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Distribution of hepatotoxic cyanobacterial blooms in Belgium and Luxembourg

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Abstract

A survey of the distribution of cyanobacterial blooms in the southern part of Belgium, in Luxembourg as well as in bordering northeastern France was carried out for 4 years (1997, 1999–2001). In the 64 cyanobacterial bloom samples collected, *Microcystis* as well as *Planktothrix* were the most frequently encountered dominant bloom formers, followed by *Anabaena, Woronichinia*, and *Aphanizomenon*. The relative frequency of (co-)dominant genera was highly correlated to the geology of the catchments. Microcystins were found in 53% of the analysed blooms and their presence was mainly assigned to *Microcystis* dominance. The highest microcystin concentration of 2231 μg g^{-1} seston DW was recorded in a sample dominated by *Woronichinia naegeliana*. Among the 6 investigated microcystin variants, MC-LR was the most frequently detected whereas MC-LY was never revealed.

Introduction

Cyanobacterial blooms occur all over the world and are responsible for many health effects on humans and animals (Carmichael, 1994, 2001; Azevedo et al., 2002). Many species are able to produce hepatotoxins and/or neurotoxins and mostly belong to the genera *Microcystis* Kützing, *Aphanizomenon* Morren, *Planktothrix* Anagnostidis et Komárek, *Anabaena* Bory de Saint Vincent, and *Cylindrospermopsis* Seenayya et Subba Raju (Sivonen et al., 1990; Carmichael, 1994; Vasconcelos, 1994; Codd et al., 1995; Willén & Mattsson, 1997; Chorus, 2001). Furthermore, like Gram negative bacteria, the cell walls of cyanobacteria present lipopolysaccharides (LPS) known to induce irritant and allergenic responses of human and animal tissues (Codd et al., 1989). This kind of problems potentially affecting human health are growing these last decades and precautionary closures of swimming areas are now regularly imposed (Ressom et al., 1994). In this human health context, a WHO expert group developed a guideline value of 20,000 cells ml^{-1} for safe practice in managing recreational waters as well as a safe level of 1.0 μg microcystin-LR equivalent l^{-1} for drinking water quality (WHO, 1998; Falconer et al., 1999). Moreover, cyanobacteria are able to produce a wide variety of chemical compounds that can alter the organoleptic water quality, such as 2-methylisoborneol (MIB) and geosmin giving drinking water an

unpleasant character (Persson, 1996). Excessive growth of cyanobacteria can thus make the water non-potable or considerably increase the costs of drinking water purification. Besides, cyanobacterial massive developments induce indirect effects like water column deoxygenation during dense blooms that can be highly deleterious for aquatic biocenoses.

The success of cyanobacteria in freshwaters of temperate regions has a multifactor origin. Synergetic combinations of favourable environmental variables and physiological advantages make cyanobacteria efficient phytoplankton competitors. For instance, the presence of heterocytes, gas vesicles and "CO_2 Concentrating Mechanism" (CCM) lead to cyanobacterial competitive superiority when nitrogen and CO_2 concentrations limit eukaryotic phytoplankton development (Reynolds & Walsby, 1975; Price et al., 1998). Furthermore, large *Microcystis* spp. colonies or bundles of filaments that characterise *Aphanizomenon flos-aquae* (L.) Ralfs reduce both herbivorous grazing and photosynthetic inactivation under high light intensities (Fulton & Paerl, 1987; Pechar & Masojídek, 1995). Water temperature, thermal stratification, light intensity, CO_2 concentration, pH as well as N/P ratio are described as the major variables involved in the control of cyanobacterial developments in freshwater ecosystems (Paerl & Millie, 1996).

So far, few data are available on the occurrence of cyanobacterial blooms and in particular on their toxicity in Belgium and Luxembourg. Van Hoof et al. (1994) demonstrated toxic and mutagenic effects of *Microcystis aeruginosa* (K.) Kützing samples by using several bioassays while Wirsing et al. (1998) reported a microcystin content of 556 $\mu g\ g^{-1}$ DW in a single Belgian *Microcystis* bloom. Willame & Hoffmann (1999) presented the geographical distribution of 21 Belgian cyanobacterial blooms as well as morphological descriptions of the main species. Consequently, the necessary toxicological data for the assessment of human health risks in these countries are lacking.

The objectives of this study were to ascertain the occurrence of cyanobacterial blooms in the southern part of Belgium, in Luxembourg as well as in neighbouring northeastern France, to determine the genera involved and to evaluate their hepatotoxic potential by the analysis of their microcystin content. Moreover, the relationship between the distribution of the dominant bloom-forming genera and of the microcystin content and the abiotic environmental variables will be analysed using two different statistical approaches.

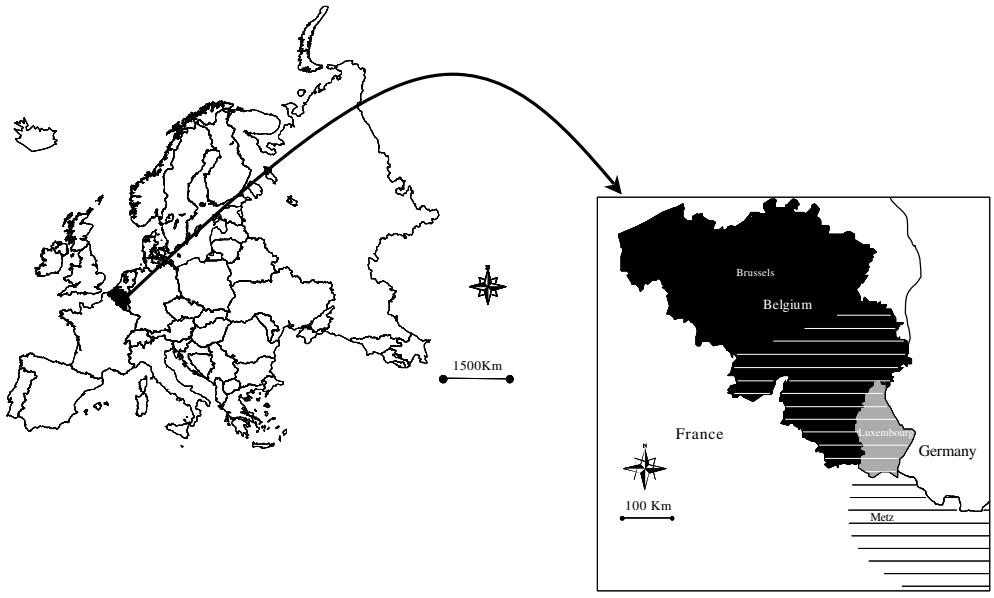

Figure 1. Map of the prospected area. Horizontal lines illustrate the region visited during 4 years (1997, 1999–2001).

Materials and methods

Study sites

During summer and autumn 1997, 1999–2001, bloom samples were collected from standing waters (ponds, lakes, reservoirs) in the central and southern part of Belgium, in Luxembourg and in some bordering northeastern French water bodies (Fig. 1).

More than 250 water bodies were visited once or up to three times and 64 cyanobacterial blooms were sampled from 49 different water bodies (Fig. 2). They are listed in alphabetical order in Table 1. The number and the location of the sampling sites intended to depict a good cross-section of representative standing waters of the prospected zones. Therefore, the sampled water bodies covered a broad range of morphometries, geological influences as well as human use (recreation, drinking water production, fisheries). Except the sampling sites 5-16-25-26-27 and 57 with a mean depth >5 m, all sampled standing waters were shallow with a mean depth inferior to 3 m.

