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Simultaneous accumulation of anatoxin-a and microcystins in three fish species indigenous to lakes affected by cyanobacterial blooms

Barbara Pawlik-Skowrońska[1,2,*], Magdalena Toporowska[1], Jacek Rechulicz[1]

[1]*Department of Hydrobiology, University of Life Sciences in Lublin, Dobrzańskiego 37,20-261 Lublin, Poland*
[2]*Polish Academy of Sciences, Centre for Ecological Research in Dziekanów Leśny, Experimental Station, Niecała 18, 20-080 Lublin, Poland*

Key words: anatoxin-a, cyanobacteria, fish, microcystins, risk assessment

Abstract

A four-year study carried out in a lake with perennial water blooms caused by toxigenic *Planktothrix agardhii* (Oscillatoriales) and *Anabaena lemmermanii*, *Anabaena flos-aquae, Anabaena* spp. and *Aphanizomenon issatchenkoi* (Nostocales) revealed that the lake-dwelling fish were threatened by simultaneous exposure to intracellular and extracellular microcystins (MCs) as well as anatoxin-a (ANTX). Higher contents of anatoxin-a and microcystins were found in livers than in fish muscles. This is the first report on ANTX accumulation in the common fish, indigenous to European freshwaters during perennial cyanobacterial blooms. Generally, the omnivorous roach (*Rutilus rutilus)* and Prussian carp (*Carassius gibelio)* accumulated higher amounts of MCs in their tissues compared to mostly predacious perch (*Perca fluviatilis)*, and similar amounts of ANTX. The long-lasting presence of MCs exceeding the safe levels for consumption was found in fish muscles. ANTX accumulation in fish muscles (up to 30 ng g^{-1} FW) suggests the probability of its transfer in a food chain.

* Corresponding author: *pawlik@poczta.umcs.lublin.pl*

INTRODUCTION

Long-term and persistent water blooms caused by a mass development of various freshwater species of toxin-producing cyanobacteria in lakes, dam reservoirs and rivers have been reported with increasing frequency (Sivonen et al. 1990; Codd 2000, Kurmayer et al. 2004; Pawlik-Skowrońska et al. 2004, 2008; Briand et al. 2002; Sotero-Santos et al. 2008; Grabowska, Mazur-Marzec 2011). Recently, the influence of secondary metabolites of cyanobacteria (including hepatotoxic microcystins and neurotoxic anatoxin-a) on ichthyofauna attracts the increasing interest due to their possible impact on human health and negative economic consequences (Welker, von Dohren 2006; Malbrouck , Kestemont 2006; Smith et al. 2008; Kopp et al. 2009; Papadimitriou et al. 2010). Microcystins and other less known metabolites of freshwater cyanobacteria are regarded as developmental toxins inhibiting different stages of fish embryogenesis (Berry et al. 2007). Accumulation of microcystins and their influence on some fish species (e.g. *Danio rerio, Carassius carpio, Cyprinus carpio*) were studied mainly under laboratory conditions (Malbrouck , Kestemont 2006; Kopp et al. 2009; El Ghazali Issam et al. 2010). However, there are very limited field studies on the dynamics of cyanotoxins (especially anatoxin-a) accumulation in ichthyofauna indigenous to Eurasian waters, which can be used as a food source. Thermolabile alkaloid anatoxin-a is considered to be less dangerous for consumers than microcystins (Bogialli et al. 2006) due to relatively short half-life in water (ca. 14 days) under natural light and pH conditions (Van Appeldoorn et al. 2007). Its strong neurotoxicity towards mammals is well known, however, it can also be a real hazard to ichthyofauna, as reported by Osswald et al. (2007) for the juvenile stages of *Cyprinus carpio* fed on *Anabaena* sp. Common species of *Anabaena* and *Aphanizomenon*

are known as ANTX producers (Sivonen et al. 1990, Pawlik-Skowrońska et al. 2004, Osswald et al. 2009, Pawlik-Skowrońska and Toporowska 2011). For example, ANTX concentration (up to 120 μg dm^{-3}) positively correlated with a density of *Anabaena* spp. occurring from June to October in a hypertrophic dam reservoir (Pawlik-Skowrońska et al. 2004). Therefore, their mass development can become a real threat to freshwater ichthyofauna and fish consumers. To our knowledge, there are no field data published on ANTX accumulation in fish living in the presence of cyanobacterial blooms. Further research is required to determine if cyanotoxins can sufficiently accumulate in aquatic food webs to affect human health.

The aim of this study was to compare the dynamics of cyanotoxin accumulation in tissues of three common species of indigenous fish inhabiting a lake with complex and perennial cyanobacterial water blooms.

MATERIALS AND METHODS

Study Area and Sampling

The study was carried out in 2006-2009 in a shallow, hypertrophic, flow-through Lake Syczyńskie (E. Poland). Species composition, abundance and biomass of cyanobacteria assemblages were examined in samples collected from the water column once a month. The abundance and biomass evaluation of phytoplankton (including cyanobacteria) was based on the algae count performed under an inverted microscope (Utermöhl 1958). For all cyanobacteria (Oscillatoriales and Nostocales) with straight filaments, 100 μm was defined as one individual. One coil of coiled *Anabaena* spp. and one colony of *Microcystis* spp. were recognized as individuals.

Fish of different age (in total 13 specimens of *Carrasius gibelio*; total length 13 – 30.5 cm; 20 *Rutilus rutilus* (3.5 – 18.5 cm) and 30 *Perca fluviatilis* (7.5 – 23.5 cm), were harvested during the study period using Norden S- REV nets, then weighed, measured and frozen (-20°C). Livers and muscles were cut out for cyanotoxin extraction and analysis.

Physico-chemical analyses

Physico-chemical characteristics of the lake water are presented in Table 1. Biogenic nutrients were determined according to Golterman (1971) and chlorophyll-*a* according to PN-ISO 10260 (2000).

Table 1

Physico-chemical characteristics of the lake water in 2006 – 2009 (annual mean values).

Parameters	2006	2007	2008	2009
Water temperature (°C)	19.4	19.4	18.4	15.8
pH	7.8	7.7	7.9	8.2
Conductivity (μS cm^{-1})	478	502	454	471
Transparency – SD (m)	0.64	1.94	0.48	0.63
N-NH_4 (mg dm^{-3})	0.463	0.262	0.382	0.427
N-NO_3 (mg dm^{-3})	0.184	0.094	0.102	0.168
P-PO_4 (mg dm^{-3})	0.095	0.117	0.064	0.078
DIN/DIP ratio	5.3	3.1 (*1.9; 14)	8.9	8.2
Chlorophyll-*a* (μg dm^{-3})	96.1	40.5	133.7	61.2
TSI_{SD}	70	57	72	67

DIN/DIP - dissolved inorganic nitrogen and phosphorus, TSI – Trophic state index (Carlson 1977), * – mean values in the first and the second half of year.

