Microbial Ecology

Distribution of Microcystins in a Lake Foodweb: No Evidence for Biomagnification

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Abstract

Microcystins, toxins produced by cyanobacteria, may play a role in fish kills, although their specific contribution remains unclear. A better understanding of the ecotoxicological effects of microcystins is hampered by a lack of analyses at different trophic levels in lake foodwebs. We present 3 years of monitoring data, and directly compare the transfer of microcystin in the foodweb starting with the uptake of (toxic) cyanobacteria by two different filter feeders: the cladoceran *Daphnia galeata* and the zebra mussel *Dreissena polymorpha*. Furthermore foodwebs are compared in years in which the colonial cyanobacterium *Microcystis aeruginosa* or the filamentous cyanobacterium *Planktothrix agardhii* dominated; there are implications in terms of the types and amount of microcystins produced and in the ingestion of cyanobacteria. Microcystin concentrations in the seston commonly reached levels where harmful effects on zooplankton are to be expected. Likewise, concentrations in zooplankton reached levels where intoxication of fish is likely. The food chain starting with *Dreissena* (consumed by roach and diving ducks) remained relatively free from microcystins. Liver damage, typical for exposure to microcystins, was observed in a large fraction of the populations of different fish species, although no relation with the amount of microcystin could be established. Microcystin levels were especially high in the livers of planktivorous fish, mainly smelt. This puts piscivorous birds at risk. We found no evidence for biomagnification of microcystins. Concentrations in filter feeders were always much below those in the seston, and yet vectorial transport to higher trophic levels took place. Concentrations of microcystin in smelt liver exceeded those in the diet of these fish, but it is incorrect to compare levels in a selected organ to those in a whole organism (zooplankton). The discussion focuses on the implications of detoxication and covalent binding of microcystin for the transfer of the toxin in the foodweb. It seems likely that microcystins are one, but not the sole, factor involved in fish kills during blooms of cyanobacteria.

Introduction

Cyanobacterial toxins are believed to be involved in the large-scale die-off of fish and waterbirds (e.g., [35, 46]. In most studies that have investigated these events direct evidence for the role of toxic cyanobacteria is lacking [41]. During periods when blooms of cyanobacteria develop, other detrimental factors may play a role, such as extreme temperatures or pH, low oxygen, or other toxin-producing microorganisms. The specific contribution of cyanobacterial toxins to fish or bird mortality in natural waters remains largely unclear [40].

Microcystins (MC) are the most widespread type of cyanobacterial toxins in freshwater systems. Microcystins covalently bind to protein phosphatases in cells of animals that take up the toxin and are potent inhibitors of these enzymes. Given that in many temperate lakes fish and birds usually do not feed directly on phytoplankton, the microcystins, if indeed responsible for the death of fish and waterfowl, must reach them via the foodweb. Only a few studies have tracked the presence of microcystin in single or multiple components of aquatic foodwebs (e.g., [2, 11, 27, 33]). Other studies have analyzed the uptake of microcystin by aquatic organisms in an experimental setting (among others, zooplankton [12, 48, 55], mussels [1, 42, 63], or fish, [5, 34]), often using

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an acutely toxic dosage and purified microcystin or cultured cyanobacteria (see [62]). These results, therefore, are not readily transferable to a lake foodweb. Hence, still relatively little is known about the concentration of microcystins and their eco-toxicological effects in the aquatic ecosystem.

Our study was triggered by several large-scale fish kills: thousands of fishes, mainly ruffe, but also smelt, died in the summers of 1994, 1995, and again in 1996 in the IJsselmeer, The Netherlands. A monitoring program was started to assess the role of blooms of potentially toxic cyanobacteria in these fish kills. The monitoring was specifically aimed at detecting the dynamics in the concentration of microcystins, not only in the phytoplankton (seston), but also in other components of the foodweb. Included were the main grazers of phytoplankton in the lake and the next trophic level, fish from different feeding guilds. We directly compared the transfer of the toxin in a food chain starting with filter-feeding zooplankton (especially *Daphnia galeata*) and one starting with the zebra mussel, *Dreissena polymorpha*. Furthermore, we studied microcystin contents in the foodweb during blooms dominated by either a filamentous (*Planktothrix agardhii*) or a colonial (*Microcystis aeruginosa*) cyanobacterium, which has implications for the types and amount of microcystin produced [15], and for the ingestion of cyanobacteria by filter feeders. Simple as this approach may seen, it provides a deeper understanding of the processes that drive the uptake and transfer of microcystin in the foodweb. In the discussion we examine the evidence for harmful effects of microcystins for different components of the aquatic foodweb.

We also query the evidence for biomagnification of microcystins in the foodweb. Biomagnification (and the related process bioaccumulation) is not used consistently in the literature [19]. We follow the definition given in [20] and [32]. Biomagnification refers to the transfer of a chemical from food to an organism, resulting in a higher concentration in the organism than in its diet. The result may be a concentration of the chemical as it moves up the foodchain. Since transfer of microcystins almost exclusively takes place via the foodweb (concentrations of dissolved microcystin are usually low [51] and may have little effect on (some) biota [31]), biomagnification rather than bioaccumulation is the relevant process to study. Lipophilicity of a chemical (as determined by the octanol-water partition coefficient; log K_{OW}) is a strong determinant of the risk for biomagnification. Since microcystin-LR has a very low log K_{OW} [10] and its depuration from biota is relatively fast [63], we hypothesize that, although microcystins will be present at different trophic levels in the lake foodweb, there will be no evidence for biomagnification of microcystin. This will moderate the harmful effects of microcystin at higher trophic levels.

Materials and Methods

Lake IJsselmeer. The lake that was studied is the large (1200 km^2), shallow (mean depth 4.5 m), eutrophic (mean summer chlorophyll *a* concentration 40–60 μg L^{-1}) Lake IJsselmeer, The Netherlands. This lake was created in 1932 when a dam sealed off the former brackish Zuiderzee from the North Sea. The main tributary of the IJsselmeer is the River Rhine; hence concentrations of phosphorus in the lake have dropped strongly in response to falling concentrations in the river. The present mean summer concentration of total phosphorus (TP) in the lake is 0.12 mg P L^{-1}, whereas it exceeded 0.3 mg TP L^{-1} in the 1970 s. Yet despite this drop in TP the lake is still turbid, and submerged macrophytes are confined to marginal areas. Blooms of cyanobacteria have been present ever since the lake turned to fresh/slightly brackish water in the 1930s. In most years *Microcystis* is the main bloom-forming genus, although *Aphanizomenon* may also be abundant. In some years *Planktothrix agardhii* is the dominant cyanobacterium in the lake. Cyanobacterial blooms and especially scum formation on the shore are perceived as a major problem by the lake management and local drinking water companies.