Sample collection and conservation

For the identification of cyanobacteria, 100 ml of dense bloom material were sampled and directly preserved in formaldehyde to a final concentration

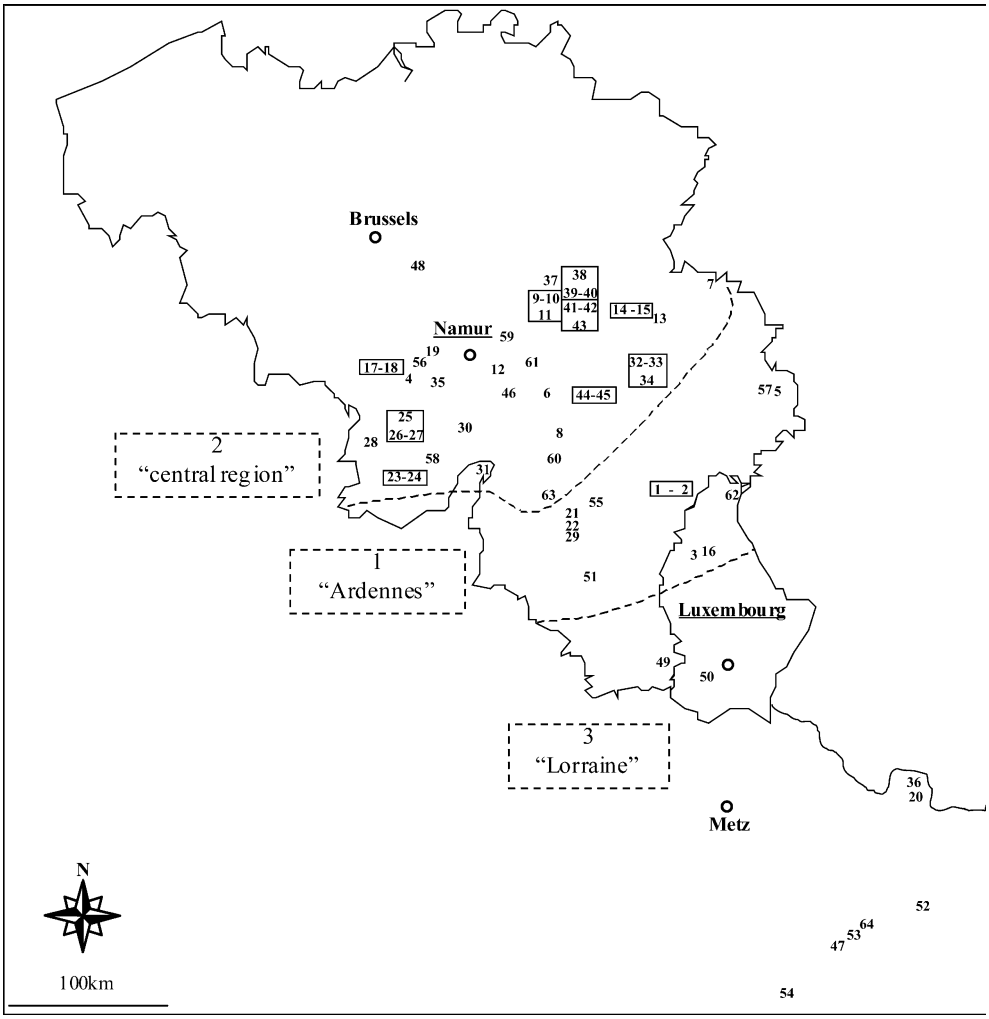

Figure 2. Geographical localisation of the 64 sampled water bodies with cyanobacterial blooms in the three regions differing by their water type. Framed numbers correspond to sites sampled several times.

Table 1. Sampling sites and dates; type, use, and trophic state of the sampled water bodies, as well as dominant cyanobacteria

No	Localization	Sampling date	Type of water bodies	Use	Trophic level	Dominant cyanobacteria
1	Aux Quatres-Chemins	28/08/1997	Pond	None	Eutrophic	*Microcystis* cf. *flos-aquae*
2	(Beho-Houffalize) BEL	12/09/2000				*Microcystis ichtyoblabe*
3	Bavigne-Beivenerbaach LUX	26/09/2001	Pre-dam	Pre-dam	Mesoeutrophic	*Anabaena planctonica*
4	Bomerée (Mont-sur-Marchienne) BEL	09/09/1999	Pond	Fishery	Hypereutrophic	*Planktothrix agardhii*
5	Bütgenbach (Bütgenbach) BEL	04/10/2000	Lake	Recreational activities	Eutrophic	*Woronichinia naegeliana*
6	Château de Barvaux-Condroz (Havelange) BEL	30/08/1997	Pond	None	Hypereutrophic	*Anabaena smithii*
7	Château de Graaf (Montzen-Plombières) BEL	21/08/1997	Pond	Fishery	Hypereutrophic	*Planktothrix agardhii*
8	Château de Hogne (Somme-Leuze) BEL	31/08/1999	Pond	None	Hypereutrophic	*Woronichinia naegeliana*
9	Château de Jehay (Amay)	22/09/1997	Pond	None	Hypereutrophic	*Anabaena spiroides*
10	BEL	23/07/1999				Coiled *Anabaena*
11		11/08/2000				*Planktothrix agardhii*
12	Château de la Neuve-Cour (Florée-Gesves) BEL	20/08/1997	Pond	None	Hypereutrophic	*Woronichinia naegeliana*
13	Château de Wégimont (Soumagne) BEL	13/08/1997	Pond	None	Hypereutrophic	*Microcystis* cf. *flos-aquae*
14	Clinique N-D des	11/08/1997	Pond	None	Hypereutrophic	*Microcystis* cf. *flos-aquae*
15	Bruyères (Grivegnée-Liège) BEL	21/08/2000				*Woronichinia naegeliana*
16	Esch-sure-Sûre LUX	03/10/2000	Reservoir	Drinking-water	Mesoeutrophic	*Planktothrix agardhii*
17	Etang de l'ancienne	09/09/1999	Pond	Fishery	Hypereutrophic	Coiled *Anabaena*
18	Abbaye d'Aulne (Charleroi) BEL	24/08/2000				Coiled *Anabaena*
19	Etang Michaux (Charleroi) BEL	25/08/2000	Pond	Fishery	Hypereutrophic	*Planktothrix agardhii*/ *Anabaena compacta*
20	Etang rouge (Insviller-Moselle) FR	06/09/2000	Pond	None	Eutrophic	*Microcystis aeruginosa*
21	Etangs de Robaiwé I (Saint-Hubert) BEL	15/09/2000	Pond	Recreational activities	Hypereutrophic	*Microcystis* sp.
22	Etangs de Robaiwé II (Saint-Hubert) BEL	15/09/2000	Pond	None	Mesoeutrophic	*Microcystis* sp.
23	Etangs de Virelles	05/09/1997	Pond	Nature conservation	Hypereutrophic	*Anabaena spiroides*
24	(Chimay) BEL	25/08/2000				Coiled *Anabaena*
25	Féronval (L'Eau d'heure-	05/09/1997	Lake	Pre-dam	Hypereutrophic	*Aphanizomenon flos-aquae*
26	Philippeville) BEL	10/09/1999				*Woronichinia naegeliana*/ *Planktothrix agardhii*/ *Aphanizomenon* sp.
27		24/08/2000		Recreational activities		*Woronichinia naegeliana*