The Carlson Trophic State Index (TSI) based on water transparency measurements (Secchi disc) was also calculated (Carlson 1977).

Cyanotoxin extraction

For determination of intra- and extracellular MCs and ANTX in the lake, water samples (0.5 – 1.0 dm^{-3}) containing cyanobacterial biomass were concentrated on Whatman GF/C filters and extracts of the biomass were prepared in 75% (v/v) methanol (Merck, pure *p.a.*) containing 0.002 M HCl using ultrasonication (3 times for 5 min., 50W, ultrasonic homogenizer Sonoplus, Bandelin).

Fish tissues (0.1 – 3 g FW of liver and 1.5 – 7 g FW of muscle) were homogenized in the acidified 75% methanol (Merck, pure *p.a.*) used in a proportion 3 ml MeOH for 1 g FW of tissue and ultrasonicated (2-3 times for 10 min). After centrifugation (14,000 rpm for 10 min. at 17°C) supernatants were collected and purified (2-3 times) with n-hexane (1:1, v/v). The hexane layers were discarded to remove the excess of lipid substances. Methanol layers were stored at -20°C until HPLC – DAD and HPLC-FLD analyses.

GC-MS analysis of microcystins

Total concentration of MCs in extracts was determined using gas chromatography/mass spectrometry (GC/MS, Varian) according to Kaya, Sano (1999) and the modified procedure described by Pawlik-Skowrońska et al. (2008). The method is based on oxidation of Adda (a specific amino-acid present in MCs) to MMPB (2-methyl-3-methoxy-4-phenylbutyric acid) and determination of MMPB as a

methyl ester. The oxidation was carried out for 4 hours with 99.8% $NaIO_4$ and 0.024 M $KMNO_4$. For derivatization, 14% BF_3 –methanol was used. As a modification, phenylbutyric acid (PB) was used as an internal standard after the oxidation step. Derivatized samples were dissolved in n-hexane and subjected to GC/MS analysis (Saturn 2000, Varian). In the EI-MS mode, the identification and quantification of MMPB methyl ester was based on ions at m/z 91, 131 and 190; for PB methyl ester 91, 104 and 146 m/z were used. Identification of MMPB and PB was confirmed by CI-MS at m/z 191 and 147, respectively. Total microcystin concentrations were expressed as equivalents of MC-LR, which was used as a standard (Alexis Biochemicals).

HPLC-DAD analysis of microcystins

HPLC- photodiode array detection system (Shimadzu) was used for microcystin detection and identification. MC-LR, -RR, -YR, -LA, -LY, - LW, -LF, -WR (Alexis Biochemicals) were used as standards and were quantified in tissue extracts at 238 nm. Extracts were separated on the LiChroCART 125-3 Purospher RP-18 column (5 µm, Merck), using a 30-100% gradient of aqueous acetonitrile (Merck) acidified with 0.05% trifluoroacetic acid, according to Lawton et al. (1994), at a flow rate of 0.7 ml min^{-1}.

HPLC-FLD analysis of anatoxin-a

ANTX was determined using liquid chromatography (HPLC, Beckman) with fluorescence detection (Shimadzu) according to James et al. (1998) and Furey et al. (2005). For ANTX derivatization, 10% NBD-F (4-fluoro-7-nitrobenzofuran; Fluka) was used. The detector parameters were as follows: excitation wavelength 470 nm, emission wavelength 530 nm. Extract separation was obtained on the RP-18 Purospher column (125 × 3 mm, 5 µm, Merck) using a TFA (0.05%) acidified acetonitrile at a flow rate of 0.6 ml min^{-1}. The detection limit (LOD) for cyanobacterial biomass and fish tissues was 5 ng g^{-1} FW. For identification and quantitative determinations, the standard ANTX (Tocris, Bioscence) was used.

RESULTS

Bloom forming cyanobacteria

The four-year study (2006-2009) revealed that the shallow Syczyńskie Lake was highly eutrophic (TSI_{SD} = 57 – 72) and the physico-chemical conditions (Table 1) supported perennial abundant development of planktonic algae (Fig. 1, Table 2). In 2006, 2008 and 2009, the annual average values of the total phytoplankton biomass (Fig. 1) were high: 34.3 – 51.1 mg dm^{-3} (maximum over 100.0 mg dm^{-3}) with the dominance of cyanobacteria (74 – 85% of the total phytoplankton biomass). In 2007, with considerable changes in the DIN/DIP ratio (Table 1), strong but only transient reduction of cyanobacteria biomass occurred (Fig. 1). Their

Table 2

Mean values and range of biomass (mg dm^{-3}) of toxin-producing, bloom-forming cyanobacteria in Lake Syczyńskie in 2006 – 2009.

Taxa	2006 AN-a: V-VIII MCs: III-XII	2007 AN-a: VI-VII MCs: I-V	2008 AN-a: IV-VIII MCs: IV-XI	2009 AN-a: V-VII MCs:I-XI
Anabaena flos-aquae *,**	0.001 0–0.01	0.07 0–0.10	0.15 0–0.690	0.15 0–0.49
Anabaena lemmermannii *	0.31 0–2.46	0.09 0–0.18	0.001 0–0.004	0.18 0–0.74
Anabaena spp.*,†	0.28 0.001–1.91	—	1.99 0.03–9.14	2.01 0.08–8.45
Aphanizomenon issatschenkoi *	0.003 0–0.01	—	0.10 0–0.32	0.017 0.01–0.02
Planktothrix agardhii **	31.24 0.04–117.30	0.23 0.03–0.50	30.61 0.01–115.42	28.61 0.01–70.94

* AN-a-producing species; ** MCs-producing species; — taxa not found;† *Anabaena* spp. = *A. spiroides, A. mendotae, A. crassa, A. heterospora, A. planctonica, A. virgurieri, A. zinserlingii, A.* cf. *heterospora, Anabaena* sp.

