR	nd[b]	0.002	nd	<LOQ	nd	nd	<LOQ	<LOQ	<LOQ
	YR	0.010	0.003	nd	0.003	0.002	0.002	nd	nd	nd
	Dem-LR	0.005	0.003	nd	0.008	0.007	0.014	0.015	nd	<LOQ
	Total	1.03	0.064	0.40	0.043	0.035	0.035	0.14	0.013	0.007
	ELISA	na[c]	0.73	1.63	0.64	0.30	0.18	<LOQ	<LOQ	<LOQ
25 m	Dem-RR	0.70	0.021	0.56	0.006	0.007	0.015	0.47	0.19	0.15
	RR	0.010	<LOQ	nd	<LOQ	nd	nd	<LOQ	<LOQ	<LOQ
	YR	0.003	nd	nd	0.002	0.001	0.005	0.019	0.009	0.020
	Dem-LR	0.003	<LOQ	nd	0.007	0.007	0.027	0.092	0.038	0.054
	Total	0.72	0.021	0.56	0.015	0.015	0.047	0.58	0.24	0.22
	ELISA	na	0.47	1.94	0.59	0.35	0.23	0.29	0.23	0.18
50 m	Dem-RR	0.001	0.022	0.066	0.001	0.018	0.012	na	0.049	0.31
	RR	nd	nd	nd	<LOQ	nd	<LOQ	na	<LOQ	<LOQ
	YR	nd	nd	nd	nd	0.001	<LOQ	na	nd	0.018
	Dem-LR	nd	nd	nd	nd	0.006	0.008	na	<LOQ	0.073
	Total	0.001	0.022	0.066	0.001	0.025	0.02	na	0.049	0.40
	ELISA	na	0.21	0.44	0.27	0.32	0.19	na	<LOQ	0.28

Results are expressed as μg/L. and were obtained from at least two independent replicates.
a The concentrations of the two isomers of the Dem-RR and Dem-LR were reported as sum.
b nd, not detected (below the LOD).
c na, not analyzed.

qualitative indication of the potential toxicity of the population was provided by the occurrence of *mcyA* and *mcyE* regions (174 and 589 bp) in all environmental samples.

The quantitative analysis on the presence of *Planktothrix mcyB* and *PC* copy numbers showed that the *mcyB/PC* ratio in surface waters was the lowest in November 2006, during the bloom (0.31) and was much higher during the other months (0.60–0.70) (Fig. 2A), when total MC content was higher. The proportion of toxic genotype increased significantly with water depth, ranging between 65 and 100% (Fig. 2B,C).

3.5. *Bacterial abundance and enzymatic activities*

Bacterial abundance ranged between 1.6×10^8 and 33.4×10^8 cells/L (Fig. 1B). In December, after the cyanobacterial bloom decline, abundances were higher in surface water and at −25 m. In March, abundance was maximal at the bottom layer, coincident with an increase in ammonia concentration, and minimal on the surface and at −25 m.

Bacteria hydrolize polymers using periplasmic or cell-surface-bound hydrolytic enzymes; the produced oligo-monomers can be directly utilized by bacteria or released in the surrounding environment (Hollibaugh and Azam, 1983; Chrost, 1992; Smith et al., 1992). Since the importance of these activities in the mobilization, transformation and recycling of organic matter in aquatic environments has been repeatedly proved (Chrost, 1992), leucine amino-peptidase (Leu), *N*-acetyl-β-D-glucosidase (chitinase, NAGA) and alkaline phosphatase (Pho) were measured. Results showed an active bacterial community in the whole water column, indicating an intense recycling role for bacteria. Since no differences were observed between layers (data not shown), data have been reported as pooled values. The monthly averages of Pho were highest in March–April and minima in October–November, whereas Leu followed an opposite trend (Fig. 3). The highest activity values corresponded to peaks at surface and −25 m. NAGA activity was much lower during the whole sampling period (Fig. 3).