28	Fourbechies (Froid-Chapelle) BEL	25/08/2000	Pond	Livestock reservoir	Hypereutrophic	*Microcystis* sp.
29	Freux (Saint-Hubert) BEL	15/09/2000	Pond	None	Mesoeutrophic	Coiled *Anabaena*
30	Ftêre (Serville) BEL	31/08/2000	Pond	Fishery	Hypereutrophic	*Planktothrix agardhii*
31	Givet (Ardennes) FR	31/08/2000	Pond	None	Hypereutrophic	*Planktothrix rubescens*
32	Gomzé-Andoumont	07/08/1997	Pond	None	Hypereutrophic	*Planktothrix agardhii*
33	(Sprimont) BEL	23/07/1999				*Microcystis wesenbergii*/ *Planktothrix agardhii*
34		25/08/2000				*Microcvslis wesenbergii*
35	Hanzinne (Florennes) BEL	02/09/1999	Pond	Fishery	Hypereutrophic	*Aphanizomenon* sp.
36	Hoste (Moselle) FR	06/09/2000	Pond	None	Hypereutrophic	*Aphanizomenon* sp.
37	Jehay étang inférieur (Amay) BEL	11/08/2000	Pond	Nature conservation	Hypereutrophic	*Planktothrix agardhii*
38	Jehay étang moyen	03/09/1997	Pond	Nature conservation	Hypereutrophic	*Microcystis aeruginosa*
39	(Amay) BEL	30/08/1999				*Planktothrix agardhii*
40		11/08/2000				*Planktothrix agardhii*
41	Jehay étang supérieur (Amay) BEL	03/09/1997	Pond	Nature conservation	Hypereutrophic	*Microcystis* cf. *flos-aquae*
42	42 (Amay) BEL	30/08/1999				*Microcystis flos-aquae*
		11/08/2000				*Planktothrix agardhii*
43						*Planktothrix agardhii*/ *Limnothrix redekei*
44	La Basse-à-l'eau (Filot-	12/08/1997	Pond	None	Hypereutrophic	*Microcystis wesenbergii*/
45	Hamoir) BEL	02/08/1999				*Microcystis flos-aquae*
						Microcystis wesenbergii/ *Microcystis flos-aquae*
46	La carpe bocqueuse (Hamois) BEL	25/08/1997	Pond	Fishery	Eutrophic	*Planktothrix agardhii*
47	Lindre FR	06/09/2000	Pond	Nature conservation	Hypereutrophic	*Planktothrix agardhii*/ *Aphanizomenon* sp.
48	Louvain-la-Neuve (Ottignies) BEL	15/09/1997	Pond	None	Hypereutrophic	*Microcystis* sp.
49	Messancy BEL	28/09/2001	Pond	Fishery	Eutrophic	Straight *Anabaena*
50	Mettendall LUX	28/09/2001	Pond	None	Eutrophic	*Aphanizomenon issatschenkoi*
51	Neufchâteau BEL	15/09/2000	Pond	Fishery	Eutrophic	*Microcystis aeruginosa*
52	Niederstinzel (Moselle) FR	06/09/2000	Pond	None	Hypereutrophic	*Planktothrix agardhii*/ *Anabaena spiroides*

Continued on p. 104

Table 1. (Continued)

No	Localisation	Sampling date	Type of water bodies	Use	Trophic level	Dominant cyanobacteria
53	Nolweilher (Moselle) FR	06/09/2000	Pond	None	Hypereutrophic	*Planktothrix agardhii*
54	Parroy (Meurthe et Moselle) FR	06/09/2000	Pond	None	Hypereutrophic	*Planktothrix agardhii*
55	Pêcherie du moulin des trois ponts (Saint-Ode) BEL	03/08/1999	Pond	Fishery	Hypereutrophic	Coiled *Anabaena*
56	Pêcherie du Poisson rouge (Charleroi) BEL	24/08/2000	Pond	Fishery	Hypereutrophic	*Planktothrix agardhii*
57	Robertville (Waimes) BEL	18/08/1997	Reservoir	Drinking-water	Eutrophic	*Microcystis aeruginosa*
58	Roly (Philippeville) BEL	05/09/1997	Pond	Nature conservation	Hypereutrophic	*Anabaena spiroides*/ *Anabaena affinis*
59	Seilles (Andenne) BEL	04/09/1997	Pond	None	Hypereutrophic	*Romeria gracilis*
60	Serinchamps (Ciney) BEL	30/08/1997	Pond	None	Hypereutrophic	*Microcystis* sp.
61	Tharoul (Clavier) BEL	20/08/1997	Pond	None	Hypereutrophic	*Planktothrix agardhii*/ *Oscillatoria limosa*
62	Weiswampach (Luxembourg) LUX	20/08/2000	Pond	Recreational activities	Mesoeutrophic	*Microcystis* sp.
63	Wellin (Saint-Hubert) BEL	14/09/2000	Pond	None	Hypereutrophic	*Microcystis* sp.
64	Zommange (Moselle) FR	06/09/2000	Pond	None	Hypereutrophic	*Microcystis novacekii*

BEL – Belgium, FR – France, LUX – Luxembourg.

of 4%. Fixed samples were stored in the dark at 4 °C until observation.

Cyanobacterial biomasses used for microcystin analyses were harvested when cyanobacterial densities allowed their sampling by means of a plankton net (mesh size of 20 μm). Concentrated bloom materials were lyophilised and conserved at −20 °C until toxin analysis. 100 ml of unfiltered as well as filtered water onto Whatman GF/C filters were preserved at −20 °C until chemical analyses.

Physical and chemical analyses

Water temperature, pH, conductivity were determined *in situ* by using WTW probes (WTW196, WTW LF323).

Ammonium, soluble reactive phosphorus (SRP), total phosphorus (TP) and silica were dosed according to standard colorimetric methods (APHA, 1995). Other ions (Ca, Mg, K, Na, SO_4, Cl, NO_3, NO_2) were analysed by ionic chromatography (DIONEX DX 500, equipped with an IonPac AG 12A or a CG 12A ion exchange column, respectively). N/P ratios were calculated as the sum of all inorganic nitrogen forms reported to total phosphorus concentrations.

Cyanobacterial identifications

Cyanobacterial identifications were performed according to Komárek and Anagnostidis's classification (Anagnostidis & Komárek, 1985; Komárek & Anagnostidis, 1986; Anagnostidis & Komárek, 1988; Komárek & Anagnostidis, 1989) using a Leica DMRX light microscope. Floras of Geitler (1932), Komárek (1958, 1991), Komárek & Hindák (1988), Komarková-Legnerová & Eloranta (1992), Komárek & Anagnostidis (1998) and Watanabe (1992, 1998) were mainly used for identification.

Microcystin analyses

Microcystin concentrations were determined in 32 bloom samples by high-performance liquid chromatography (HPLC) equipped with a diode array detector.

For HPLC analysis, 10 mg freeze-dried material was extracted in 1 ml of 75% aqueous methanol. The samples were sonicated for 15 s in the Misonix ultrasonicator (Farmingdale, NY, USA) with an ultrasonic probe (100 W, diameter 19 mm with "spike") and liquid processor XL. Then, the extracts were centrifuged twice at $11000 \times g$ for 10 min at 4 °C. The supernatants were collected and evaporated in the SC110A Speedvac® Plus, ThermoSavant (Holbrook, NY, USA). The samples were dissolved in 1 ml of 75% aqueous methanol before HPLC analysis.

The samples were analysed using an Agilent 1100 series (Waldbronn, Germany) composed with quaternary pump, autosampler and thermostated column compartment. Chromatographic separation was achieved on a Merck Purospher Star RP-18e column (55 × 4 mm; 3 μm) with a C_{18} guard column (4 × 4 mm). The determination of microcystins by HPLC-DAD was performed using a gradient mobile phase of H_2O + 0.05% trifluoroacetic acid (TFA) (eluent A) and acetonitrile (ACN) + 0.05% TFA (eluent B) and diode-array (detection at 200–300 nm). The linear gradient conditions were as follows: 25% B at 0 min, 70% B at 5 min, 70% B at 6 min, 25% B at 6 min. Sample volume was 20 μl, flow rate 1 ml min^{-1} and column temperature 40 °C. Microcystins in the cyanobacterial extracts were identified against microcystin standards. Except for the samples 13-14-38-41-44-57 for which only qualitative investigations of MC-LR and MC-RR were performed, six microcystin variants were quantified (MC-LR, MC-RR, dmMC-RR, MC-LY, MC-LW, MC-YR). Microcystin concentrations were expressed as μg MC per dry weight of bloom materials.