The foodweb of the IJsselmeer can be characterised in the following way. Phytoplankton is the main primary producer. Of the primary production about one fifth is consumed by zooplankton, of which *Daphnia galeata, Daphnia cucullata*, and a hybrid species of these two are the main grazers. About one third of the primary production is consumed by *Dreissena*. The remainder of the production is channeled toward detritus and the microbial loop. The most abundant planktivorous fish in the lake is smelt (*Osmerus eperlanus*), a relic from the time when the IJsselmeer was still a brackish bay. Ruffe (*Gymnocephalus cernua*) is the main species feeding on benthic organisms, whereas roach (*Rutilus rutilus*) is the only fish species that feeds on zebra mussels. Larger perch (*Perca fluviatilis*) and pikeperch (*Stizostedion lucioperca*) are the most common predatory fishes. The IJsselmeer is a wetland of international importance under the Ramsar Convention. It hosts >1% of the biogeographic populations of 20 bird species (as much as 35% of the NW European population of the greater scaup *Aythya marila*). Large numbers of these diving ducks feed on *Dreissena* in autumn and winter. Various birds are piscivorous, e.g., cormorants (*Phalacrocorax carbon*) and goosanders (*Mergus merganser*).

Lake Sampling. It is known that phytoplankton distribution and abundance in the IJsselmeer is subject to large spatial and temporal variation [58]. To include some of this variation, the lake was sampled at biweekly

intervals between May and October 1997–1999 at three locations in the southern and three locations in the northern half of the IJsselmeer. Usually the chlorophyll concentration and the density of cyanobacteria are lower in the south and higher in the north. This is a consequence of a reverse gradient in the density of zebra mussels, which are more abundant in the south because of the ample availability of suitable substrate [23].

The following variables were included in the monitoring program (i) chlorophyll *a*; (ii) ash-free dry weight of the seston; (iii) phytoplankton abundance and composition; (iv) zooplankton abundance and composition; (v) standard egg production (SEP) of *Daphnia*; and (vi) microcystin concentrations in various compartments of the foodweb. For phytoplankton analysis a total volume of 25 L of lake water was collected from different depths in the water column; a 50 mL subsample was fixed with GAPF (0.1% glutaraldehyde / 0.01% paraformaldehyde) for subsequent analysis. Phytoplankton was analysed both microscopically and on the EurOPA flowcytometer, which distinguishes cyanobacteria from eukaryotic algae and is able to handle large colony-forming species [14]. For zooplankton analysis 25 L of water collected from different depths was concentrated using a plankton-net with a 30 μm mesh size. Zooplankton was resuspended in 45 mL tap water and fixed with 5 mL 37% formaldehyde. For SEP analysis 200 L of water was collected with a submersible pump and a plankton net of 300 μm mesh size. Tests showed that the pump did not affect swimming behavior or mortality of *Daphnia*. *Daphnia* was collected with a Pasteur pipette and fixed with 5 mL of a saturated sucrose solution, containing 37% formaldehyde. From 100 individuals the length and the number of eggs was determined. SEP was calculated using linear regression analysis as the average number of eggs for an animal with a standard length of 1 mm. Chlorophyll *a* and ash-free dry weight contents (AFDW) were analyzed according to standard (ISO) laboratory procedures. Mussels were collected with a net and carefully cleaned with a brush under running tapwater. Animals of all size classes were stored in the freezer at −20°C, and occasionally in liquid N_2 until further analysis. Fish was caught at three moments during the summer of 1999 (June, July, and September) and at three different locations in the lake (south, middle, north). From the catches a total of 25 individuals per species (perch, ruffe, and smelt) were collected. The length and weight of the fish were noted. Fish livers were prepared free and stored in the freezer at −80°C until analysis of microcystin. The ash free dry weight content of zooplankton, mussels and fish was determined on the same samples that were prepared for microcystin analysis.

Microcystin Analysis. A subset of the samples (1996 samples only) was screened for the presence of neurotoxins, but since these were never found only results on microcystins will be presented here. Microcystin was analyzed (i) dissolved in water; (ii) in the seston (the fraction including phytoplankton); (iii) in zooplankton; (iv) in mussels, and (v) in fish livers. The concentration of dissolved microcystin was measured according to [39]. Microcystins were extracted in 70% v/v aqueous methanol and separated on reverse-phase HPLC with photodiode array detection, according to the method described by [29]. The HPLC was equipped with a μBondapak C18 column; flow rate was 1 mL min^{-1}; temperature of the column was 40°C. For retention times between 15 and 40 min a spectrum between 200 and 300 nm was recorded every 2 s. Microcystins were identified on basis of a characteristic spectrum between 237 and 242 nm. The number of different microcystins was determined by the number of peaks that eluted with different retention times and showed a typical microcystin UV spectrum. Hence microcystins could be differentiated but not identified on basis of HPLC retention times. All different microcystins were transformed to microcystin-LR equivalents for quantification of the total microcystin content. Microcystin-LR (Sigma Chemicals) was the only standard that was used.

Microcystin analysis of the seston was performed on material collected on GF/F filters, freeze dried, and extracted in 5 mL methanol after a 30 min treatment in an ultrasonic bath at 0°C. The extract was dried under N_2 flow at 30°C, resuspended in 70% methanol, and transferred to HPLC vials.

Zooplankton from which the microcystin content was analyzed was collected from 2 m depth, by filtration of 600 L of lake water over a plankton net with a 250 μm mesh size; the zooplankton was resuspended in 2 L tap water. Before the analytical procedure could be started samples had to be prepared to avoid contamination with phytoplankton, which would probably contain microcystin. The following steps were followed: (i) the water was left standing overnight in measuring cylinders at *in situ* lake temperature and under dimmed light, allowing detritus to sink to the bottom and buoyant cyanobacteria to float to the top; (ii) scums of cyanobacteria were removed, and the remainder of the sample (but excluding the detritus) was rinsed carefully with tapwater and transferred to clean beakers; (iii) the water containing the zooplankton was flushed with CO_2, by which treatment the zooplankton was sedated and sank to the bottom of the beakers — the overlying water was removed; (iv) these steps were repeated until zooplankton was free of cyanobacteria (checked microscopically).

The methodology for microcystin analysis in zooplankton was developed and tested with *Daphnia magna* spiked with microcystin-LR. For this 100 μL of a 6.7 μg mL^{-1} MC-LR stock solution was added to a laboratory population of *Daphnia magna*, reared on the green alga

Scenedesmus quadricauda. Zooplankton was disintegrated by a treatment of 60 min in an ultrasonic bath at 0°C. Further treatment was similar to that of the seston. Three washing steps with methanol were included to free the samples from impurifications. In trials with *Daphnia* a recovery rate of 96% was found for microcystin-LR, whereas the chromatograms showed few other peaks that could interfere with the microcystin analysis.

The methodology for zebra mussels was closely comparable to that for zooplankton. Mussels from the IJsselmeer were thoroughly cleaned, freeze dried, and homogenized in a mortar. To 2 g of freeze dried material 100 µL of a 6.7 µg mL^{-1} MC-LR stock solution was added. For mussels spiked with microcystin-LR, a recovery rate for microcystin-LR of 79% was found. In the case of the mussels more potentially disturbing peaks were found in the chromatograms, which could be separated from the microcystin peaks by analysis of the spectra.

Extraction of microcystin from fish livers followed a similar procedure. Livers were extracted in 70% methanol for a minimum period of 12 h at room temperature. This procedure was repeated once. Extracts were pooled, centrifuged, and purified in a repeated washing step with hexane. Further purification was achieved by using a solid-phase extraction column (Isolut C_{18}; Sopachem). Microcystin was eluted from these columns with 70% methanol acidified with trifluoracetic acid. The recovery rate of 100 µL of a 6.7 µg mL^{-1} MC-LR stock solution was estimated once using six livers of each species. An average recovery rate of 68% was found. All microcystin measurements were corrected for their respective recovery rates.