4. Discussion

The presence of cyanobacteria in freshwater lakes represents a potential threat for human health. Since factors and processes involved in *P. rubescens* growth and cyanotoxin production are not adequately known, it is at present difficult to define both environmental markers as predictors of future blooms, as well as early markers in cyanobacteria populations able to indicate bloom activity will occur. Their availability would have a critical role in preventing human exposure and adopting measures for risk prevention. In this study, by applying an integrated approach, we have provided a scientific basis for the elaboration of tailored monitoring programs on parameters relevant for risk assessment and management. The same approach could be extended to other toxic species, whose toxicity can be modulated by various environmental factors.

The lake is characterized by a low anthropic pressure. Data on nutrient concentrations are typical of a deep oligotrophic lake (Quiros, 2003) and bacterial abundances, as a marker of

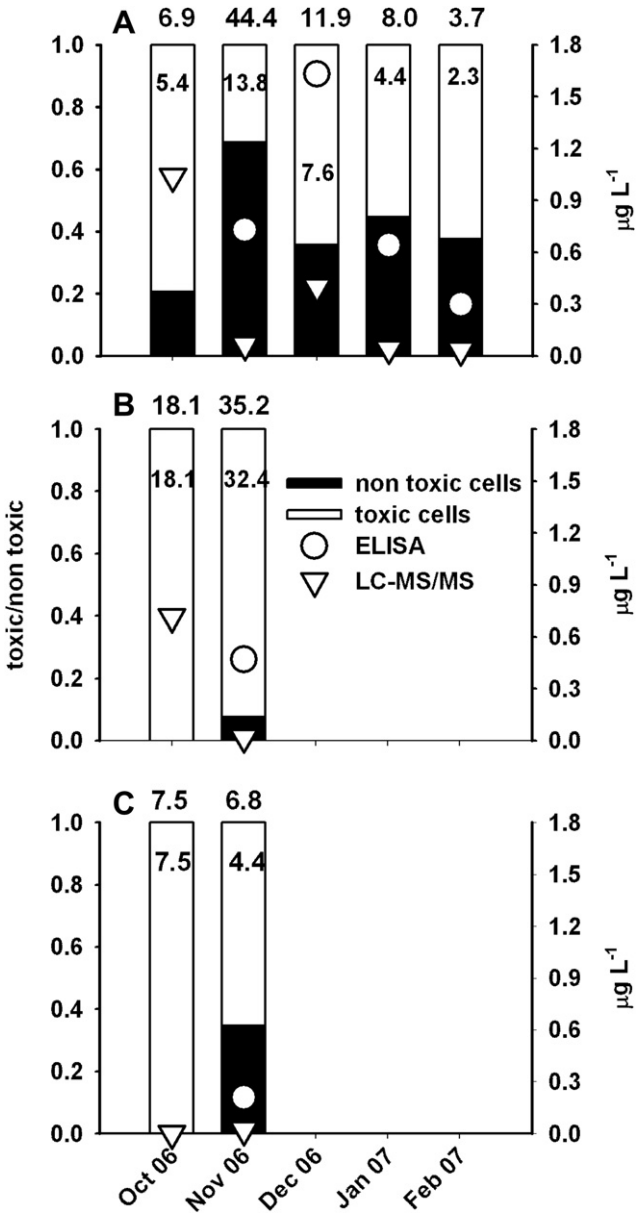

Fig. 2 – Microcystin levels (ELISA and LC-MS/MS right y-axis) and toxic cells/whole population ratio (left y-axis), reported as empty and close fraction of each bar. The three panels show results measured in (A) surface water, (B) −25 m, (C) −50 m. Numbers on top of each bar are total cells per liter (×10⁶); numbers inside the bars are toxic cells per liter (×10⁶).

trophic status (Porter et al., 2004), were in the range of oligotrophic/mesotrophic lakes (Porter et al., 2004). On the other hand, preliminary data showed high values of N-tot and P-tot (~1000 mg/kg dw) in sediments. N and P release from sediments is the result of very complex processes (Søndergaard et al., 2003), which although continuous and long-lasting, can be quantitatively limited and very slow. This seems to be the case in Lake Gerosa, with undetectable or extremely low ortophosphate levels. P limitation conditions are also suggested by the Pho/Leu ratio >1 most of the time (Sala et al.,