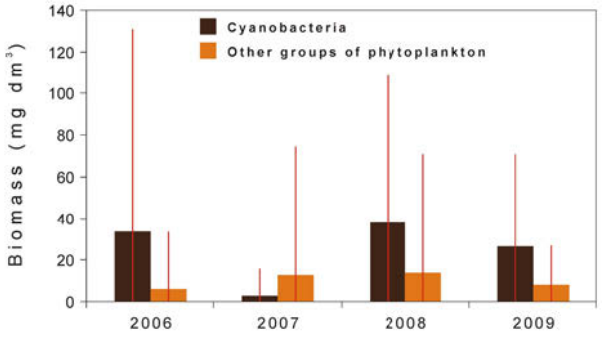

Fig. 1. Biomass of cyanobacteria and other taxonomic groups of algae in Lake Syczyńskie in 2006 – 2009. Annual average (columns), and minimum and maximum values (bars).

average contribution (17%) in the total biomass of phytoplankton was about 5 times lower than in other years. Toxin-producing cyanobacteria, which induce water blooms, were from Oscillatoriales (*Planktothrix agardhii* (Gom.) Anagh. et Kom.) and Nostocales (*Anabaena lemmermannii* Richt., *Anabaena flos-aquae* Breb. ex Born. et Flath., *A. planctonica* Brunnth., *A. heterospora* Nygaard, *A. spiroides* Kleb., *A. mendotae* Trelease, *A. zinserlingii* Kosinskaja, *A. virguieri* Denis et Frémy, *A. crassa* (Lemm.) Kom.-Legn. et Cronb., *Anabaena* sp. and *Aphanizomenon issatschenkoi* (Usač.) Prošk. Lavr. In 2006, 2008 and 2009, *P. agardhii* (Table 2) reached the highest biomass (average values ranged within 28.6 – 31.2 mg dm^{-3}, maximum up to 115.4 – 117.3 mg dm^{-3}). It was the main bloom-forming species (especially in summer and autumn) and the producer of microcystins. There was a strong positive correlation between *P. agardhii* abundance and total MC concentration (R^2 = 0.80 – 0.98 in 2006-2008; 0.53 in 2009). Generally, ANTX-producing Nostocales had smaller biomass, which significantly changed over the years and seasons (Table 2). They were most numerous in spring-summer periods. For example, the average biomass of *Anabaena flos-aquae* increased during the study period from 0.001 mg dm^{-3} in 2006 to 0.15 mg dm^{-3} in 2008 – 2009. High biomass of *A. lemmermannii* found in 2006 (0.31 mg dm^{-3}) fluctuated in subsequent years. Total average biomass of other *Anabaena* species increased from 0.28 mg dm^{-3} in 2006 to 2.01 mg dm^{-3} in 2009. They were not found in the lake in 2007, similarly to *Aphanizomenon issatschenkoi* the highest biomass of which was observed in 2008 (Table 2).

Anatoxin-a and microcystins in cyanobacteria

Intracellular cyanotoxins found in the lake during abundant occurrence of cyanobacteria are presented in Table 3. Their contents are related to cyanobacteria taxa that revealed the highest positive correlations between their biomass and cyanotoxin production. The obtained data suggest that within 2006-2008, ANTX was produced by different species of *Anabaena* and *Aphanizomenon issatschenkoi*. For example, in 2006 the dominant *A. lemmermanii* seemed to be the main ANTX producer, while in 2008-2009 − various *Anabaena* species together with *Aphanizomenon issatschenkoi*. The highest positive correlation ($y = 2E - 06x + 0.0003$; $R^2 = 0.99$) between *Anabaena* spp. abundance and intracellular ANTX concentration in water was recorded in 2006. Summer appearance of *Aphanizomenon issatschenkoi* also accounted for ANTX production. In 2008, also a strong positive correlation between its abundance and ANTX concentration was found ($y = 7E - 06x + 1.318$; $R^2 = 0.67$). In spring 2006, the surface scum was created in 99% by *A. lemmermannii* producing exclusively high amounts of ANTX (5.88 mg dm^{-3}), while in the surface scum created in 98% by *A. flos-aquae*, besides intracellular ANTX (0.39 mg dm^{-3} of scum) also MCs (0.73 mg dm^{-3}) were present.

Both extracellular and biomass-bound (intracellular) ANTX and MCs were detected in the lake (Figs. 2, 3). Intracellular ANTX was detected in lake water in spring and summer periods (with the highest concentrations of 2.28 µg dm^{-3} and 1.43 µg dm^{-3}, respectively), but the extracellular form was found only in 2008 (0.23 – 0.49 µg dm^{-3}). In the lake affected by year-long blooms caused by a mixture of cyanobacterial species, ANTX and MCs were mostly present in water at the same time. However, MC

Table 3

The content (mean and range of values) of intracellular cyanotoxins (mg g^{-1} FW) in the biomass of the bloom-forming cyanobacteria that revealed the highest positive correlation with toxin production.

Taxa	Toxin	2006 AN-a: V-VIII MCs: III-XII	2007 AN-a: VI-VII MCs: I-V	2008 AN-a: IV-VIII MCs:IV-XI	2009 AN-a: V-VII MCs: I-XI
Anabaena spp. *Aphanizomenon issatschenkoi*	AN-a	*49.01 0.17-193.93	0.02 0.004-0.04	#4.27 0.36-9.30	&5.93 0.07-19.88
P. agardhii	MCs	1.43 0.15-7.96	1.29 0.31-15.38	0.61 0.56-2.53	0.57 0.20-1.90

Anabaena spp. = *A. lemmermannii, A. flos-aquae, A. spiroides, A. mendotae, A. crassa, A. heterospora, A. planctonica, A. virguieri, A. zinserlingii, A.* cf. *heterospora, Anabaena* sp.; * - dominant *A.lemmermannii* (V); # -dominant *A. flos-aquae* (IV); & - dominant *A. lemmermannii* (V), *A. planctonica* (VII)

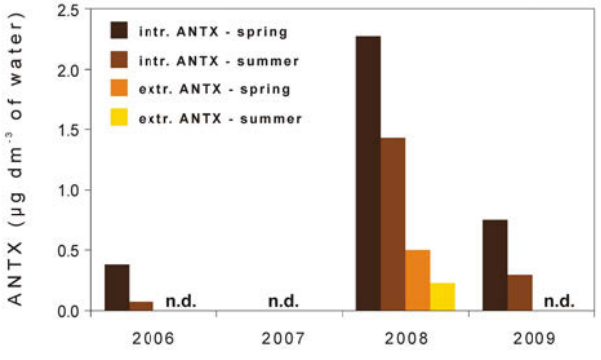

Fig. 2. Season average concentrations (n = 6) of intracellular and extracellular anatoxin-a detected only in spring and summer 2006 – 2009 in Lake Syczyńskie; n.d. – not detected.