During the course of the study new insights about microcystin analysis emerged, especially in animal tissue samples. Therefore a small selection of samples from the seston, zooplankton, mussels, and fish were analyzed for the presence of microcystin using LC-MS (as well as HPLC and ELISA) at the laboratory of J. Meriluoto at Åbo Akademi University in Turku, Finland (see [52]). In these analyses we also screened the samples for the presence of detoxication conjugates of microcystins. Covalently bound microcystins were extracted from mussel tissue and analyzed using the MMPB method. This method is based upon detection of 2-methyl-3-methoxy-4-phenylbutyric acid, an oxidation product of microcystins. The method was modified from [50] and performed as follows: The pooled mussel material was oxidized with 0.1 M $KMnO_4$ and 0.4 M $NaIO_4$ at pH 9 by shaking overnight at room temperature, then acidified with 1 M H_2SO_4 to pH < 3 and extracted with Supelco (Bellefonte, PA, USA) C_{18} solid-phase extraction cartridges. MMPB was detected on a LC-MS. Although the results of these analyses will be published elsewhere (see [13]) the consequences of the analyses in Finland for the monitoring of microcystins in the foodweb of the IJsselmeer (where standard MeOH extraction and HPLC-UV analysis was used routinely) will be discussed.

Histological Analysis of Fish Livers. Liver tissue was embedded in paraffin and stained with haematoxylin and eosin. The tissue was examined microscopically and histological abnormalities were recorded.

Statistics. Linear regression analyses were used to test SEP against (i) the percentage cyanobacteria in the seston; (ii) the microcystin concentration in the seston; and (iii) the microcystin content of zooplankton (all at $P < 0.05$).

Variation in microcystin concentrations in fish livers were tested using a type III general linear model (GLM). Prior to analysis data were fourth-root transformed to meet the assumptions of normality, homoscedasticity, and sphericity of GLM analyses for repeated measurements. ''Summer months'' (June, July and September) were used as a within-sample factor. A Sidák posthoc test was used for pairwise multiple comparisons of the effect of fish species (smelt, ruffe, perch) as the between-sample factor [43].

Logistic regression (computation of Wald statistic at $P < 0.05$) was used to test for the relation between the presence and absence of liver damage (as the binary response variable) and microcystin contents of fish liver (as the continuous predictor) [43].

Results

Seston and Plankton. Results from seston analysis on the flow cytometer indicated that the vast majority of the particles did not contain chlorophyll A. This implies that for non selective filter feeders only a small percentage of their food is made up of (toxic) cyanobacteria. The percentage phytoplankton in the seston varied between 15% and 25%. Cyanobacteria were the dominant group in the phytoplankton with a percentage that varied around 60%, but reached maximum values of 75–90% in September. Maximum densities of cyanobacteria approached 10^6 particles mL^{-1} toward the end of the summer. Differences in the average and maximum density of cyanobacteria showed little variation between years; concentrations were usually higher in the north than in the south (data not shown). Microscopic analysis showed that although non toxic cyanobacterial genera and known producers of microcystin coexisted, species belonging to the latter group dominated in all 3 years, mainly *Microcystis*, which dominated in 1997 and 1998 and *Planktothrix*, which dominated in 1999. Other genera, including *Anabaena* and *Coelospherium*, were found in smaller numbers.

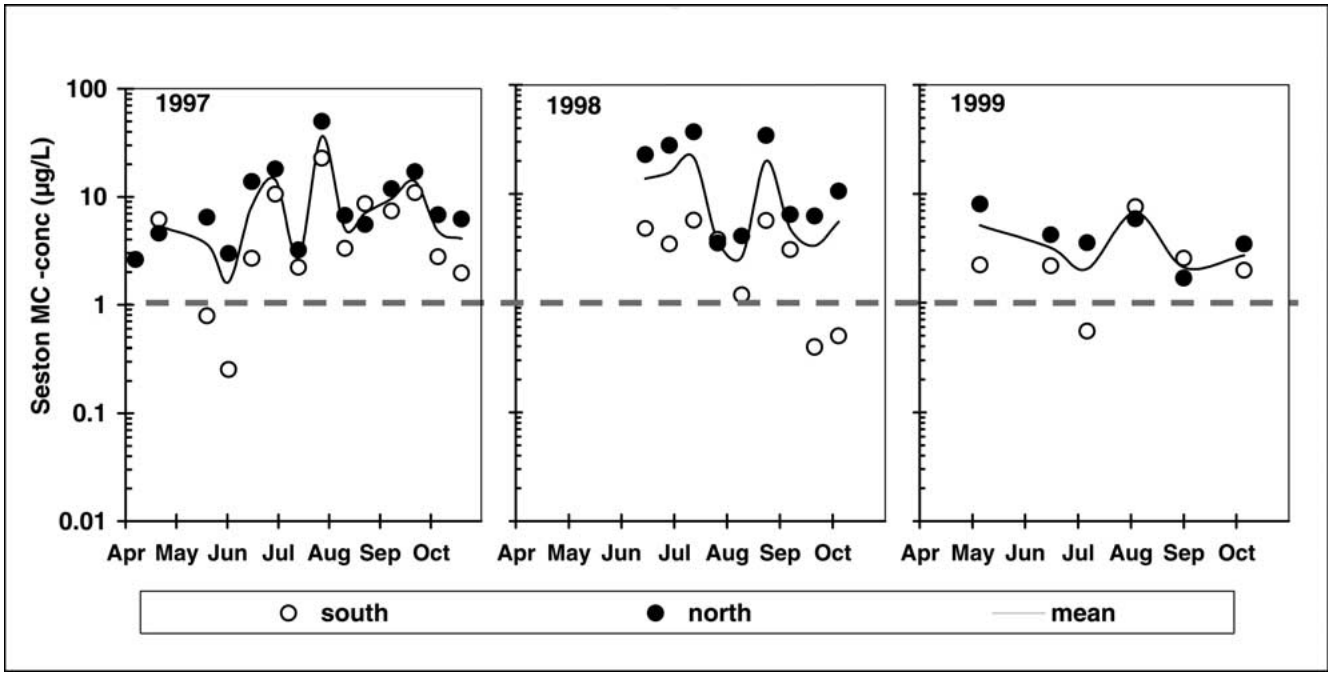

Figure 1. Microcystin concentration in the seston during three consecutive summers (1997–1999) in the southern (open symbols) and northern (closed symbols) part of the IJsselmeer. The line indicates the average for the whole lake. The dotted horizontal line indicates the microcystin level that equals the LD_{50} for *Daphnia pulex*, exposed to toxic *Microcystis* [45].

Zooplankton consisted of cladocerans, copepods, rotifers and "others" (in June mainly *Dreissena* larvae; later in summer also vorticellids). The number of cladocerans, which are commonly seen as the most efficient grazers in the zooplankton, was lowest in 1997, somewhat higher in 1998, and highest in 1999. In most years cladocerans (dominant species *Bosmina coregoni*, *Bosmina longirostris*, *Chydorus* sp., *Ceriodaphnia* sp., *Diaphanosoma brachyurum, Daphnia galeata*, and *Daphnia galeata* x *cucullata*) peaked in August (within the monitoring period of May – October), in 1999 with a maximum of 4000 ind. L^{-1} (data not shown).