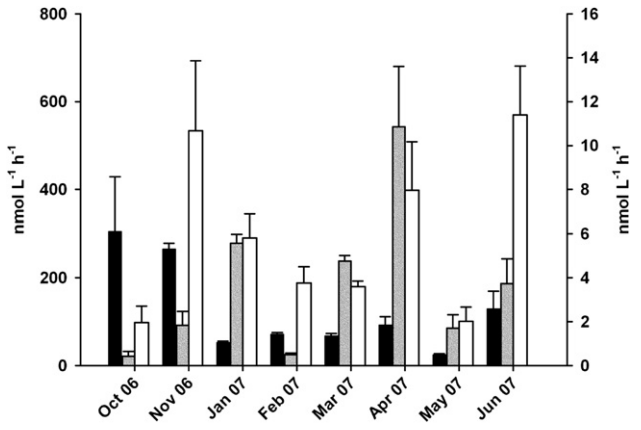

Fig. 3 – Seasonal dynamic of ectoenzyme activities. Data from the three depths have been pooled and results are expressed as averages ± SE. Black and grey bars, Leu and Pho, left y-axis; white bars, NAGA, right y-axis; note the different scale.

2001): indeed, phyto- and bacterio-plankton can overcome P limitation by expressing high rates of Pho. In this case, the observed high activity seems mainly attributed to phytoplankton rather than to *P. rubescens* or the bacterial community, since it significantly correlates with chlorophyll-*a* levels ($r = 0.53$, $n = 21$, $p < 0.01$, not shown), except during cyanobacteria blooming. Therefore, *P. rubescens* can either bloom in October–November by regulating its buoyancy (Oliver, 1994), moving between optimal light intensity (top) and nutrients (bottom), or by previous phosphorus uptake in excess of the amount immediately needed for growth.

Leu and NAGA activities appear to be important for *P. rubescens* and bacteria to overcome nitrogen limitation. Indeed, on the surface and at −25 m, where nitrogen was mainly present in its organic form, a significant negative correlation between Leu and NAGA activities (expressed as moles of processed nitrogen) and nitrate ($r = 0.78$, $n = 27$, $p < 0.001$; Fig. S3) was evidenced. Worm and Søndergaard (1998) showed that *Microcystis*-associated bacteria and/or *Microcystis* spp. cells during a bloom accounted for >50% total Leu. Analogously, the high Leu observed in October–November, during the cyanobacterial bloom, could be partially due to *Planktothrix*- or particle-attached bacteria. In the latter case, the low efficiency of attached bacteria in the uptake of hydrolyzed monomers (Smith et al., 1992) can partially make them available to other planktonic organisms and to *Planktothrix* as well. Zotina et al. (2003) demonstrated that *P. rubescens* is able to utilize organic N by direct uptake of aminoacids and other small organic compounds in conditions of low irradiance.

P. rubescens was the only cyanobacterium present, showing high seasonal variation on the surface and at −25 m, but constantly present on the bottom at significant levels. This is typical of *P. rubescens*, which has been described to form metalimnetic layers in deep stratified and oligo-mesotrophic lakes, differently from its green variety *P. agardhii*, that prefers shallow, nutrient-rich, well-mixed water-bodies (Barco et al., 2004). The cyanobacterial density negatively correlates to N-tot/P-tot ratio ($r = 0.68$, $n = 27$, $p < 0.001$, not shown); the correlation improved when the analysis excluded bottom values (Fig. 4A). Although other reports indicate that an N/P ratio exceeding 16 is necessary for *P. agardhii* to take dominance and bloom (Teubner et al., 1999) or that phosphate content shows the highest positive correlation with cell density (Catherine et al., 2008), our findings indicate that low levels of nutrients are not a marker for low risk of cyanobacterial proliferation. Indeed, *P. rubescens* started to bloom in the absence of dissolved nitrate, likely utilizing nitrogen released by bacterial enzyme activities.