Statistical analyses

Cluster analysis was performed with the unweighted pair-group method arithmetic averages (UPGMA) by using Primer 5 software (Clarke & Warwick, 2001). Cluster analysis was done with untransformed values of Ca-SO_4-Mg concentration as well as conductivity.

Statistical analyses were used to investigate the relationships between physical–chemical variables and dominant cyanobacterial genera (presence/absence) on one hand, and between physical–chemical data and seston microcystin contents on the other hand. Rarely dominant species such as *Romeria gracilis* (Koczw.) Koczw., *Limnothrix redekei* (Van Goor) Meff. and *Oscillatoria limosa*

Gom. were not taken into account in the statistical analyses.

Canonical Correspondence Analysis (CCA) was performed with the CANOCO software (Ter Braak & Smilauer, 1998). CCA allows the interpretation of relationships between tested matrices. It is a linear, direct method that assumes unimodal distribution of genera (or toxin contents) along environmental gradients. Monte Carlo permutation tests were used to determine the statistical significance of each variable ($p < 0.05$). Abiotic matrices (physical and chemical data) were standardised. The biotic matrix (presence/absence of cyanobacteria) was not transformed. The ordination method could not be employed on the toxin matrix since the number of toxin data was too low in comparison to the abiotic matrix to support this statistical approach.

The so-called BIO-ENV procedure carried out with Primer 5 software (Clarke & Warwick, 2001) correlates similarity matrices of biota (or toxins) and environmental variables (Clarke & Ainsworth, 1993). It is an exploratory, non-linear technique that does not involve hypothesis-testing procedure but tries to find the best combinations of environmental variables correlated to biotic (or toxins) matrix using Spearman Rho (ρ) coefficient. For this purpose, abiotic and toxin matrices were square root transformed and corresponding similarity matrices were calculated on the basis of normalised Euclidean distances. The similarity matrices of the untransformed biotic data was realised by using the Bray-Curtis coefficient.

Results

The conductivity of the studied water bodies presenting a cyanobacterial bloom greatly varied between a minimum of 63 μS cm^{-1} and a maximum of 1123 μS cm^{-1}, reflecting the geological diversity of the catchments. The cluster analysis realised according to the conductivity and the conservative elements Ca, Mg, SO_4 revealed three main water types corresponding to the three main geological areas of the prospected zone: the "Ardennes" region (cluster 1), the central region with the "Région Nord", "Sillon Sambre-et-Meuse", "Ardenne Condruzienne", "Condroz", "Famenne", "Calestienne" (cluster 2), and the "Lorraine" region (cluster 3) (Bellière & Groessens, 2000–2001) (Fig. 3). In the "Ardennes" region, water was characterised by a low mineralisation and calcium content (median conductivity: 142 μS cm^{-1}; median Ca concentration: 4.6 mg l^{-1}), whereas mineralisation and calcium concentrations were highest in the "Lorraine" region (median conductivity: 871 μS cm^{-1}; median Ca concentration: 80.9 mg l^{-1}). The central region included several geological catchments character-

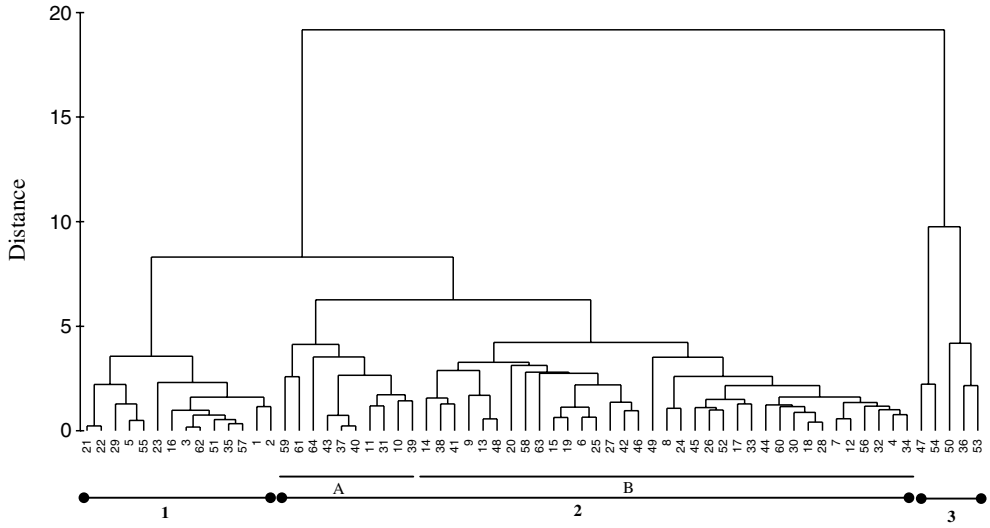

Figure 3. Clustering analysis performed on Ca–SO_4–Mg concentrations as well as on conductivity values.

ised by calcareous (''Région Nord, Condroz, Calestienne'') and acidic areas (''Ardenne Condruzienne, Famenne''). Sampling sites of the central region mostly belong to the calcareous ones. Their waters had ionic characteristics that occupied an intermediate position related to the other two regions (median conductivity: 346 μS cm^{-1}; median Ca concentration: 17.6 mg l^{-1}). Concerning the central region, 2 water subclusters (2A, 2B) were distinguished on the basis of the clustering analysis; these two types mainly differ by their conductivity, Ca and Mg concentrations. The regional differences in the three main water types are also reflected in Na, K and Cl concentrations (Fig. 4).

pH was always in the alkaline range and sometimes reached quite high values (up to 9.7). On the basis of total phosphorus concentrations and OECD (1982) trophic levels, the majority of the water bodies could be considered as hypertrophic since almost all TP concentrations were superior to 100 μg P l^{-1}, whereas most SRP concentrations were below 20 μg P–PO_4 l^{-1} with a median of 9 μg P–PO_4 l^{-1} (Fig. 4). Nitrogen forms including nitrate, ammonium and nitrite had moderate concentrations, with median concentrations of 115 μg l^{-1} N–NO_3, 25 μg l^{-1} N–NH_4 l^{-1} and 10 μg l^{-1} N–NO_2, respectively. Consequently, N/P ratios were usually below 25.

During the period of observations, 64 cyanobacterial blooms from 49 localities were collected (Table 1). Blooms were encountered in all prospected regions, but were more frequent in the central part of Belgium (''Région Nord, Sillon Sambre-et-Meuse, Ardenne Condruzienne, Condroz, Famenne, Calestienne'') (Fig. 2).

Microcystis, *Planktothrix* and *Anabaena* were the genera most frequently found as (co-)dominant in surface scums. They (co-)dominated respectively in 34, 34 and 20% of the investigated water bodies. *Aphanizomenon* and *Woronichinia* Elenkin were rarely observed and (co-)dominated in 9% of the samples (Fig. 5). Furthermore, blooms (co-)dominated by *Limnothrix redekei* (Van Goor) Meffert, *Oscillatoria limosa* Gomont as well as an atypical *Romeria gracilis* (Koczw.) Koczw. bloom were recorded only once in sampling sites 43-61-59, respectively.

According to the Monte Carlo permutation tests, CCA gave Ca, SO_4, Mg and conductivity as the only explicative variables influencing the dominance of *Aphanizomenon* and *Woronichinia* (Fig. 9). *Aphanizomenon* dominance was positively correlated to high values of these four variables whereas *Woronichinia* dominance appeared improved at low values. The other 12 variables could not be interpreted since they were statistically not significant and so, no correlations of *Microcystis*, *Anabaena* and *Planktothrix* dominance with any variables could be done.

BIO-ENV analysis performed with the whole physical and chemical data set, gave a low Spearman coefficient ($\rho = 0.11$) for the best variables combination that included Ca, conductivity and TP as the main explicative variables in the cyanobacterial genera dominance (Table 2).