Microcystin in the Seston. Concentrations of dissolved microcystin were extremely low on every occasion (data not shown). Microcystin was found in nearly every sample of the seston. The number of microcystins varied between 1 and 12. Microcystin LR, if present, made up <11% of the total microcystin content. Microcystin concentration of the seston — expressed as LR equivalents — reached maximum values in the northern half of the lake in 1997 and 1998; concentrations in the south and lakewide in 1999 were lower (see Fig. 1). Microcystin content on basis of ash free dry weight varied between 0 and 3600 µg microcystin g^{-1} AFDW (≈0–2400 µg g^{-1} DW). Means and ranges of microcystin concentrations are shown in Table 1.

To asses the risks for zooplankton from microcystin in the seston we have drawn a horizontal line in figure 1 at a microcystin-LR concentration of 1 µg L^{-1}. This value equals the LD_{50} for *Daphnia pulex* exposed to toxic *Microcystis* [45]. Other groups in the zooplankton may differ in their sensitivity to microcystin, and other (sublethal) effects are also possible, but the line in Fig. 1 still may serve for visualizing the potential ecological impact of microcystin in the seston on zooplankton. This approach suggests that microcystin concentrations in the IJsselmeer nearly always exceeded the level where negative effects on zooplankton are to be expected, for instance on *Daphnia* fecundity. To check whether this was the case the SEP of *Daphnia* was monitored in 1997 and 1998. SEP varied between 1.6 and 3.6 eggs per animal. When plotted a negative relationship was observed between SEP and the percentage cyanobacteria in the seston (data not shown), this relationship, however, was not significant ($R^2 = 0.04$; $P = 0.75$ for 1997 and $R^2 = 0.09$; $P = 0.43$ for 1998). In addition no significant relationships were found between the microcystin content of the seston and SEP ($R^2 = 0.20$; $P = 0.45$ for 1997 and $R^2 = 0.04$; $P = 0.60$ for 1998), between the microcystin content of cyanobacteria — calculated on basis of the flowcytometer data on particle densities — and SEP ($R^2 = 0.16$; $P = 0.50$ for 1997 and $R^2 = 0.03$; $P = 0.46$ for 1998) or between the microcystin content of the zooplankton and SEP ($R^2 = 0.01$; $P = 0.87$ for 1997 and $R^2 = 0.03$; $P = 0.67$ for 1998).

Table 1. Summer average microcystin contents of different foodweb compartments μg MC g^{-1} AFDW, except where indicated μg L^{-1} for the phytoplankton/seston

	1997		*1998*		*1999*	
	South	*North*	*South*	*North*	*South*	*North*
Phytoplankton (μg L^{-1})	6.5	12	3.2	17	2.8	4.5
Range	0.41–45	0.14–68	0.24–7.4	0.13–50	0.21–10	0.95–9.8
Phytoplankton	1088	1203	396	689	437	378
Range	291–3690	20–3912	30–990	7–1436	42–1089	73–939
Zooplankton	157	211	114	140	63	89
Range	0–556	0–763	0–430	4–1352	0–226	0–408
Dreissena	12	7	2	3	8	4
Range	4–27	1–18	0–11	0–18	0–30	1–8
Perch liver					24	
Range					17–51	
Ruffe liver					54	
Range					9–194	
Smelt liver					218	
Range					59–874	

The second row for each trophic level/species shows the range of microcystin contents that were measured. Averages from samples taken in the southern and the northern part of the lake are shown separately because the IJsselmeer commonly shows a south-to-north increase in cyanobacterial density. For fish such a distinction is not made; numbers are based upon lake-wide averages. For more details see Materials and Methods.

Microcystin in Zooplankton and Mussels. Microcystins were found in 80% of all zooplankton and 89% of all *Dreissena* samples. The concentrations in zooplankton usually exceeded those in mussels (Fig. 2); the highest concentrations that were found in zooplankton approached 1000 μg g^{-1} AFDW. Seasonally averaged values, using data from all sampling stations, varied between 63 and 211 μg g^{-1} AFDW in zooplankton (approximately 57 – 192 μg g^{-1} DW) and 2 and 12 ug g^{-1} AFDW in the mussels (approximately the same range on basis of DW, caused by low ash content of bivalves). See 4 Table 1 for an overview of microcystin contents in the different trophic compartments

The horizontal lines in Fig. 2 point out whether the microcystin concentration in the zooplankton is likely to affect the health of planktivorous fish. The lines are calculated on basis of (i) the known diet of planktivorous fish in the IJsselmeer [38], and (ii) toxicological data from a study on rainbow trout [54]. In this study it was found that a dose >550μg microcystin-LR per kg body weight (BW), administered by gavage eight times at 12 h intervals (i.e., a daily dose of 1100 μg microcystin-LR per kg BW), induced modest to severe liver damage in the trout (but no mortality). The microcystin concentration that potentially induces damage in IJsselmeer fish is lower for juvenile than for adult fish, because juvenile fish take in more food, relative to their body weight (respectively 10% and 1% DW BW^{-1} day^{-1}). Toxic effects on adult fish are to be expected in 40% of all cases where microcystin concentrations in the zooplankton were measured; for juvenile fish toxic effects are nearly always expected.

Microcystin in Fish. Microcystin concentrations were measured in the livers of three fish species: perch, ruffe, and smelt. The data are shown in Fig. 3. Microcystins were found in all fish livers, with concentrations varying in a wide range between 9 and 874 μg g^{-1} AFDW (Table 1). Fish was caught at three moments in the summer. Concentrations did not differ significantly between dates ($F = 0.508$; $P > 0.05$). The microcystin concentrations in smelt livers were significantly higher than in ruffe or perch ($F = 20.17$; $P < 0.05$). These fishes have a different diet. Smelt is planktivorous, whereas ruffe is benthivorous and perch is planktivorous when small, but predatory when larger, and the latter was the case for the fishes caught in this study.

Histological abnormalities that could potentially be ascribed to microcystin were found in 37% of the livers that were studied. For smelt, half of the livers studied showed abnormalities; for the other species, especially ruffe, this percentage was lower. The histopathological findings ranged from inflammation to degeneration and necrosis (Fig. 4). However, none of the livers from fish caught in the IJsselmeer were enlarged or darker in color as was observed in gavage experiments with perch and microcystin (authors unpublished data). In the lake no significant relationship was observed between the degree of liver abnormalities and the microcystin content (figure 5; microcystin concentrations in healthy livers did not differ significantly from that in damaged livers (Wald statistic = 4.63; $P > 0.05$).