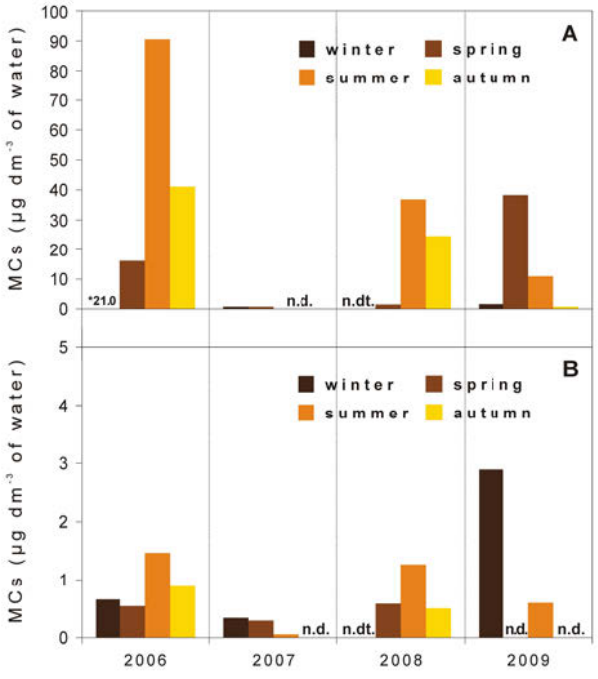

Fig. 3. Season average concentrations (n = 6) of intracellular (A) and extracellular (B) microcystins in 2006 – 2009 in Lake Syczyńskie; n.d. – not detected, n.dt. – not determined, * – detected only in trace concentrations (ng dm^{-3} of water).

concentrations (Fig. 3) were considerably higher than ANTX (Fig. 2) and mostly (apart from winter and spring 2007) intracellular MCs (Fig. 3A) dominated over the extracellular form (Fig. 3B). HPLC-DAD analysis of phytoplankton biomass revealed the presence of four MC isoforms: MC-YR, MC-LR, MC-LA and dmMC-RR that dominated quantitatively. Intracellular MC concentrations in lake water (Fig. 3A) varied in a very wide range: from as low seasonal concentrations as 0.02 µg dm^{-3} in winter to as high as 90.21 and 40.89 µg dm^{-3} in summer and autumn 2006. Average intracellular concentration of MCs related to *P. agardhii* biomass (Table 3) decreased approximately three times: from 1.43 mg g^{-1} FW in 2006 to 0.57 mg g^{-1} FW in 2009. Similarly to ANTX, the lowest level of MCs was found in 2007 when the essential decrease in cyanobacteria biomass occurred. Concentrations of extracellular MCs (Fig. 3B) changed in a narrower range (from 0.04 to 2.87 µg dm^{-3} of the lake water). The benthic filamentous cyanobacterium *Oscillatoria limosa* Ag. ex Gom., continuously inhabiting the lake, also produced MCs (0.19 mg g^{-1} DW of the benthic mat).

Cyanotoxins in fish tissues

As a consequence of long-term blooms caused by producers of ANTX and MCs, both cyanotoxins were accumulated in the indigenous fish inhabiting the lake, i.e. omnivorous Prussian carp (*Carassius gibelio*) and roach (*Rutilus rutilus*) as well as mostly predacious perch (*Perca fluviatilis*) (Figs. 4, 5, 6, 7). For the first time ANTX was detected (Fig. 8) in tissues of fish subjected, under field conditions, to extra- and intracellular forms of cyanotoxins produced by different species of Nostocales. Generally, higher contents of ANTX and MCs were found in livers than in muscles, regardless of fish species (Figs. 4, 7). Severe necrotic damage in fish livers was frequently observed (Fig. 9). Mostly, the concentrations of ANTX and MCs were higher in tissues of the omnivorous than predacious fish. However, in a few cases of young perch, cyanotoxins' accumulation was higher than in Prussian carp (Figs. 5, 7) and roach (Figs. 5, 6, 7). ANTX accumulation in all fish species (Figs. 4, 5) was generally higher in 2008-2009 than in 2006-2007, consistently with its occurrence in the lake. The contents of ANTX detected in fish muscles (Fig. 5) were much lower than in livers (Fig. 4). Its contents in muscles of Prussian carp in 2006 ranged from 5.9 to 13.9 ng g^{-1} FW, while in roach and perch examined in 2008-2009 from 0 to 36 ng g^{-1} FW (Fig. 5). The variability of MCs contents in fish tissues was also independent of fish species (Figs. 6, 7). MCs detected in livers of Prussian carp ranged from 0.02 to 3.12 µg g^{-1} FW, in roach from 0 to 7.24 µg g^{-1}, and in perch from 0 to 8.81 µg g^{-1} FW (Fig. 6). MC contents in fish muscles (Fig. 7) were ca. 20 times lower than in livers (Fig. 6). Like in the cyanobacterial biomass, dmMC-RR dominated quantitatively in fish tissues, however, MC-LR, MC-YR, MC-LA and MC-LY were also detected.

DISCUSSION

Cyanotoxin producers and accumulation in fish

Water blooms caused by toxigenic cyanobacteria are a worldwide problem related to water eutrophication (Rücker et al. 1997, Becker et al. 2004, van Apeldoorn et al. 2007). Hepatotoxic microcystin production by freshwater cyanobacteria, such as *Planktothrix agardhii, Microcystis* spp. and some *Anabaena* species is a common phenomenon (Sivonen et al. 1990, Briand et al. 2002, Van Apeldoorn et al. 2007, Pawlik-Skowrońska et al.