When the analysis was limited to the toxic fraction within *P. rubescens* population, independently of the depth, a better negative correlation with N/P ratio was obtained (Fig. 4B). A similar correlation was observed between total nitrogen and the *mcyA*-positive *P. agardhii* cells in a French lake (Briand et al., 2008). A positive correlation was found between the toxic/non-toxic cells ratio and total P ($r = 0.66$, $n = 9$, $p < 0.05$, not shown), suggesting a competitive advantage for non-toxic cells at decreasing P concentration. Contrasting results have been described so far about the influence of P and N on the occurrence of toxic genotype (Briand et al., 2008; Yoshida et al., 2007); the discrepancies are likely due to the observation of different species (*Microcystis* vs *Planktothrix*), and to different background nutrient concentrations.

The high toxic fraction observed in Lake Gerosa (30–100%, Fig. 2) seems to be typical for *Planktothrix* spp.: in *P. agardhii* the *mcy*-positive fraction is the same (31–100%) (Kurmayer et al., 2004; Briand et al., 2008). On the contrary in *Microcystis* populations the toxic fraction is generally lower (0.5–38%) and stable during the period of seasonal population growth (Kurmayer et al., 2004; Yoshida et al., 2007). Previously, it was found that almost all filaments in a *P. rubescens* population contain genes for MC synthesis (Kurmayer et al., 2004). Our data confirm that the toxic fraction is high but variable, particularly during the bloom. Indeed, the number of toxic cells is linearly dependent on cell density (Fig. 4C), except for the superficial bloom population, in which the fraction of toxic cells attained the lowest value. The inverse dependence of MC-producing genotype on cell density in superficial waters has been previously described for *P. agardhii* in France (Briand et al., 2008). It has been hypothetically related to reduced light conditions, due to the self-shading generated by high biomasses. The hypothesis was based on the observation that at low light non-toxic *Microcystis* in culture are better competitors than *mcy*-positive individuals (Kardinaal et al., 2007). On the contrary, the highest percentage of toxic cells occurred below the thermocline in Lake Gerosa; furthermore, the bloom density in November at −25 m was as high as in surface water, with a higher percentage of toxic cells. Therefore, environmental factors other than light might be involved, including a competitive advantage of toxic cells at lower biomass, in conditions unfavorable for their growth. Alternatively, the over-representation of non-toxic individuals may be related to higher growth rate compared to MC-producers, or to the energy cost of MC synthesis for cells, mainly devoted to division. The potential protective role of MC in preventing grazing does not seem to be the selective pressure (Rantala et al., 2004); nevertheless, it cannot be excluded