Biomagnificaton of Microcystin. When all microcystin monitoring data are combined (see Fig. 6) it is

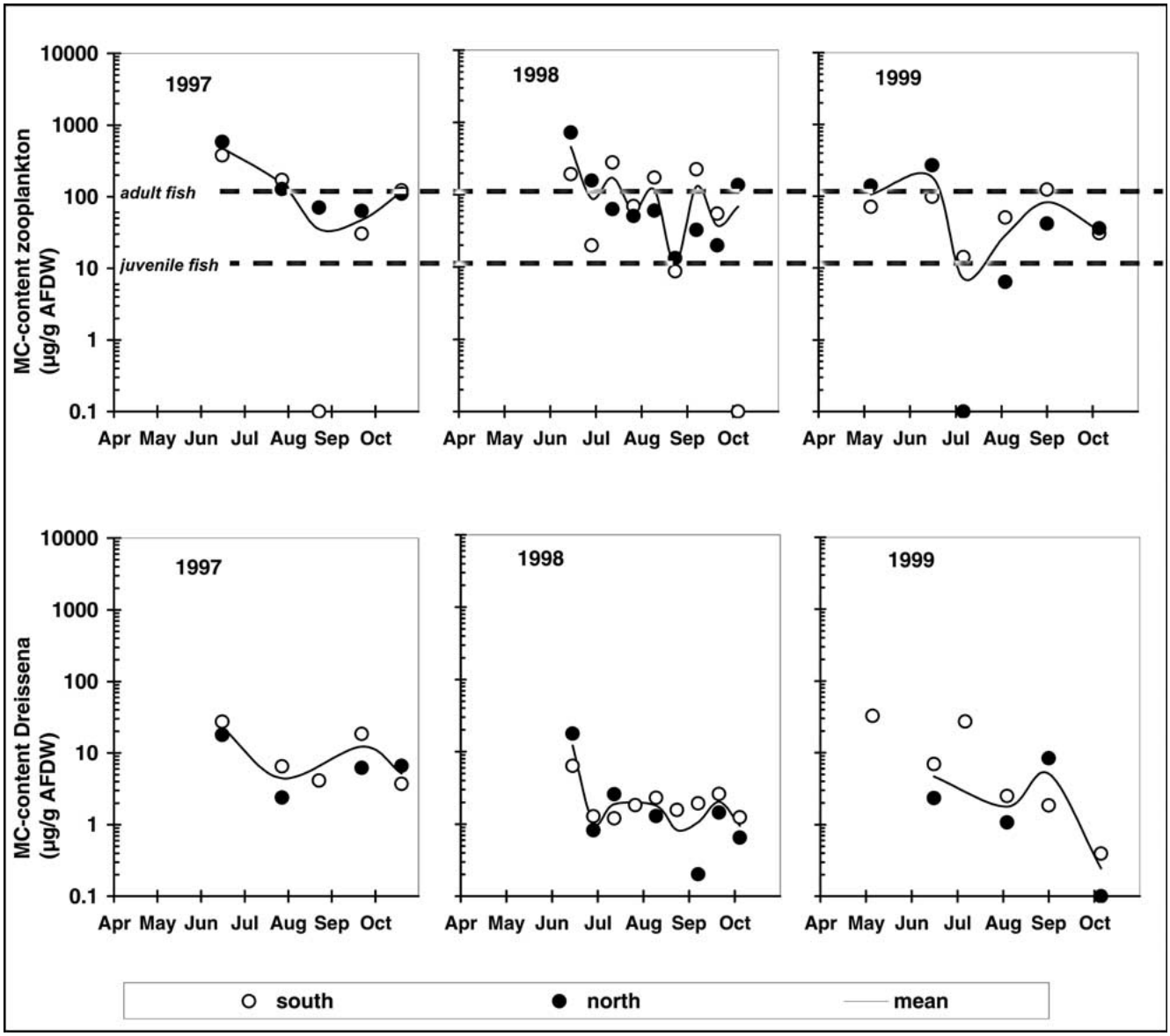

Figure 2. Concentration of microcystin in the zooplankton and *Dreissena* during three consecutive summers (1997–1999) in the southern (open symbols) and northern (closed symbols) part of the lake. The line indicates the average for the whole lake. The two horizontal lines indicate the microcystin level where, based upon the diet of (zooplanktivorous) fish species in the IJsselmeer and a study on the effects of microcystin in rainbow trout [54], damaging effects are to be expected for juvenile and adult fish.

clear that microcystin in none of the foodweb compartments exceeded the concentration in the phytoplankton (seston) that produces the toxin. Biomagnification factors averaged over all available data are shown in Table 2. There is one case where microcystin content of an organism exceeded that in its diet: microcystin levels in smelt livers were higher than those in zooplankton. This probably merely reflects the specific accumulation of microcystins in the livers of exposed animals (see discussion). Hence in general no evidence for biomagnification was found, either in single samples or averaged over the season. What is remarkable in Fig. 6 is the relatively high amount of microcystin in zooplankton compared to *Dreissena*, and the relatively high amount in smelt livers compared to perch and ruffe.

Discussion

Microcystin in the Foodweb. Our results on the concentrations of microcystin in the foodweb of the IJsselmeer are summarized in Table 1. Other studies found microcystins in comparable ranges of microcystin-LR equivalents (see especially [51]), among others 1–2000 µg g^{-1} DW in the seston [40], up to 1387 µg g^{-1} DW in lake communities of zooplankton [27, 55], up to 20 ng g^{-1} wet weight (WW) in zebra mussels [2], and > 60 µg g^{-1}

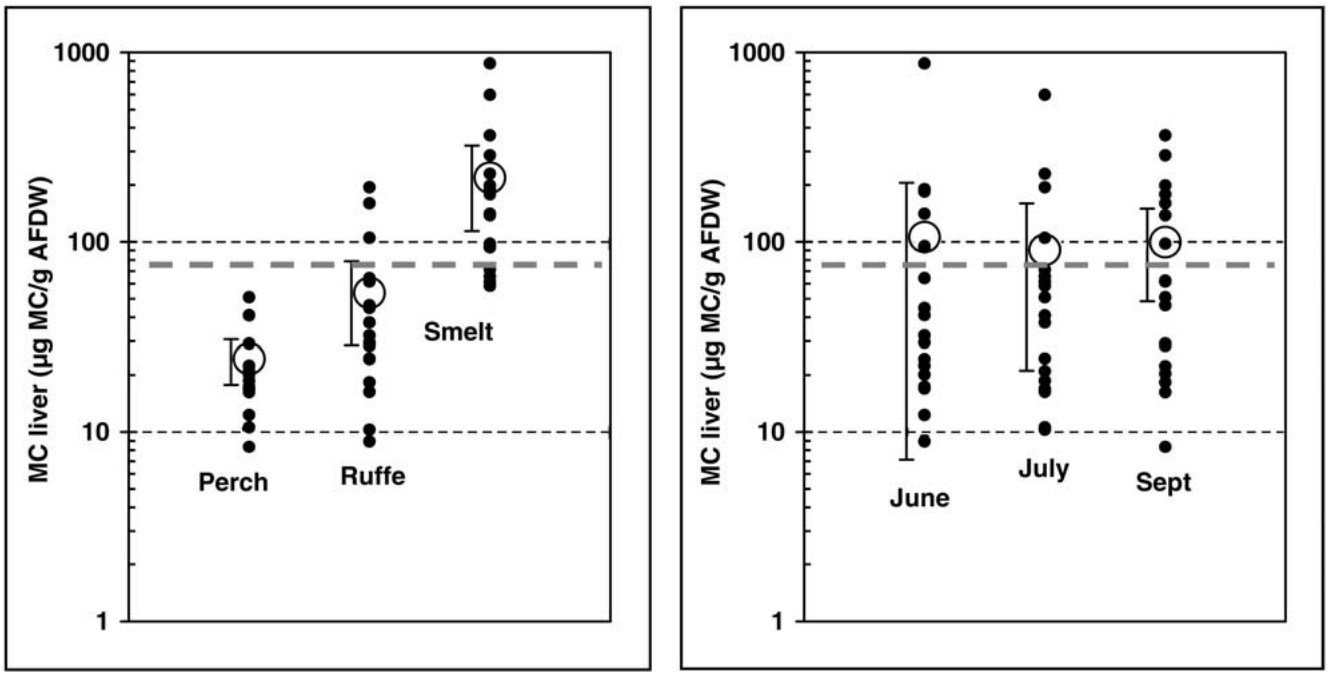

Figure 3. Microcystin contents of fish livers for three species (perch, ruffe, and smelt), caught at three moments of the cyanobacterial growing season. Shown are individual contents and the average ±95% confidence limits. Smelt have a significantly higher level of microcystin in their livers than do ruffe or perch. The horizontal line indicates a microcystin content that could induce adverse effects on piscivorous birds.