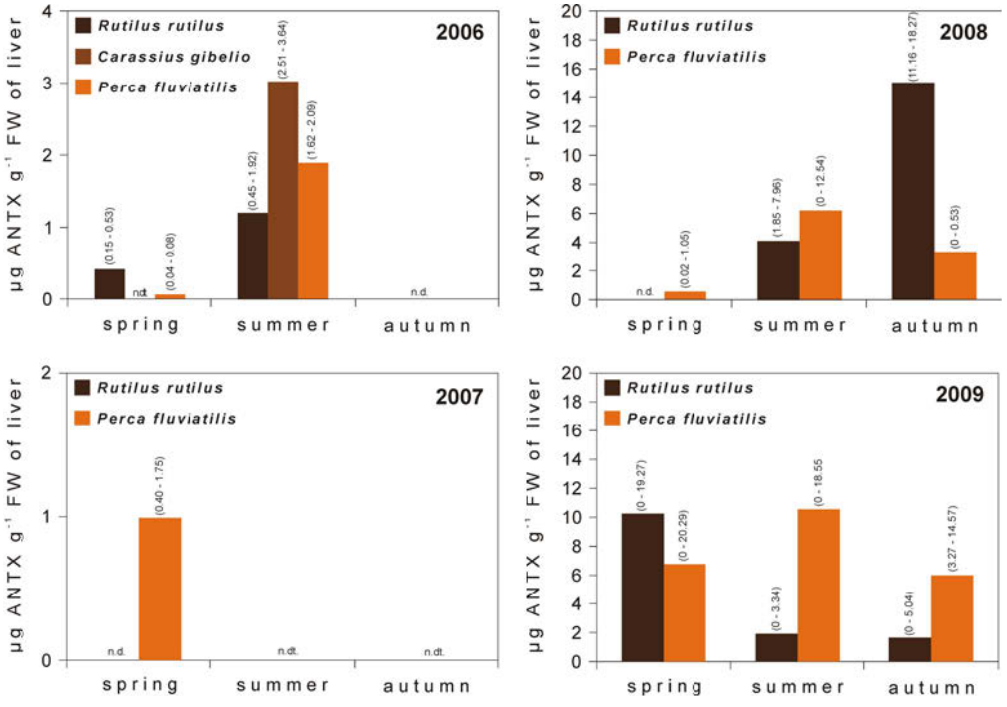

Fig. 4. Anatoxin-a accumulation in livers of fish inhabiting Lake Syczyńskie in 2006 – 2009. Concentrations are presented as mean (n = 3-4), and minimum and maximum values (in parenthesis); n.d. – not detected. n.dt. – not determined.

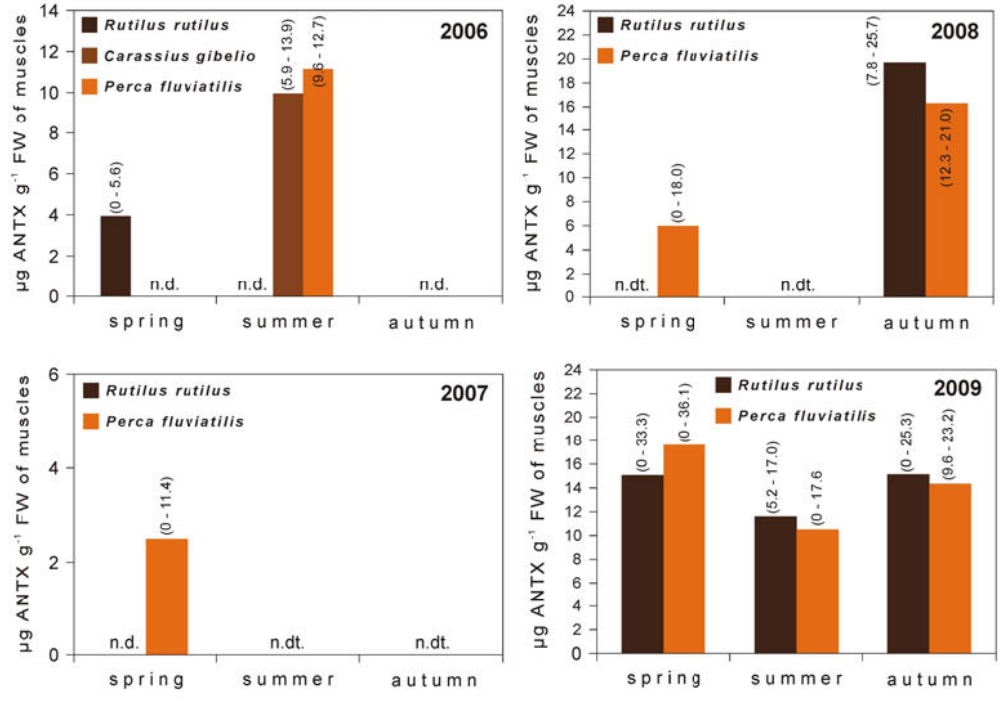

Fig. 5. Anatoxin-a accumulation in muscles of fish inhabiting Lake Syczyńskie in 2006 – 2009. Concentrations are presented as mean (n = 3-4), and minimum and maximum values (in parenthesis); n.d. – not detected. n.dt. – not determined.

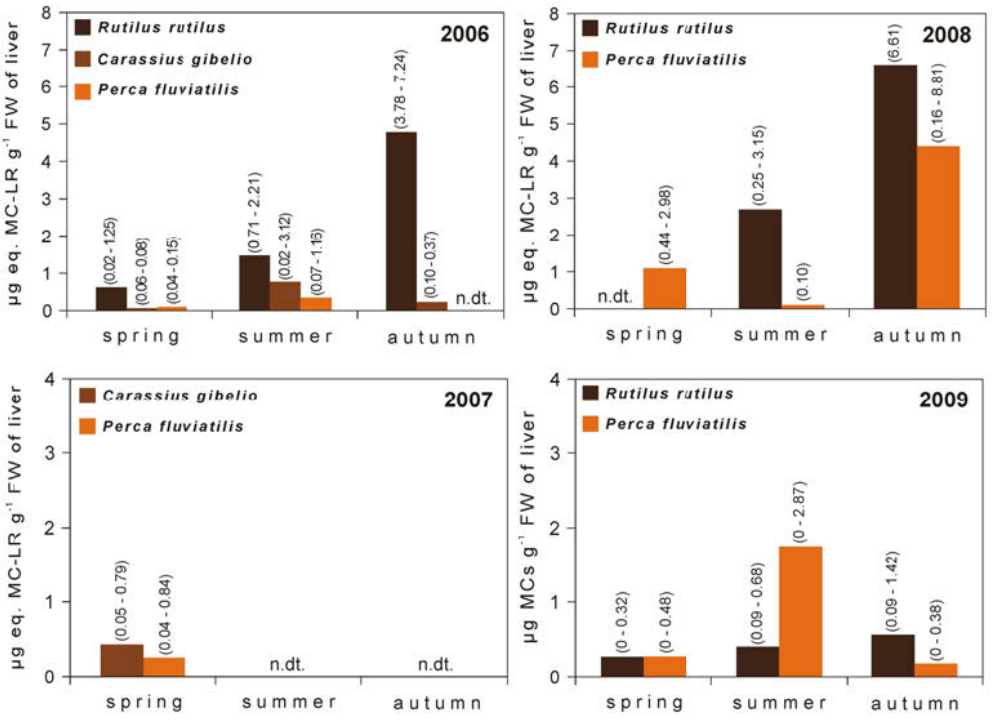

Fig. 6. MCs accumulation in livers of fish inhabiting Lake Syczyńskie in 2006 – 2009. Concentrations are presented as mean (n = 3-4), and minimum and maximum values (in parenthesis); n.d. – not detected. n.dt. – not determined.