The relative frequency of the (co-)dominant genera (Fig. 6) showed that *Microcystis* was the most abundant genus in waters with low ion charge (cluster 1: ''Ardennes'' region) while the highest frequencies of *Planktothrix* were recorded in the waters with the highest conductivities (clusters 2A and 3). Likewise, *Aphanizomenon* and *Woronichinia* were most frequently observed as (co-)dominant in the high conductivity waters (cluster 3: ''Lorraine'' type) and low conductivity waters (cluster 1: ''Ardennes'' and cluster 2A: central type), respectively.

BIO-ENV analysis performed on the different clusters lead to significant Spearman coefficients for variable combinations of clusters 1-2A-3 whereas analyses performed on clusters 2(A + B) and 2B appeared less informative (low ρ coefficient) (Table 2).

Microcystins were detected in all the studied regions and in 53% of the analysed cyanobacterial blooms (Fig. 7). Of the microcystin-containing blooms, 47% were (co-) dominated by *Microcystis*, followed by *Planktothrix* and *Woronichinia* with 29% and 18%, respectively. Finally, 12% of the samples containing microcystin were (co-) dominated by *Anabaena* whereas 1 out of the 3 *Aphanizomenon* bloom samples contained microcystin. The minimum concentration, measured in total seston was 31 μg g^{-1} DW (at a detection limit of 5 μg g^{-1} DW) while the maximum revealed in a *Woronichinia* bloom amounted to 2231 μg g^{-1} DW (Fig. 8). Total microcystins concentration mean amounted to 266 μg g^{-1} DW with a median of 31.0 μg g^{-1} DW. In the blooms presenting

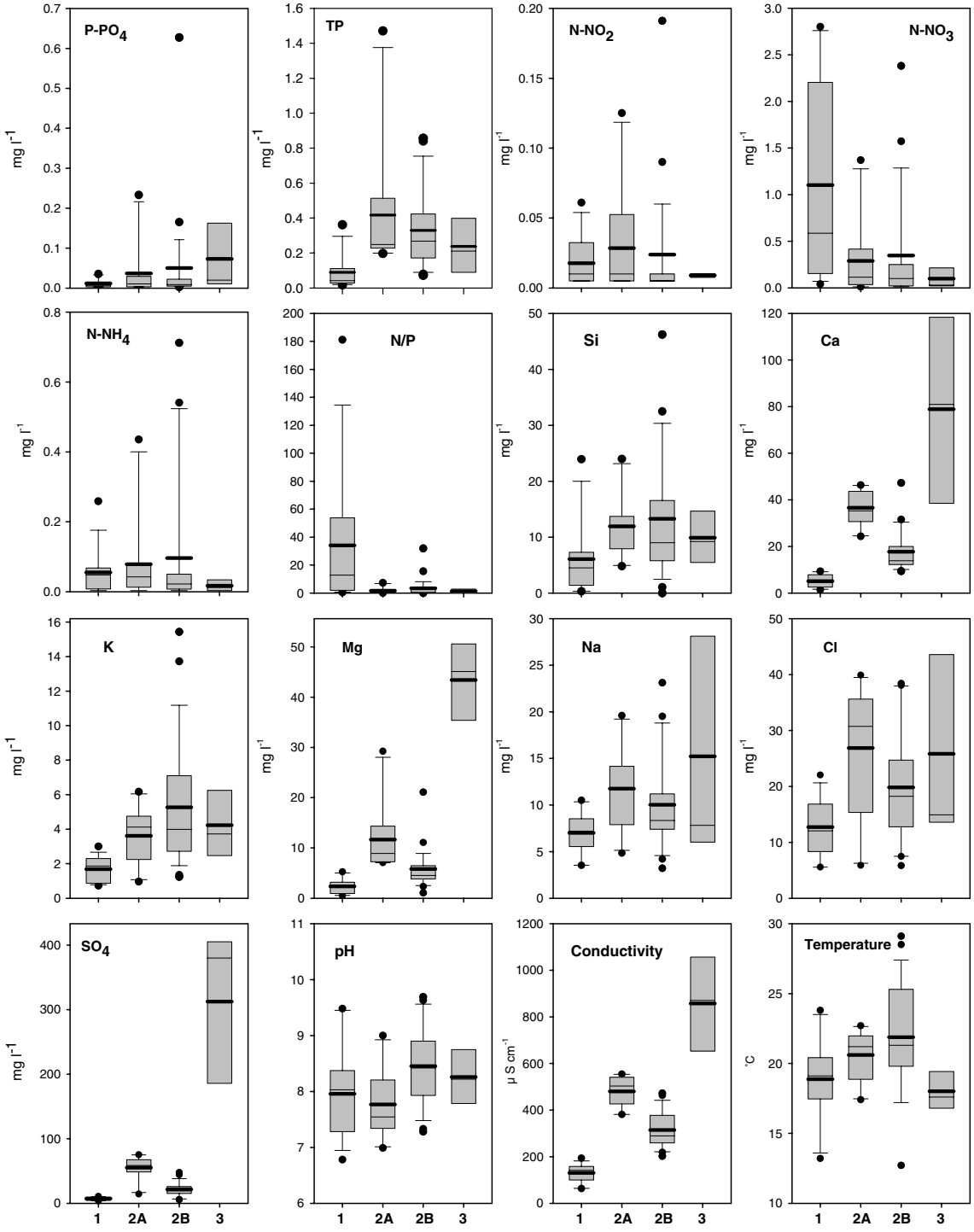

Figure 4. Box-plots of physical and chemical variables of sampled water bodies dominated by cyanobacteria in the 4 distinguished water (sub-)types. Fine line: median. Bold line: mean. Grey box: 25th–75th percentile. Vertical lines: 10th–90th percentile. Dark points: extreme values.

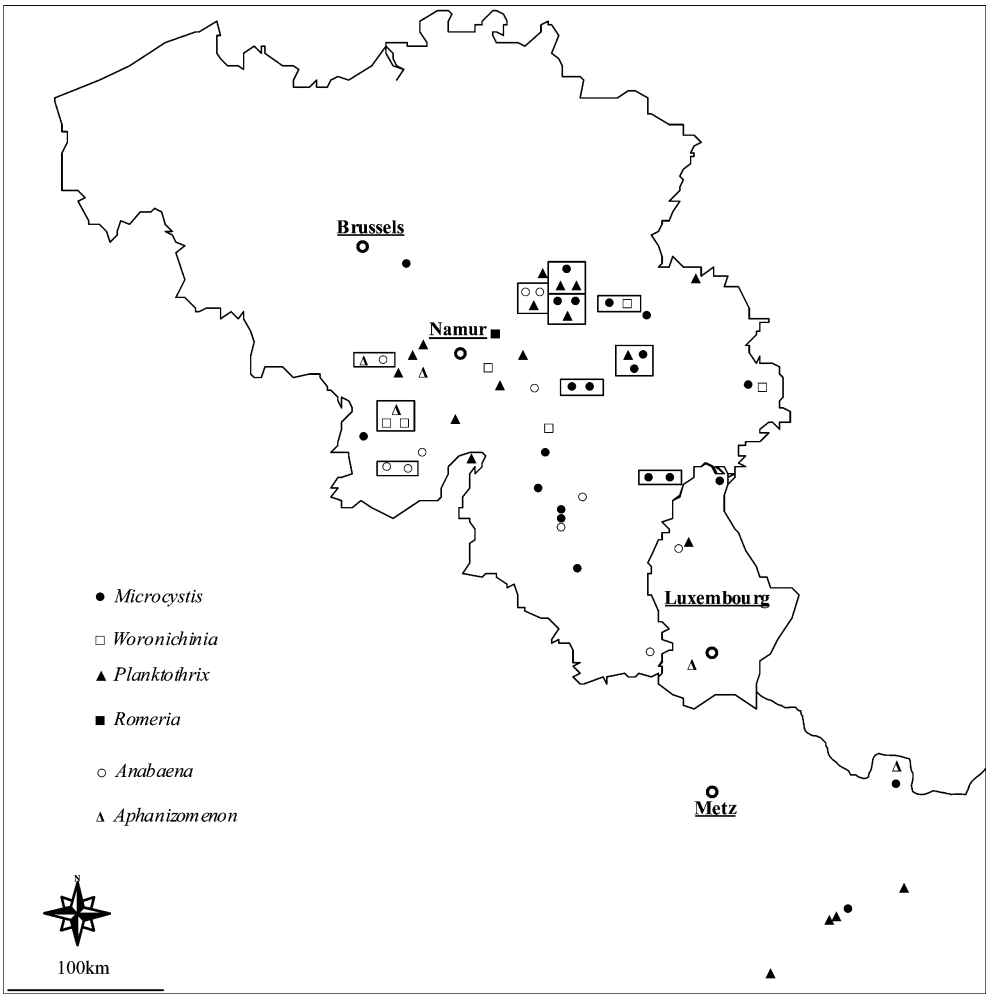

Figure 5. Geographical distribution of the dominant cyanobacterial genera forming blooms.