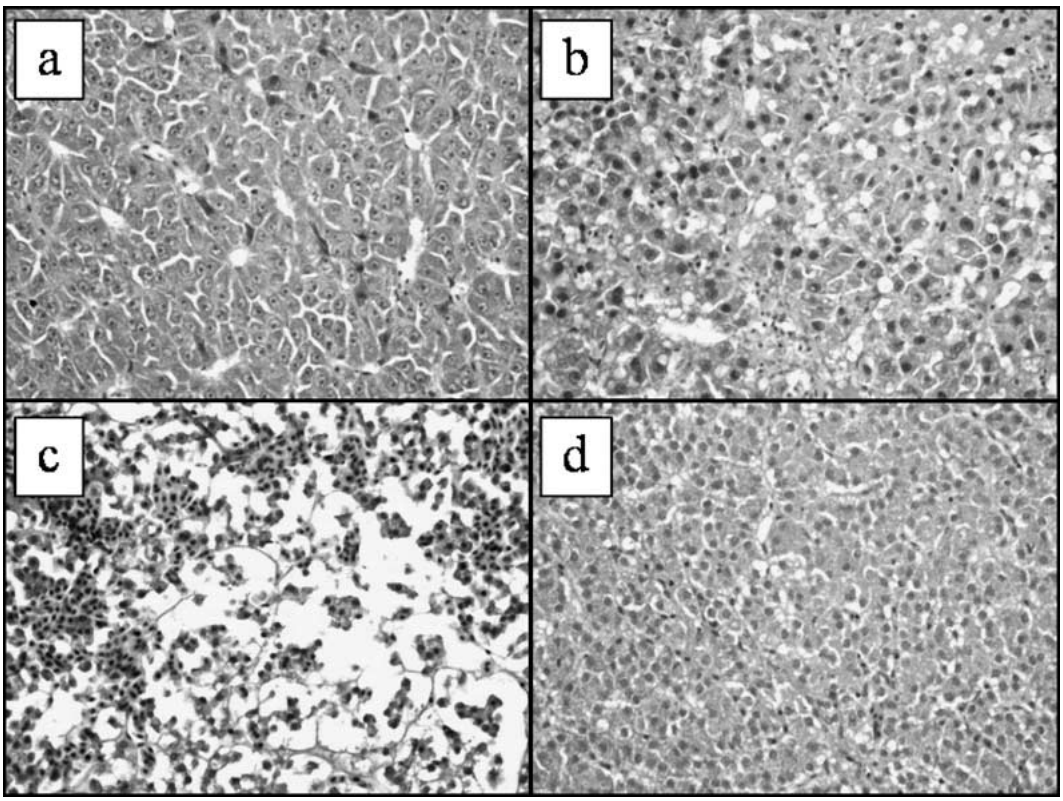

Figure 4. Histopathology of fish livers (magnification 20×): (a) perch caught in the IJsselmeer; (b) perch administered a moderate dose of microcystin via gavage; (c) perch administered a high dose of microcystin via gavage; (d) healthy, nonexposed perch. In (a) and (b) degeneration of liver tissue is visible because openings between cells appear. Some hepatocytes appear enlarged and darkened and are mildly degenerated. In (c) the liver tissue is necrotic and has completely lost its integrity.

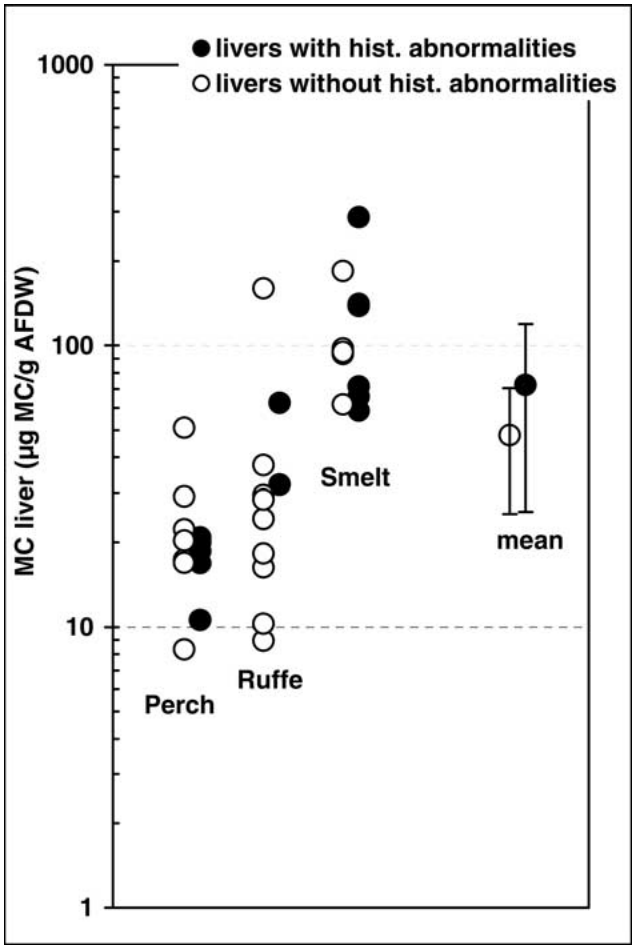

Figure 5. Microcystin content of fish livers for three fish species. Indicated is whether histological analysis did or did not find histological abnormalities that could be ascribed to microcystin exposure (cf. Fig. 4). Livers without abnormalities are indicated by open symbols, livers with abnormalities by closed symbols. On the right-hand side of the figure the average ±95% confidence limits for all livers that were examined are given. There is no relationship between microcystin content and the occurrence of abnormalities.

DW in the hepatopancreas of the freshwater clam *Unio douglasiae* [63]. A range of 10–130 µg g^{-1} DW was reported for freshwater clams and mussels from a number of studies, mentioned in [49]. Concentrations of microcystin in fish livers reached an average of 6.3 and a maximum of 31.1 µg g^{-1} tissue in a study on *Tilapia rendalli* [11]. Livers of phytoplanktivorous silver carp fed with natural *Microcystis* cells accumulated up to 17.8 g microcystin-RR g^{-1} DW (lower in muscles and blood) [62].