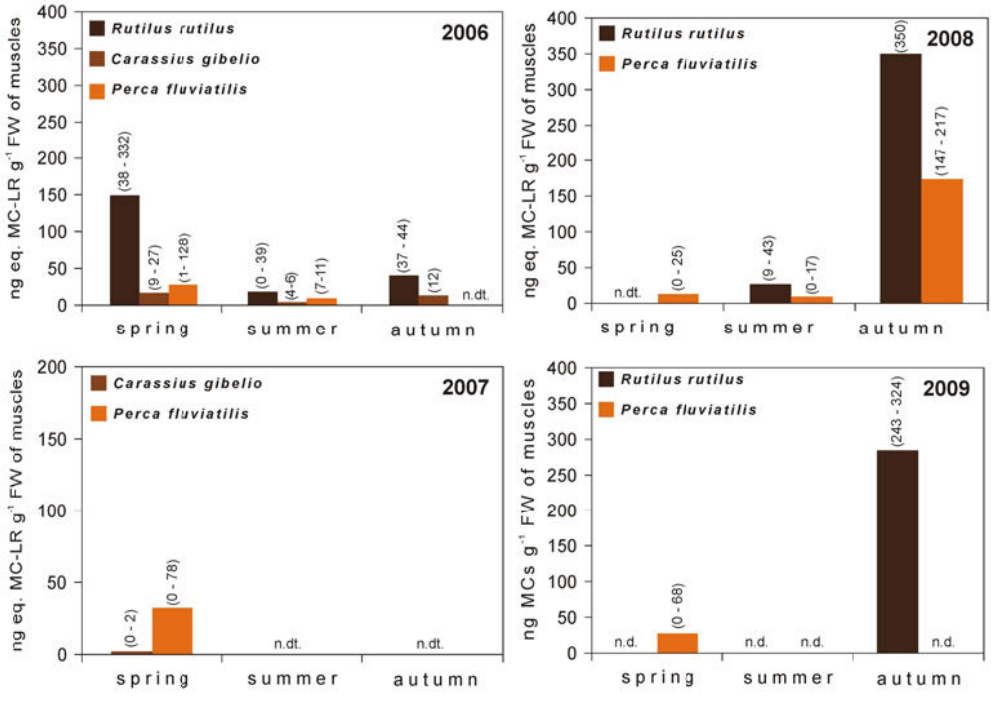

Fig. 7. MCs accumulation in muscles of fish inhabiting Lake Syczyńskie in 2006 – 2009. Concentrations are presented as mean (n =3- 4), and minimum and maximum values (in parenthesis); n.d. – not detected. n.dt. – not determined.

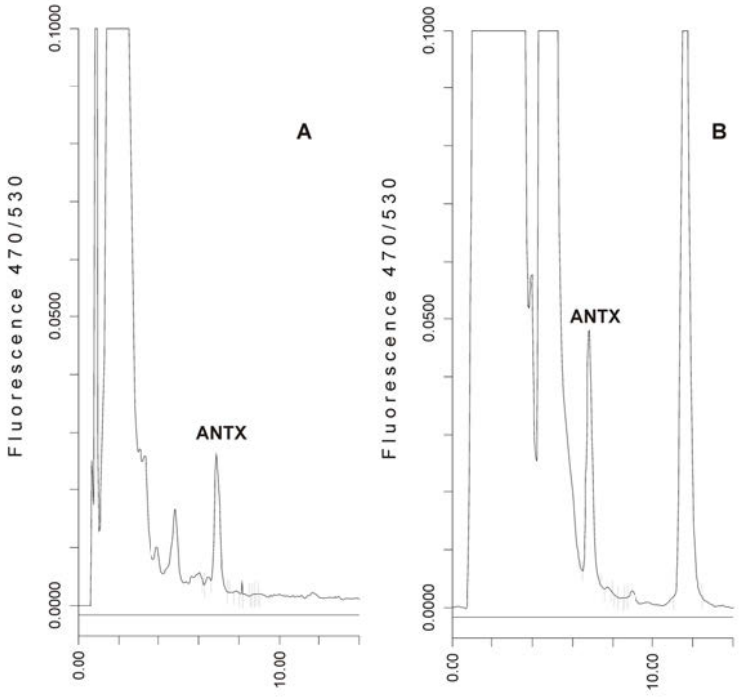

Fig. 8. Chromatograms corresponding to analysis of standard ANTX (A) and roach liver sample (B).