microcystin, concentrations varied from 31 to 118 $\mu g\ g^{-1}$ DW when *Microcystis* was dominant or (co-) dominant, from 31 to 972 $\mu g\ g^{-1}$ DW when *Planktothrix* was (co-)dominant, from 47 to 67 $\mu g\ g^{-1}$ DW when *Anabaena* was dominant, and from 571 to 2231 $\mu g\ g^{-1}$ DW when *Woronichinia* was dominant. The only *Aphanizomenon* bloom, which presented microcystins had a content of 42 $\mu g\ g^{-1}$ DW. In most blooms, several (up to 6) microcystin variants were present. Microcystin-LR was the most frequent microcystin variant found. It was detected in 64% of the analysed blooms, followed by microcystin-YR (55%), whereas MC-LY was never revealed (Fig. 8). The share of microcystin-LR in relation to total microcystins ranged between 0 and 100% (mean: 47%, median: 47%).

BIO-ENV analysis performed on physical and chemical/total microcystin matrices gave an elevated Spearman coefficient of 0.5 for the best variables combination that included N/P, Mg and Si as the main explicative variables effecting microcystin contents (Table 2).

Discussion

Distribution of dominant bloom-forming cyanobacteria in relation to environmental factors

As many other countries (see the monograph of the WHO by Chorus & Bartram, 1999), Belgium and Luxembourg do not escape to toxic cyanobacterial

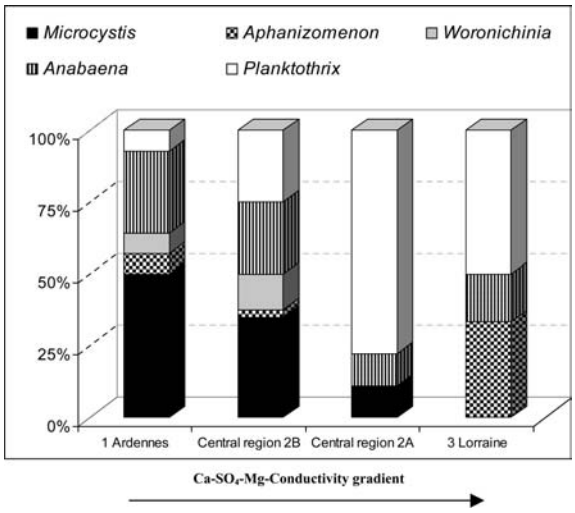

Figure 6. Relative abundance of dominant cyanobacterial genera within the 4 water (sub-)types defined on the basis of clustering analysis.

bloom problems. *Microcystis*, *Planktothrix, Aphanizomenon, Anabaena* and *Woronichinia* were the dominant bloom-forming genera. *Limnothrix*, frequently reported from northern Germany (Wiedner et al., 2001) was only observed once while the expanding *Cylindrospermopsis* (Padisák, 1997) was not observed at all.

The geographical distribution of the most common bloom-forming genera seems to follow a north–south pattern, with heterocystous genera, especially *Anabaena* dominating in northern Europe, *Planktothrix* (*Limnothrix*)-*Microcystis* in northern Germany, and *Microcystis* in blooms in the central and southern parts of Europe. Thus, Skulberg et al. (1994) revealed that *Anabaena* was the most common bloom-former in South Norway where they nearly dominated 50% of the investigated blooms. In Swedish lakes, Willén & Mattsson (1997) recorded a higher frequency of *Anabaena* spp. than other bloom-forming species. Furthermore, Sivonen et al. (1990) demonstrated that the frequency of blooms containing *Anabaena* spp. was the highest in Finland. In Germany, *Microcystis* (in the southern state of Baden-Wurttemberg) and *Planktothrix* (in the northern part) were the most common bloom-formers (Fastner et al., 1999; Wiedner et al., 2001). *Planktothrix* spp. dominated blooms seem to be mainly confined to the latter part of Europe, since they were only rarely recorded dominant in Portugal (Vasconcelos, 1994) and were also less abundant in Norwegian, Swedish and Finnish blooms (Sivonen et al., 1990; Skulberg et al., 1994; Willén & Mattsson, 1997). By contrast, *Microcystis* was the most frequently dominating genus encountered in Slovak (Maršálek et al., 2000) and Portuguese (Vasconcelos, 1994) freshwater blooms.

The cyanobacterial bloom assemblages in the studied area seem thus to occupy an intermediate position with *Microcystis* dominating most of the blooms, but with a significant number of blooms dominated by heterocystous taxa and by *Planktothrix*.

Table 2. BIO-ENV analyses showing the best variable combinations evaluated by Spearman coefficient for physico-chemistry/cyanobacterial dominance and physico-chemistry/total microcystin concentration

	Matrix	Best variable combination	Spearman coefficient
Physico-chemistry/cyanobacterial dominance	Complete data set	TP-Ca-conductivity	0.11
		Ca-conductivity	0.10
		SO_4-Ca-temp.-PO_4	0.10
	cluster 1	PO_4-NH_4-K-N/P-temp.	0.55
	cluster 2 (A + B)	PO_4-NH_4-K-TP	0.11
	cluster 2A	NO_3-K-N/P-temp.	0.54
	cluster 2B	pH	0.06
		PO_4-pH	0.06
		PO_4-Si-pH	0.06
	cluster 3	TP-pH-Temp.	0.44
Physico-chemistry/total microcystin concentration		N/P-Mg-Si	0.50