Entrance of microcystin into the foodweb has to occur via ingestion of toxic cyanobacteria. It is therefore relevant to ask whether the two most important grazers in the IJsselmeer, *Daphnia galeata* and *Dreissena polymorpha*, would ingest colonial and filamentous (toxic) cyanobacteria (or would they reject them?). Zebra mussels seem capable of efficiently sorting particles on the pallial organs [4]. *Dreissena* in the Great Lakes has been shown to remove *Microcystis* colonies from the plankton, but viable cells were rejected via the pseudofaeces and were not ingested [22, 56]. This may promote blooms of toxic *Microcystis*, but this effect is probably dependent of the nutrient concentration in water bodies, especially of total phosphorus [44]. Overall it seems likely that selective rejection of toxic cyanobacteria by *Dreissena* is one of the factors that lead to low microcystin contents of these mussels. Assessment of the potential of zooplankton to filter large cyanobacteria may be hindered by a lack of experimental field-based studies (as stated in [17]), although much work has been done over a long period of time (e.g. [6]). Opinions differ on the capability of *Daphnia* to select and avoid cyanobacteria without interference of the uptake of preferred food particles [12, 18], but the lack of an efficient selection mechanism in *Daphnia* may explain why microcystin contents in Daphnia were higher than in *Dreissena*.

Whereas assimilation of toxic cyanobacteria hardly has any negative consequences on freshwater mussels [2], life history experiments have shown that exposure of *Daphnia* to toxic cyanobacteria does have severe consequences. Microcystins are already efficient at low intake rates and rapidly cause the death of cladocerans [48]. A role for toxic compounds other than microcystin should not be discounted, however. Inhibition of feeding seems independent of the presence of microcystins in cyanobacteria [47], and compounds other than microcystins may even have direct toxic effects [30].

Although the food chain starting with *Dreissena* hardly accumulates microcystin, it is possible that microcystins are transported to the benthic community via pseudofeces of the mussels [2]. In the Ilsselmeer ruffe may be exposed to microcystins via this route. Importantly however, even when fish consume food that contains microcystin, little of this will actually be taken up or accumulate in the body. Only 1.7% of microcystin given via gavage to rainbow trout actually reached the liver [53]; hence microcystins in fish may be excreted rapidly [11, 34]. And yet, despite the many barriers for the uptake or accumulation of microcystin we found hepatic lesions in 37% of the fish livers that were analyzed. A similar high percentage was found in a population of carp that feeds directly on cyanobacteria [8]. Our study demonstrates the occurrence of extensive liver damage in fish exposed to microcystin after vectorial transport of the hepatotoxins through the foodweb.

Limitations of the Study. Our results indicate harmful levels of microcystin in biota. However, there are some (''technical'') limitations which imply that our results should be interpreted with caution: (i) microcystins

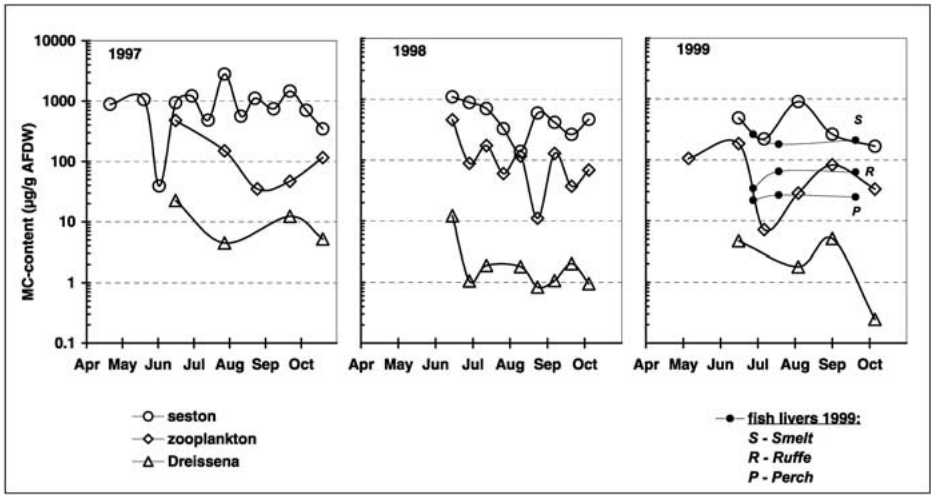

Figure 6. Concentration of microcystin in phytoplankton, zooplankton, mussels, and the livers of three fish species during three consecutive summers (1997–1999; fish 1999 only). Data averaged over all six sampling stations.

in animal tissue were determined with HPLC after MeOH extraction, which does not include covalently bound microcystin, nor does it distinguish properly between microcystin and its detoxication products [36]; (ii) the identity of the microcystins in the seston and the foodweb compartments of the IJsselmeer remained unknown; the concentration of the toxins is expressed as microcystin-LR equivalents, whereas other microcystins can be much less toxic than microcystin LR; and (iii) ecotoxicological studies that relate cyanobacterial toxin concentrations in the field to toxic effects for the relevant species are often not available.

The uptake of cyanobacterial toxins triggers the activation of detoxication systems. The capacity for detoxication of microcystin (formation of Cys and GSH conjugates) appears to be widespread among aquatic organisms [41] and it may be a relevant mechanism that reduces the transfer of microcystins in the foodweb. We were unable to detect conjugates in zebra mussels from the IJsselmeer, using LC-MS. If this is a representative result it seems unlikely that the error made by using the HPLC is too large, but this remains to be studied.

It has been shown for a number of aquatic organisms that the great majority of microcystin in the tissues was bound covalently in a complex with protein phosphatases [60, 61]. These microcystins were not detected after standard MeOH extraction. It is likely that zooplankton, *Dreissena*, and fish (livers) from the IJsselmeer contained covalently bound microcystin that did not show up in our analysis. In a test (using the MMPB method) the maximum amount of microcystin that was covalently bound in *Dreissena* from the IJsselmeer (as a percentage of the total amount of toxin) was 38% [13]. Hence the fraction of bound microcystin, although still considerable, was (much) lower than in earlier studies [53, 60, 61]. An important but unanswered question is whether the covalent microcystin–PPase complex is still as toxic as unbound microcystin. Toxicity of the complex may be in the order of the microcystin–glutathione conjugates (Karlsson, pers. comm.). It has been put forward that nodularin may be more available to higher levels in the foodweb than the related microcystin because nodularin does not bind covalently to protein phosphatase [24]. Hence also covalent binding of microcystin may reduce the transfer of the toxin to higher trophic levels.

In the Usselmeer the maximum contribution of microcystin-LR to the total microcystin concentration was only 11%. Microcystin-LR is among the most toxic variants, with an LD_{50} in mice (after intraperitoneal injection) of 50 µg $g^{-1}kg^{-1}$ [9]. Although other microcystins can be equally toxic, some variants are clearly less toxic in mouse bioassays, including [D-Asp^3] microcystin-RR (LD_{50} of 250 µg kg^{-1}) that has been found to be the major variant produced during *Planktothrix* blooms [15]. However, because of differences in lipophilicity and polarity, intraperitonial LD_{50} cannot be used directly to rank toxicity of microcystins taken up with the food. For instance, there were great differences between the uptake and transportation of microcystin-LR and microcystin-RR in silver carp. Despite a massive accumulation of microcystin-RR (and not of microcystin-LR), the fish appeared unharmed [62].