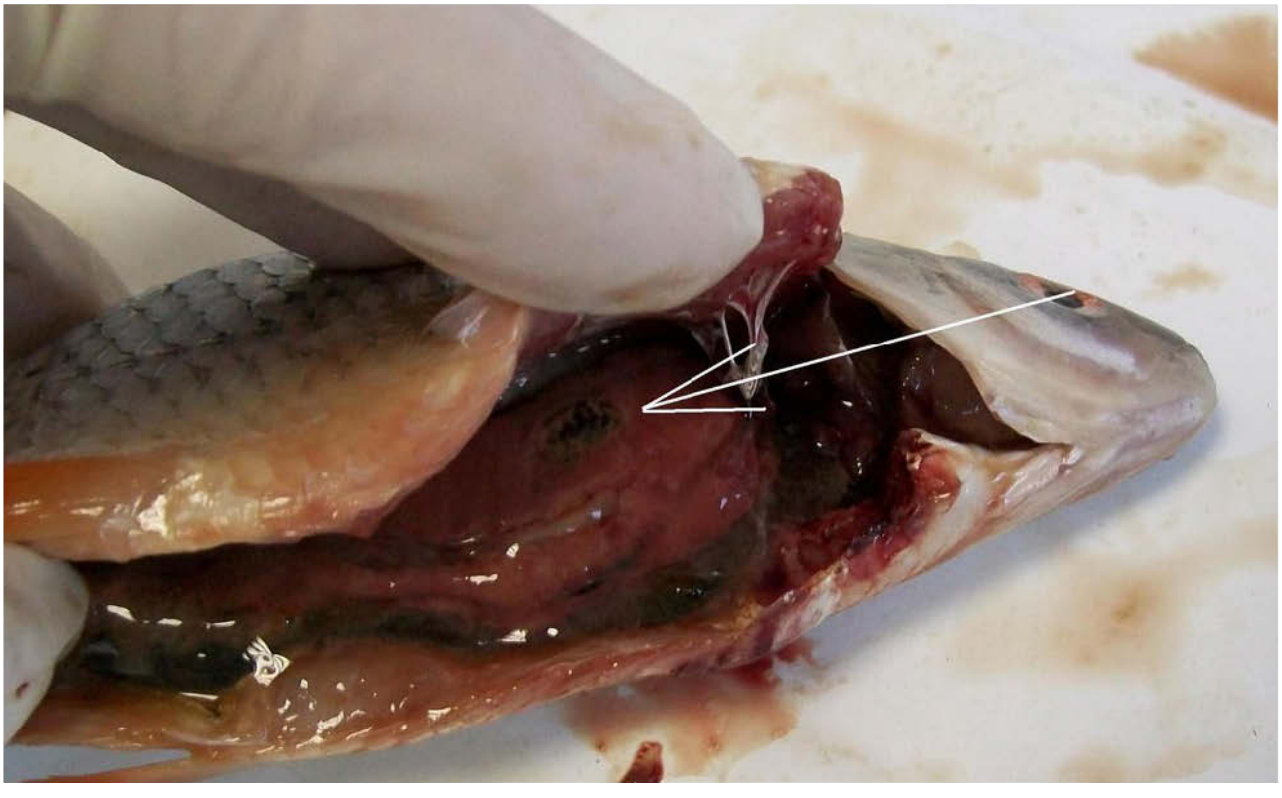

Fig. 9. Liver necrotic damage (arrow) in fish (roach) from Lake Syczyńskie affected by blooms of toxigenic cyanobacteria.

2008). However, ANTX presence in water reservoirs has been less frequently reported (Bumke-Vogt et al. 1999, Pawlik-Skowrońska et al. 2004, Osswald et al. 2009b, Pawlik-Skowrońska and Toporowska 2011). As shown in this study, water blooms in the hypertrophic lake were created every year by different cyanobacterial species that developed intensively over the same periods. Among them, several *Anabaena* spp. and *Aphanizomenon issatchenkoi* appeared to be very important ANTX- producers. Recently, some strains of *Aphanizomenon issatschenkoi* found in German lakes (Ballot et al. 2010) produced as much as 2.35 mg ANTX g^{-1} FW. Despite the extensive investigation of cyanobacterial metabolites (Carmichael 1992, Mazur-Marzec 2006, Welker and Döhren 2006) and their influence on living organisms (Malbrouck and Kestemont 2006, Wilson et al. 2006), there is still insufficient information on their accumulation, effects and transfer in aquatic food webs. Only in recent years, the influence of microcystins on ichthyofauna of European waters has been investigated (Ibelings et al. 2005, Kopp et al. 2009, Papadimitriou et al. 2010). However, there is still very limited information on ANTX accumulation in fish in natural, although polluted, environment. Exclusive laboratory experiments on juvenile carp feeding on *Anabaena* spp. or rainbow trout exposed to extracellular ANTX have been reported so far (Osswald et al. 2007, 2011). There are no reports on accumulation of MCs and ANTX in common, freshwater fish, such as roach, Prussian carp and perch, which differ in feeding strategy (Kamjunke et al. 2002) and constitute a component of human diet.

To our best knowledge, this is the first field study on accumulation of ANTX and hepatotoxic MCs in tissues of European freshwater fish living in water bodies permanently affected by heavy cyanobacterial blooms. We confirmed that in highly eutrophic water bodies several toxin-producing species of Oscillatoriales and Nostocales may simultaneously reach high biomass and occur together with other potentially toxic species of Chroococcales, such as *Microcystis* spp. or *Woronichinia* sp. (Toporowska and Pawlik-Skowrońska, data not presented). In Lake Syczyńskie, *Anabaena* spp. and/or *Aphanizomenon issatschenkoi* – the main ANTX producers developed intensively in spring-summer seasons almost every year, whereas MC-producing *P. agardhii* formed a year-long blooms (Pawlik-Skowrońska et al. 2008, Toporowska et al. 2010). ANTX production by the cyanobacteria in Lake Syczyńskie was generally higher than that reported by Osswald et al. (2009b) in Portuguese freshwaters (up to 24.62 μg g^{-1} DW), but similar to production reported by Sivonen et al. (1989,1990) for bloom material from Finland (12 – 4,360 μg g^{-1} DW). Besides the abiotic factors responsible for mass development of particular cyanobacterial taxa (Bradburn et al. 2012), the intrinsic capabilities of different cyanobacterial populations account for the cyanotoxin production (Rantala-Ylinen et al. 2011). It is known that both producing and non-producing strains can develop in the same water body (Kurmayer et al. 2004). As shown in this work, however, several toxin producers may contribute to water blooms at the same time being a real threat to aquatic fauna. The obtained results showed that under unstable environmental conditions, at temporal fluctuations in the structure and abundance of cyanobacterial assemblages, a considerable variability in concentrations of cyanotoxins occurs both in water and in fish tissues. Both omnivorous and carnivorous fish exposed to extracellular and intracellular cyanotoxins accumulated MCs and ANTX at the same time. Ibelings et al. (2005) reported higher accumulation of MCs in planktivorous smelt than in benthivorous ruff and carnivorous perch living in a water reservoir with blooms of *M. aeruginosa* and *P. agardhii*. Opposite results on the highest accumulation of MCs in livers of carnivorous fish, lower in omnivorous and the lowest in planktivorous species (Xie et al. 2005) indicated possible MCs biomagnification in a food chain. Our study revealed that MCs were accumulated in higher concentrations in livers than in fish muscles. A similar observation was reported by Xie et al. (2004) in liver and muscle (17.8 and 1.77 μg g^{-1} DW, respectively) of silver carp and by Li et al. (2004) in experiments with carp fed on *Microcystis* sp. The concentrations of MCs in livers and muscles of the European fish found in this work changed within a similar range like in the Asian phytoplanktivorous *Hypophthalmichthys molitrix*, herbivorous *Parabramis pekinensis*, omnivorous *Cyprinus carpio*, *C. auratus* and carnivorous *Culter ilishaeformis*, *C. erythropterus*, *Pseudobagrus fulvidraco*, *Coilia ectenes* from water bodies affected by blooms caused by *Microcystis*, *Anabaena* and *Planktothrix* (Xie et al. 2005). However, the concentrations of MCs in fish living in Lake Syczyńskie were sometimes much higher than in the Prussian carps (maximum 250 – 350 ng g^{-1} FW in livers and 50 – 70 ng g^{-1} FW in muscles) obtained from Greek waters with lower MCs concentrations (Papadimitriou et al. 2010). The highest amounts of MCs as well as ANTX in fish from Lake Syczyńskie