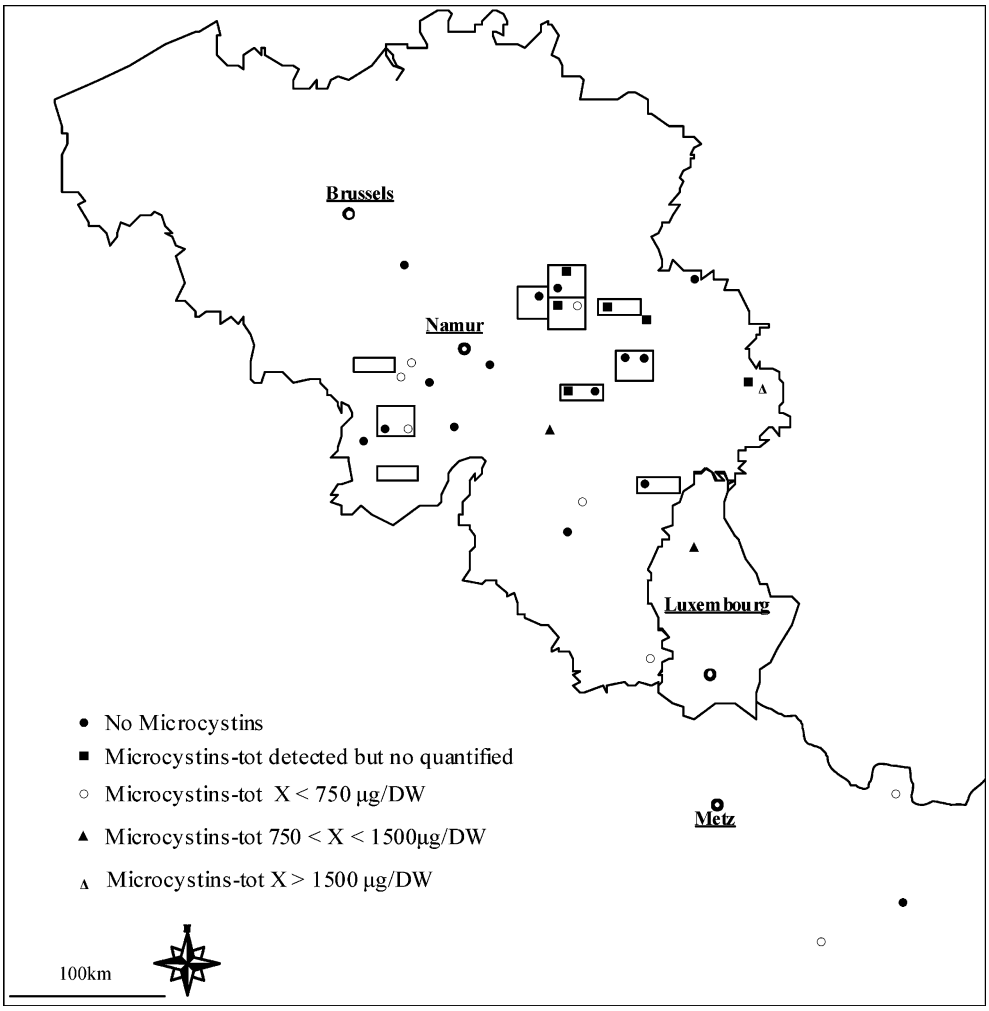

Figure 7. Geographical distribution of microcystin-containing blooms.

Bloom-forming genera commonly found in Europe are also responsible of massive developments throughout the world. Surveys carried out in South America, Africa, Australia and Asia pointed out that most of bloom-forming cyanobacteria are cosmopolitan. Indeed, *Microcystis*, *Anabaena*, *Aphanizomenon* and *Planktothrix* are dominant in temperate but also in tropical habitats (Bowling, 1994; Hadas et al. 1999; Komárek et al., 2002; Shen et al., 2003). Nevertheless, their relative abundance is variable according to the country investigated. For instance, in Australia, blooms were mostly located in the southeastern part of the continent corresponding to the wettest and coolest area. Despite of the widespread occurrence of *Planktothrix agardhii* throughout the world, *P. agardhii* was rarely encountered in Australia. This may be due to the high temperature and turbidity of Australian water bodies. This fact reflects that environmental conditions influence the distribution and the species composition of the cyanobacterial blooms. Nevertheless, cares have to be taken in the interpretation of the geographical blooms localisation since the "distribution map" remains incomplete, especially in tropical and subtropical areas. Other cyanobacteria such as *Cylindrospermopsis* (Branco & Senna, 1994; Bouvy et al., 2000) that remains unobserved in Belgium and Luxembourg had a more confined occurrence. Nonetheless, this tropical genus is now extending from its native area and invading mid-latitude areas (Druart & Briand, 2002; Briand et al., 2004).

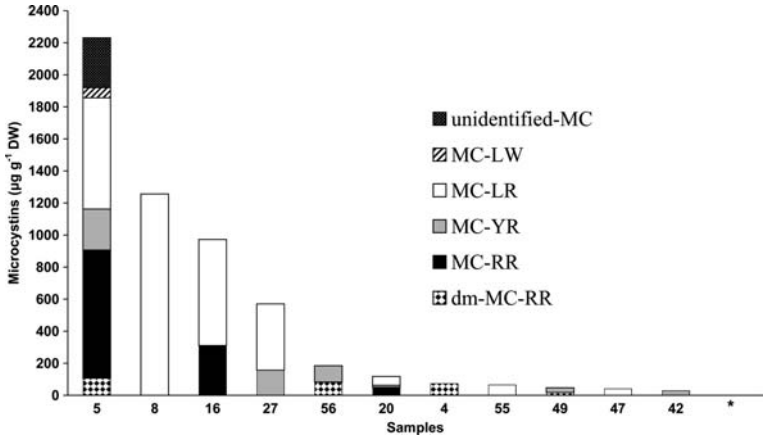

Figure 8. Concentrations of the 6 analysed microcystin variants (dmMC-RR/MC-RR/MC-YR/MC-LR/MC-LW/MC-LY) in the bloom samples. * – samples site 1-7-10-12-26-28-29-30-32-33-35-39-45-48-52 for which no microcystins were revealed.

According to our survey and despite that some bias associated to the sampling strategies cannot be excluded, the results suggest significant differences in the geographical distribution of the dominant bloom-forming genera. The CCA carried out on the 64 cyanobacterial bloom samples and the physical and chemical variables revealed conductivity, Ca, SO_4, and Mg as significant explicative variables effecting the dominance of *Aphanizomenon* and *Woronichinia* (Fig. 9). *Aphanizomenon* dominance was positively correlated to highly mineralised and calcium–magnesium–sulphate rich waters, whereas *Woronichinia* dominance is favoured by low values of these variables. No significant correlations were obtained between the dominance of the genera *Microcystis*, *Anabaena* and *Planktothrix* and the abiotic variables by CCA. The BIO-ENV procedure performed on the whole data set also indicates, despite a rather low Spearman coefficient ($\rho = 0.11$), the role of conductivity and Ca. Furthermore, the BIO-ENV procedure included TP in the best variable combination having an effect on cyanobacterial genera dominance (Table 2).

These results point out the central role of parameters related to the geology, which determines the natural water types, on the composition of the cyanobacterial bloom assemblages. These findings are in agreement with those of Reynolds & Petersen (2000), who showed that the distribution of dominant bloom-forming cyanobacteria in Irish lakes was strongly related to the geology of the catchments and more particularly to fertile calcareous waters. Furthermore, *Aphanizomenon* was commonly described as dominant in hard water lakes (Konopka, 1989; Zhang & Prepas, 1996) and was also frequent in some brackish waters like the Baltic Sea (Lehtimäki et al., 1997). The dominance of *Aphanizomenon* in such environments underlines its ability to thrive in waters with high ion strength suggesting the importance of this factor on the distribution of *Aphanizomenon*. Few data are available on the ecological requirements of *Woronichinia naegeliana*. This species is commonly described as an autumnal species able to form blooms at lower temperature than other bloom-forming cyanobacteria (Wilk-Woźniak, 1998; Wilk-Woźniak & Mazurkiewicz-Boroń, 2003). According to CCA, its development seems to be positively influenced by low ionic charges.