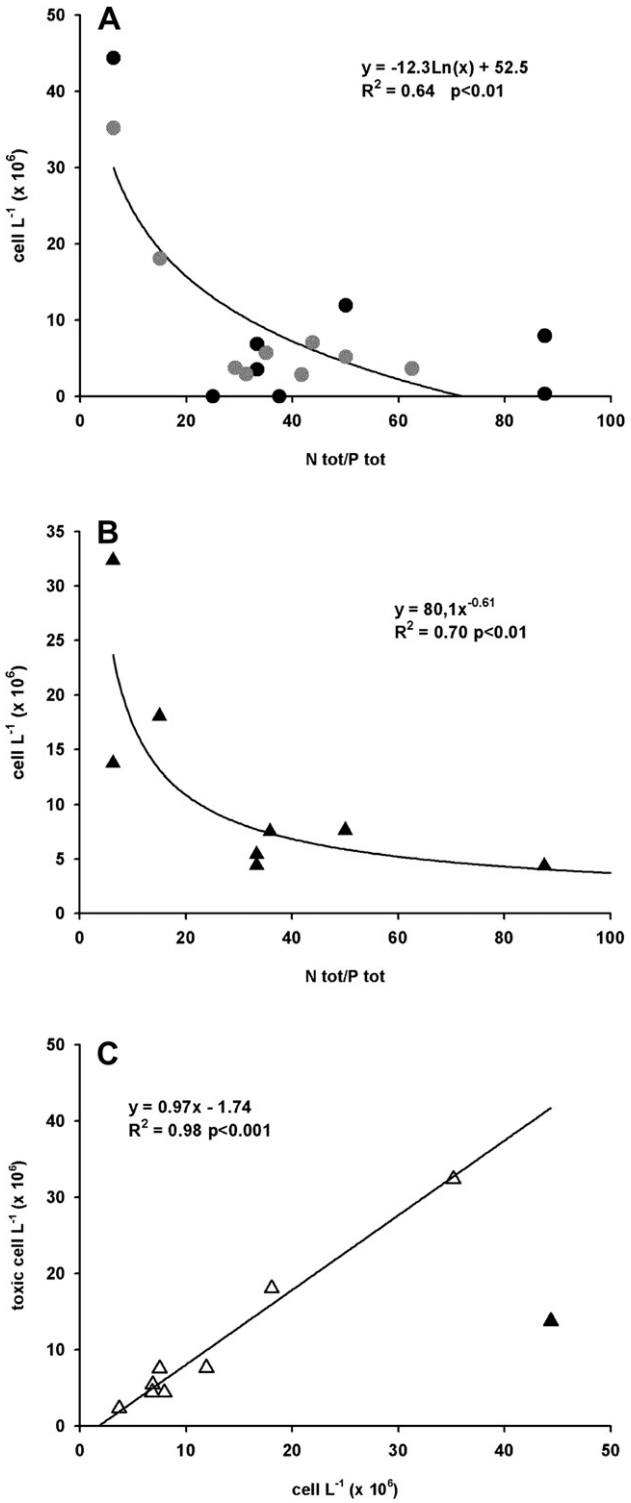

Fig. 4 – Relationships of *P. rubescens* abundance with different parameters. (A) Correlation of *P. rubescens* population with N/P ratio; black circles, surface; grey circles, −25 m. Regression line has been calculated combining the data from surface water and −25 m. (B) Relationship of toxic cells vs N/P; data from the three depths. (C) Relationship of toxic cells vs the whole population; regression line has been calculated excluding the 'outlier' value, corresponding to the superficial bloom in November 2006.

that maintaining a fraction of MC-producing cells over time may represent an advantage for the species, in terms of biological diversity.

The dynamics of toxic cells within the total *P. rubescens* population only partially explain the shift in the peak of toxin production, occurring 1 month before and one later than maximum cell density: indeed, in November, although the percentage of toxic cells was the lowest, the absolute number of *mcyB*-positive cells was the highest (Fig. 2). A MC peak just before the cyanobacteria biovolume maximum has been reported in *Planktothrix*-dominated freshwater Dutch lakes (Janse et al., 2005), and for *P. agardhii* in a French lake. In the latter case, on a statistical basis, only about 50% of the variation in MC concentration was accounted for by the presence of *mcyA*-positive cells (Briand et al., 2008). Therefore other factors in addition to toxic/non-toxic ratio should be responsible for this phenomenon. The lowest total MC production found in November (Table 2) may be attributed to the increased presence of mutants unable to synthesize MC within the *mcyB*-positive cells, under conditions allowing blooms to occur (Ostermaier and Kurmayer, 2009). Alternatively, the expression of *mcy* genes might have been down-regulated by environmental factors, as has been shown for low light intensity, with decreasing levels of *mcy* transcripts (Kaebernick et al., 2000). Nonetheless, the MC levels in Lake Gerosa were quite low (<2 μg/L), when compared with values reported for lakes in the Netherlands (up to 140 μg/L) (Janse et al., 2005), France (up to 10.8 μg/L) (Jann-Para et al., 2004), or Italy (up to 14.2 μg/L) (Messineo et al., 2006), all infested by *P. rubescens*.