Risks of Microcystins for Fish and Birds. Our conclusion that planktivorous fish in the IJsselmeer is exposed to harmful doses of microcystin (see horizontal line in Fig. 2) is largely based upon experimental work with rainbow trout [53], which is absent in the IJsselmeer. We performed experimental studies with dominant fish species from the lake and administered different doses of microcystin, both intraperitoneally and via gavage (authors unpublished data, manuscript in preparation). The LD_{50} that we found for perch after intrapentoneal injection (1500 µg kg^{-1} BW) was much

higher than those reported for mammals, and also somewhat higher than those for carp or rainbow trout [26]. However, when microcystin was administered orally (up to 1150 ug microcystin kg^{-1} BW given by gavage eight times over 96 h, i.e., a total dose of 9200 μg kg^{-1} BW) no mortality was seen, although histopathology of the livers showed that microcystins were having severely detrimental effects for the perch used in the study. On basis of the experiments we conclude that mortality of fish exclusively through the uptake of microcystin via their food seems unlikely.

Assessment of the risk for piscivorous birds and diving ducks with the respective microcystin concentrations found in fish and mussels is not straightforward, either. For instance the lower limit for the sensitivity of waterbirds for microcystin is unknown (see also [28]). One of the main fish-eating birds in the IJsselmeer is the cormorant. Assuming (i) a body weight of 2.6 kg; (ii) a daily intake of 400 g fish per day; (iii) a contribution of the liver of 1.5% of the fish wet weight; and (iv) an AFDW percentage of 23.5% of the liver wet weight, a cormorant would consume 1.4 g AFDW fish liver per day. Taking into account a NOAEL 4 (no observable adverse effect level) of 40 μg kg^{-1} bodyweight (based upon a study with mice and microcystin-LR, given orally [16]) a critical concentration of 75 μg g^{-1} AFDW fish liver is derived (see horizontal line in Fig. 3). Most of the microcystin concentrations in ruffe and perch livers are below this level, but those in smelt livers nearly all exceed it, up to 12 times (see Table 1). The LD_{50} by gavage in mice is on the order of 5000 μg kg^{-1}. Hence it seems unlikely that waterfowl actually die from exposure to microcystin, although liver damage of piscivorous birds certainly seems possible.

Biomagnification of Microcystin. Biomagnification relates the concentration of a chemical in an organism to that in its diet, bioaccumulation to that in the water. Uptake via the food is the main route for transfer of microcystins in the foodweb. We found no evidence for biomagnification, as hypothesized in the introduction. The highest microcystin concentrations outside the seston were found in the livers of smelt, and those exceeded the microcystin levels in zooplankton, suggesting that biomagnification had taken place. However, this may merely be a reflection of the specific accumulation of microcystin in livers. Comparing microcystin concentrations in whole organisms (zooplankton, mussels) to those in selected organs gives a skewed representation of biomagnification [20]. As an example, see microcystin concentrations in *Dreissena* and its predator, goby, in Lake Erie [2].

In the literature there is often speculation that bioaccumulation (read biomagnification) of cyanobacterial toxins takes place (e.g, [33, 37, 57, 61]), and that this would strengthen the risk for aquatic organisms higher up in the foodweb. In reality, however, very few studies provide data that support biomagnification of microcystin (but see [59] for an example where microcystin in zooplankton exceeds that in phytoplankton). Accumulation of another hepatotoxin produced by cyanobacteria, cylindrospermopsin, to values much higher than those in the environment was found in the hemolymph of the swan mussel *Anodonta cygnea* exposed to *Cylindrospermopsis* under experimental settings, showing the potential for bioaccumulation in the field [49]. Indeed the accumulation of microcystin in *Anodonta grandis simpsoniana* to values that exceeded the concentration of toxin in the phytoplankton has been demonstrated [42].

Conclusions: Evidence for Harmful Effects of Microcystin in the Foodweb? The fact that microcystins are found in the livers of fish caught from the IJsselmeer clearly indicates that transfer of microcystins within the foodweb takes place, despite the absence of biomagnification. We were, however, unable to link the microcystin contents in foodweb components to their potential effects. For instance we measured microcystin in the liver of various fish species and observed histological abnormalities that could be ascribed to microcystin, but the two were not matched. There are several possible explanations for the lack of such relations in the field. Neither covalently bound microcystins nor detoxication products were included, and this may obscure the real concentrations of ''active'' toxins in biota. Also, a variety of compounds from cyanobacteria other than microcystin has been shown to be toxic to aquatic organisms and should be included in the eco-toxicological assessment (e.g, [25]).

The value of the present study is that (i) for the first time it has measured the concentration of microcystin in a number of foodweb compartments during the cyanobacterial growing season and over a number of consecutive years; (ii) the data permit a comparison of the transfer of microcystin via food chains starting with two different filter feeders and produced by two morphologically different cyanobacteria; (iii) the data enabled us to draw a conclusion on biomagnification of (unbound) microcystin in the foodweb: it does not occur of the IJsselmeer (but of course we must be careful in extrapolating from our own study to lakes in general). Zooplankton or zebra mussels may act as a vector for the transfer of cyanobacterial toxins in the foodweb, but through rapid excretion, decomposition, and transformation these biota may also act as a sink (see [21]). This will reduce the exposure of higher trophic levels to microcystin produced at the base of the foodweb.

Our study was initiated by a number of fish kills. Is it likely that these were caused by microcystins? Fish from the IJsselmeer did not die in our experiments, even when exposed to high doses of microcystin via gavage. Hence

Table 2. Biomagnification factors of microcystin in foodweb components of the IJsselmeer

	1997		*1998*		*1999*	
	Seston	*Diet*	*Seston*	*Diet*	*Seston*	*Diet*
Zooplankton	16	16	24.6	24.6	19	19
Dreissena	0.9	0.9	0.5	0.5	1.4	1.4
Perch liver					5.9	11
Ruffe liver					13.2	120
Smelt liver					53.5	286

The factor is determined as the average microcystin content in biota (μg MC g^{-1} AFDW) as a percentage of the average value in the seston (MC in organism/MC in seston × 100) and as a percentage of the average value in the diet of the respective organisms (MC in organism/MC in diet × 100). All available data were averaged for the 3 years shown in Table 1. ''Diet'' is seston for zooplankton and *Dreissena*, zooplankton for planktivorous smelt, smelt for piscivorous perch, and pseudofeces of *Dreissena* for benthivorous ruffe. We did not analyze microcystin contents of benthic macrofauna and for this reason we use an estimate of the amount of microcystin in pseudofeces of *Dreissena* (which may act as the vector for transport of the toxin to the benthic community). In experiments with *Dreissena* fed with toxic *Microcystis* PCC 7820 we found that pseudofeces contained 7.5 times more MC (on basis of DW) than the mussels that produced it (unpublished data). We use this conversion factor to calculate microcystin in pseudofeces from IJsselmeer mussels containing 30 μg MC g^{-1} DW (see Table 1—1999). Microcystin does not exceed the level in the seston in any of the foodweb components. Smelt liver contained 2.9 times the concentration in its diet and ruffe 1.2 times the concentration in pseudofaeces of *Dreissena* (however, see discussion about skewed representation of biomagnification when MC levels in whole organisms are compared to that in a selected organ).

we would conclude that it is more likely that fish kills are caused by a multitude of stress factors that co-occur during blooms of cyanobacteria than by microcystin. Harmful, sublethal effects of microcystins, however, seem highly likely for many biota in the lake. Examples of such effects can be found in [3] or [7] where chronic exposure to microcystin in fish resulted in reduced growth and disturbed reproduction.

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