In contrast to CCA, the BIO-ENV analysis could also be applied to matrices with a reduced size and therefore allowed the determination of the best variables combination involved in the control of dominant cyanobacterial bloom formers within each of the four water types (Table 2). Significant Spearman coefficients were obtained for variable combinations of clusters 1, 2A, and 3, whereas analyses performed on clusters 2 and 2B appeared non informative (low ρ coefficient). Cluster 1 (the ''Ardennes'' water type) was characterised by a significant combination ($\rho = 0.55$) of 5 variables (PO_4, NH_4, K, N/P, temperature) explaining the genera distribution inside the group. For cluster 2A (the central water type), NO_3, K, N/P and temperature were revealed as the best variable

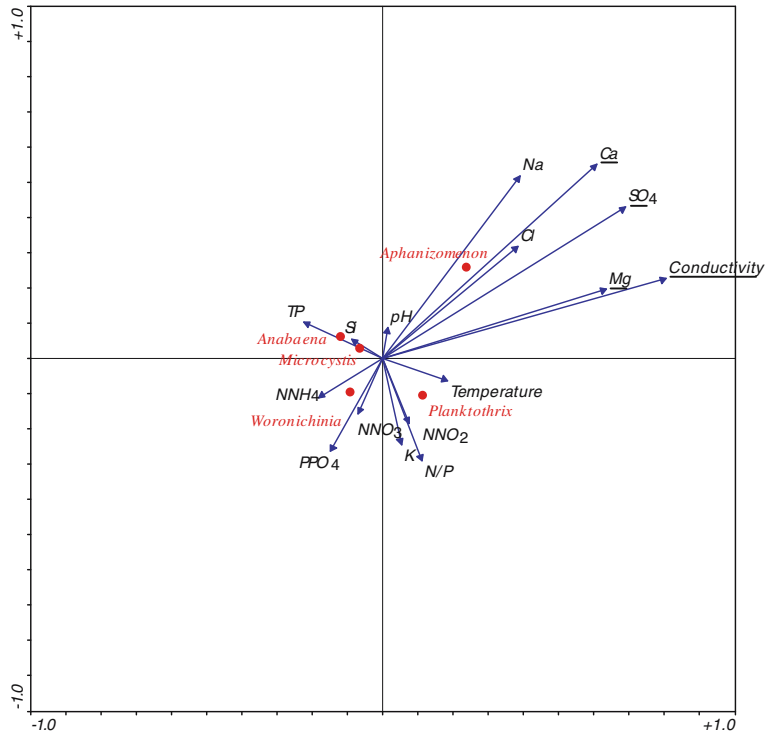

Figure 9. Canonical correspondence ordination of the main (co-)dominant cyanobacterial genera (points) as well as environmental variables (arrows). Cumulative percentage variance of species-environment relation axis 1–2 : 67.6. Statistical significant variables are underlined (according to Monte Carlo Permutation tests – $p < 0.05$).

combination in the explanation of the genera distribution ($\rho = 0.54$). TP, pH as well as temperature represented the best variable combination obtained on cluster 3 by BIO-ENV analysis ($\rho = 0.49$).

Consequently, in the mostly (hyper)eutrophic shallow water bodies of the studied area, the ion strength seems to act as the main shaping force of the cyanobacterial assemblages, while other key factors such as phosphorus, nitrogen and temperature play a secondary role within each water (sub-)type.

Besides the catchment characteristics, climatological variables such as temperature and precipitation assuredly also play a major role on the cyanobacterial dominance. These variables could also contribute to the differences of the cyanobacterial flora between the three regions (“central region, Ardennes, Lorraine”) since these areas have contrasted climatological regimes (D.G.A.T.L.P., 2003). For instance, mean temperature recorded in July in the “Ardennes region” is 2 °C inferior to those of the central and the “Lorraine region” (15 °C against 17 °C). Furthermore, annual rainfall can reach more than 1400 mm in the “Ardennes region” whereas values recorded in the central region rarely overcome 1000 mm.

Frequency of microcystin-producing genera

Microcystin was observed in 53% of the analysed bloom samples. This percentage is within the range of results from other European surveys, e.g. 66% in Denmark (Henriksen, 1996), 66% in Germany (Fastner et al., 2001), 60% in Portugal (percentage based on the combination of hepatotoxic tests (i.p. mouse) and microcystin analysis results) (Vasconcelos, 2001), although higher ratios may occur, e.g. 90% in Czech Republic (Maršálek et al., 2001).

Of all analysed samples (co-)dominated by *Microcystis* spp., 47% were found to contain microcystins while for the other genera it ranged from 12 to 33%. The majority of the samples

where microcystins were pointed out were dominated by *Microcystis* (47%), followed by *Planktothrix* (29%), *Anabaena* (12%), *Woronichinia* (18%), and *Aphanizomenon* (6%). When analysed at a European scale, the frequency of genera dominating hepatotoxic, respectively microcystin-containing blooms varies from region to region. In southern Norway, Skulberg et al. (1994) showed that almost 44% of the lakes with toxigenic cyanobacteria leading to a hepatotoxic response by standard mousse bioassay were dominated by *Microcystis* followed by *Anabaena*, whereas blooms of *Planktothrix* and *Snowella-Woronichinia* revealed reduced toxic properties (less than 15%). *Microcystis* and *Anabaena* are also known as the most common genera found in hepatotoxic blooms in Finland (Sivonen et al., 1990). In Norway, Utkilen et al. (2001) showed that hepatotoxic cyanobacterial blooms were most frequently associated to *Anabaena* dominance. In Danish lakes, microcystins occurred most frequently in samples dominated by *Microcystis* followed by those containing several cyanobacterial taxa (Henriksen, 1996). In Germany, *Planktothrix* was found to be the most common dominant taxon in samples containing microcystins in many parts of northern Germany, whereas in the southern state of Baden-Wurttemberg, *Microcystis* was clearly the most important genus in toxic blooms. In Central (e.g. Slovakia and Czech Republic: Maršálek et al., 2000; 2001) and southern Europe (e.g. Portugal: Vasconcelos, 1994), *Microcystis* was the dominant microcystin producer in more than two third of the samples. Consequently, although *Microcystis* appears as the main hepatotoxic/microcystin producing genus throughout Europe, other genera may represent a significant part in the toxigenic blooms.

Microcystin concentrations and variants

In the analysed samples, total microcystin concentrations per seston biomass ranged from 31 to 2231 $\mu g\ g^{-1}$ DW and were similar to findings from most other countries (Chorus, 2001). The maximum microcystin concentration obtained in a *Woronichinia naegeliana* dominated bloom was 4 times higher than the one measured by Wirsing et al. (1998) in a Belgian *Microcystis* bloom. In our survey, the order of decreasing median of microcystin concentrations associated to (co-)dominant genera is *Woronichinia* (median: 1256 $\mu g\ g^{-1}$ DW), followed by *Planktothrix* and *Microcystis* (identical median: 75 $\mu g\ g^{-1}$ DW), *Anabaena* (median: 57 $\mu g\ g^{-1}$ DW) and *Aphanizomenon* (median: 47 $\mu g\ g^{-1}$ DW). These results are in agreement with data from Denmark (Henriksen, 2001) and Germany (Chorus, 2001) where the order of decreasing median concentrations is *Planktothrix* > *Microcystis* > *Anabaena*.

It is hazardous to correlate the observed seston microcystin concentrations to the environmental variables and to identify physiological responses of seston microcystin content to abiotic factors, which are often put forward on the basis of culture studies (e.g. temperature, light, nutrients, etc.) (Sivonen, 1990; Lee et al., 2000; Wiedner et al., 2003). To this end it would be necessary to determine within the seston biomass the cellular microcystin production of the microcystin-producing cells. These results obtained in the present study suggest that species composition is the overriding variable governing microcystin concentrations in the blooms of the studied area. The strong positive correlation (Spearman coefficient $\rho = 0.50$) obtained with N/P, Si, and Mg by the BIO-ENV procedure (Table 2) probably indicates that these variables have a lower importance on microcystin production compared to their effects on species composition.

As in other European water bodies, microcystin-LR was the most frequently encountered microcystin variant (Sivonen & Jones, 1998). However, this may be simply due to the fact that microcystin-LR is often the only analysed variant. In 55% of the analysed samples, microcystin-LR amounted to less than 50% of total microcystins and in 36% of the hepatotoxic samples microcystin-LR was not present at all. These data indicate that the toxicity potential cannot be simply based on microcystin-LR concentration and that other microcystins should not be neglected in the analysis of cyanobacterial bloom samples.

Conclusions

This study shows for the first time the extent of microcystin-containing cyanobacterial blooms in

southern Belgium and Luxembourg. Quite high microcystin concentrations were found in water bodies used for recreational purposes or for drinking water production and hence revealed a potential threat to human health. Data obtained highlight that the monitoring of cyanobacterial blooms should be on the agenda of the administrations responsible for water quality management in both countries.

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