The higher values measured with the immunological assay as compared to LC-MS/MS, may be attributed to the occurrence of unspecific congeners not detected with our LC-MS/MS method, but recognized by the ELISA kit. The latter method reacts with the aminoacid ADDA, regardless of the molecules where it is incorporated; thus, it is not able to discriminate among different congeners, and gives only a semi-quantitative estimation of total MC content, expressed as MC-LR equivalents. Nevertheless, for monitoring purposes, it has the advantage of not failing to detect unknown or unexpected MC congeners. Among the seven congeners investigated, Lake Gerosa samples never contained MC-LR, -LW and LF, whereas MC-RR and -YR were sporadically present at very low levels. The demethylated forms of MC-LR and MC-RR, the latter being by far the most abundant (Table 2), are typically produced by *P. rubescens* (Barco et al., 2004; Hoeger et al., 2007). However, the identification of new congeners from water blooms of *P. rubescens* (Grach-Pogrebinsky et al., 2004) and the different pattern of congeners production reported for *Planktothrix* leave the possibility open that variants are produced, other than those tested. In addition, the relative production of different congeners is not the same over time, as confirmed by the variability of the ratio between Elisa- vs LC-MS/MS-measured MC levels, ranging between 4 and 40 during the period November 2006–January 2007 (when values were far from the LOQ). The changing of congener pattern production due to genetic or environmental factors is supported by the observation that specific *mcyB* variant correspond to the biosynthesis of different MC congeners (Kurmayer and Gumpenberger, 2006) and that increasing irradiance

stimulates the production of more toxic MC congeners in *P. agardhii* (Tonk et al., 2005).

Microcystins immunochemically detected in Lake Gerosa and expressed as MC-LR equivalents were in some cases slightly higher than the guideline defined by WHO for MC-LR in drinking water (WHO, 2004); however the LC-MS/MS method applied to the same samples evidenced that MC-LR was never found. The demethylated MC-RR which was the most abundant congener, shows a three- to five-fold lower acute toxicity than MC-LR (Zurawell et al., 2005). Therefore, based on its dynamic and MC levels, *P. rubescens* occurrence in Lake Gerosa does not hamper bathing activities and crop irrigation (Funari and Testai, 2008), and does not appear to represent a real risk for human health (Funari and Testai, 2008).

However, the seasonal dynamics may drastically change with environmental conditions and tailored monitoring activities should be carried out on a regular basis in order to take possible risks under control.

5. Conclusion

When freshwater lakes are a possible source of human and livestock exposure, it is our opinion that tailored monitoring plans should be elaborated on the basis of preliminary studies. These should be carried out by using an integrated approach, such as the one described in this paper, aimed to characterize the water body. Our data provide clear indications that cyanobacterial density, the surveillance of which remains crucial in routine monitoring activities, might not be a fully reliable parameter to protect human health. Indeed, the ratio between strains producing MCs and the whole *P. rubescens* population varies with time and environmental conditions, with a not yet clarified trend. In addition, when *P. rubescens* is the dominant species and the lake is used as a source for drinking water, it is also important to analyze samples below the thermocline, where the proportion of MC-producing cells is higher than on the surface. Hence, the quantitative estimate of toxic genotype abundance is more relevant than cell counts for assessing the potential risk. Furthermore, in a monitoring programme it is important to include an unspecific biological method together with a more sophisticated LC-MS/MS detection, to have an indication of both a 'global' cyanotoxin content and a profile of the produced congeners, with different toxic potential.

Studies like this should then be repeated every few years, in order to (i) follow possible changes in the lake's general trophic status; (ii) identify the environmental factors triggering blooms in order to make predictions; (iii) verify the efficiency of mitigation measures adopted in order to prevent cyanobacterial proliferation and/or human exposure; and (iv) when necessary, carry out an appropriate risk assessment.

Conflict of interest statement

The authors have no conflicts of interest with regards to this manuscript.

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Appendix A. Supplemental material

Supplementary information for this manuscript can be downloaded at doi:10.1016/j.watres.2009.10.045.

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