were found at the time of the highest cyanobacterial biomass or soon after reaching the maximum levels. It indicates that concentrations of cyanotoxins in fish tissues are closely connected with their amounts in phytoplankton biomass. However, due to passive exposure to cyanotoxins released into water (Xie et al. 2005), it is also probable that their accumulation in fish can be substantial during periods with high concentrations of extracellular cyanotoxins (Pawlik-Skowrońska et al. 2008). Recently, Osswald et al. (2011) reported that juvenile rainbow trout accumulated in the whole body considerable amounts of ANTX (e.g. 3.9 μg g^{-1} FW) only after 96 h of experimental exposure to 132 μg dm^{-3} extracellular toxin). This value is comparable with ANTX levels detected in muscles of fish living for a longer time in Lake Syczyńskie. As reported by Osswald et al. (2011), ANTX bioaccumulation in fish is a dose-dependent process and permanent exposure may cause a constant presence of the toxin in tissues.

The lower contents of MCs and ANTX in muscles than in livers of different fish may result from different toxin persistence under natural conditions and different efficacy of cyanotoxin detoxification processes in the organism. Accumulated microcystins can be metabolized into less harmful compounds after conjugation with glutathione (GSH) *via* glutathione- *S*-transferase (Pflugmacher et al. 1998) or with cysteine (Zhang et al. 2009) resulting in microcystin excretion or physiological degradation. Previous studies of Cazenave et al. (2006) demonstrated that cyanotoxins induce oxidative stress reduced by the activity of antioxidant enzymes, such as glutathione peroxidase. The higher accumulation of cyanotoxins in juvenile (*versus* adult) organisms observed by Lance et al. (2006) in laboratory experiments in freshwater snail *Lymnea stagnalis* may be due to a less developed and therefore less competent immune system (Dikkeboom et al. 1985), with a consequently less efficient detoxification. ANTX is decomposed to non-toxic dihydroanatoxin-a and epoxyanatoxin-a (Harada et al. 1993) and its half-time in water reservoirs is shorter than that of MCs (Hardy 2008). However, as recently reported by Rymuszka and Sierosławska (2010) and Osswald et al. (2009a), ANTX may cause apoptosis of immune cells and skeletal malformations in fish larvae. In addition, ANTX –producing Nostocales, such as *Anabaena* and *Aphanizomenon*, can be easily digested and assimilated by fish, contrary to MC- producing *M. aeruginosa* and *P. agardhii* (Kamjunke et al. 2002, Kolmakov and Gladyshew 2003). Detoxification of MCs under natural conditions may be a long-term process dependent on many environmental and biological factors, like temperature (Ozawa et al. 2003), animal species (Zhang et al. 2009), a developmental stage (Oberemm et al. 1997) and time of animal exposure. For example, Adamovsky et al. (2007) stated that MCs can be eliminated from fish tissues in a couple of weeks after transferring the fish to clean water. However, fish inhabiting eutrophic water systems with perennial cyanobacterial blooms do not have this option. ANTX detoxification in fish remains unknown (Smith et al. 2008). As it was stated in this work, MCs were present in fish year-long, but their highest concentrations occurred mainly after the most intensive *P. agardhii* development. Degradation of MCs may be inhibited at low water temperatures (Ozawa et al. 2003), which was also proved in our previous study carried out in winter (Pawlik-Skowrońska et al. 2008) when high concentrations of extracellular MCs (up to 11 μg dm^{-3}) prevailed over intracellular forms. Therefore, cyanotoxins may be present in fish tissues also in a subsequent spring, a year after the most intensive cyanobacterial bloom.

Risk Assessment

A daily tolerable dose of MCs for humans was established by WHO (1999) as 0.04 μg kg^{-1} body weight. It means that a 60 kg person, consuming 300 g fish muscles contaminated with 0.15 μg g^{-1} FW per day (as stated in muscles of perch and roach in this work) can absorb 15 times more of MCs than WHO standards suggest. Generally, a long-term high risk of MCs and ANTX consumption was observed in the lake. Recently, Chen et al. (2009) reported accumulation of MCs in serum of fishermen consuming fish and seafood contaminated with MCs. Contrary to MCs, not much is known about the accumulation of ANTX, its effects and the detoxification process in fish (Osswald et al. 2007, 2011). This is the first field study showing ANTX accumulation in tissues of ichthyofauna inhabiting the lake with long-lasting cyanobacterial blooms. Osswald et al. (2007) showed in laboratory experiments that at 10^7 individuals of *Anabaena* spp. dm^{-3}, the level of ANTX in a body of juvenile carp was 0.073 μg g^{-1} FW and all fish died between the 26^{th} and 29^{th} hour of exposure. Fish in our study were much bigger and older, and contained temporally higher amounts of ANTX in livers (up to 20 μg g^{-1} FW), as well as detectable concentrations

(up to 30 ng g^{-1} FW) in edible mussels. The high variability of ANTX contents in fish tissues may be connected with large differences in their age and size. ANTX accumulation in the whole body of juvenile rainbow trout, exposed to ANTX for only 96 h, was essential (Osswald et al. 2011). This indicates possible human exposure through contaminated fish ingestion. Usually short-term blooms of different ANTX producers may be hazardous to fish because of fast and intensive accumulation of ANTX, and nothing is known about its detoxification in organisms of consumers. However, the year-long blooms of MC-producers, such as *P. agardhii* (Pawlik-Skowrońska et al. 2008), which may overwinter in a vegetative state as benthos, represent stronger threat due to longer time of exposure and accumulation. So far, there is no limit for a daily tolerable dose of ANTX for humans, however its accumulation in consumable fish may indicate a need for this.

CONCLUSIONS

European freshwater fish inhabiting water reservoirs with multispecies blooms of toxin-producing *Planktothrix*, *Anabaena* and *Aphanizomenon* simultaneously accumulate in their tissues both anatoxin-a and microcystins. Similarly to MCs, ANTX produced by cyanobacteria may affect indigenous ichthyofauna under natural conditions, however, the evaluation of ANTX threat to fish consumers needs further studies.

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