

RESEARCH

Open Access



# Cyanobacteria and their secondary metabolites in three freshwater reservoirs in the United Kingdom

Daria Filatova<sup>1</sup>, Martin R. Jones<sup>2</sup>, John A. Haley<sup>3</sup>, Oscar Núñez<sup>4,5</sup>, Marinella Farré<sup>1</sup> and Elisabeth M.-L. Janssen<sup>2\*</sup> 

## Abstract

**Background:** Bloom-forming cyanobacteria occur globally in aquatic environments. They produce diverse bioactive metabolites, some of which are known to be toxic. The most studied cyanobacterial toxins are microcystins, anatoxin, and cylindrospermopsin, yet more than 2000 bioactive metabolites have been identified to date. Data on the occurrence of cyanopeptides other than microcystins in surface waters are sparse.

**Results:** We used a high-performance liquid chromatography–high-resolution tandem mass spectrometry/tandem mass spectrometry (HPLC–HRMS/MS) method to analyse cyanotoxin and cyanopeptide profiles in raw drinking water collected from three freshwater reservoirs in the United Kingdom. A total of 8 cyanopeptides were identified and quantified using reference standards. A further 20 cyanopeptides were identified based on a suspect-screening procedure, with class-equivalent quantification. Samples from Ingbirchworth reservoir showed the highest total cyanopeptide concentrations, reaching 5.8, 61, and 0.8 µg/L in August, September, and October, respectively. Several classes of cyanopeptides were identified with anabaenopeptins, cyanopeptolins, and microcystins dominating in September with 37%, 36%, and 26%, respectively. Samples from Tophill Low reservoir reached 2.4 µg/L in September, but remained below 0.2 µg/L in other months. Samples from Embsay reservoir did not exceed 0.1 µg/L. At Ingbirchworth and Tophill Low, the maximum chlorophyll-a concentrations of 37 µg/L and 22 µg/L, respectively, and cyanobacterial count of  $6 \times 10^4$  cells/mL were observed at, or a few days after, peak cyanopeptide concentrations. These values exceed the World Health Organization's guideline levels for relatively low probability of adverse health effects, which are defined as 10 µg/L chlorophyll-a and  $2 \times 10^4$  cells/mL.

**Conclusions:** This data is the first to present concentrations of anabaenopeptins, cyanopeptolins, aeruginosins, and microginins, along with microcystins, in U.K. reservoirs. A better understanding of those cyanopeptides that are abundant in drinking water reservoirs can inform future monitoring and studies on abatement efficiency during water treatment.

**Keywords:** Cyanotoxin, Cyanopeptide, Water quality, Mass spectrometry, Suspect-screening, Surface water, Natural toxin, Emerging contaminant

## Background

Cyanobacteria, also known as blue-green algae, are prokaryotes that can thrive under diverse environmental conditions and occur globally in aquatic and terrestrial habitats [1]. Adaptations that facilitate their survival and provide them with competitive advantages include the presence of a wide range of pigments (e.g., chlorophyll-a, phycocyanin, phycoerythrin

\*Correspondence: elisabeth.janssen@eawag.ch

<sup>2</sup> Department of Environmental Chemistry, Swiss Federal Institute of Aquatic Science and Technology (Eawag), 8600 Dübendorf, Switzerland  
Full list of author information is available at the end of the article

absorbing across a wide light spectrum), buoyancy, nitrogen fixation, production of dormant cells, and formation of colonies or filaments [2]. These advantages allow cyanobacteria to form high-density communities termed, blooms [3]. One major issue associated with bloom-forming cyanobacteria is the production of bioactive secondary metabolites, some of which are identified as toxins. These cyanotoxins include microcystins, cylindrospermopsin, anatoxins, and saxitoxins. Microcystins are heptapeptide molecules that contain a characteristic Adda moiety [(2*S*,3*S*,4*E*,6*E*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid], or derivatives thereof, that is linked to their hepatotoxic effects [4]. Microcystins are produced by multiple cyanobacteria genera, including *Microcystis*, *Dolichospermum* (previously *Anabaena*), *Planktothrix*, *Nostoc*, *Oscillatoria*, and *Anabaenopsis*. While more than 300 microcystin variants have been identified to date [5], a few microcystins are routinely included in the analysis of surface waters. Anatoxin-a is the most widely studied of the known anatoxins and it has a potent neurotoxic activity. Anatoxin-a binds to nicotinic acetylcholine receptors in peripheral nerve cells, which can be lethal by respiratory arrest [6]. *Anabaena* is the main producing genus of anatoxin-a besides *Planktothrix*, *Oscillatoria*, *Microcystis*, *Aphanizomenon*, *Cylindrospermum*, and *Phormidium* [7]. Cylindrospermopsin is a cyclic guanidine alkaloid that blocks protein synthesis, which can affect liver, kidney, thymus, and heart functions [4, 8]. Cylindrospermopsin is known to be produced by *Cylindrospermopsis*, *Aphanizomenon*, *Anabaena*, and *Lyngbya* genera [9]. Due to the toxicological risks posed by various cyanobacterial metabolites, guideline values have been introduced by several countries (such as the EU, USA, Canada, Brazil, Australia, South Africa, China, and Japan) to protect the public from exposure to cyanotoxins [10]. The World Health Organization (WHO) has set a guideline threshold value of  $2 \times 10^4$  cyanobacterial cells/mL for recreational waters that would pose a relatively low probability of adverse health effects, which may correspond to a concentration of 20 µg/L microcystins for a *Microcystis* bloom [4]. WHO has set a guideline value of 1 µg/L for total microcystins-LR (MC-LR) in drinking water [11]. However, recently, an update of the WHO guideline has been finalized, and this value was modified. Now, not only MC-LR is considered, but also a total microcystins' content. The value for lifetime is 1 µg/L and for short-term events 12 µg/L in drinking water [12]. Additionally, threshold values for cylindrospermopsin, anatoxin-a, and saxitoxins are now also included, those are 3, 30, and 3 µg/L in drinking water [13–15]. Microcystins and anatoxin-a have been

regularly reported in European waters with concentrations up to 100 and 14.4 µg/L, respectively, during seasonal studies in the recent years (e.g., Italy, Poland, Czech Republic, Germany, Russia, France, and Netherlands [16] and references herein). Many freshwater bodies in the United Kingdom (U.K.) frequently experience cyanobacterial blooms and high microcystin concentrations [17, 18]. One study reported that 53% of 117 samples from cyanobacterial blooms reached microcystin concentrations above 0.2 µg/L and 13% of samples exceeded the WHO alert level of 20 µg/L [19]. Anatoxin-a has been detected in several lakes in Ireland reaching a maximum of 112 µg/L [20].

Beyond these regulated cyanotoxins, more than 2000 secondary metabolites from cyanobacteria have been structurally identified to date [5]. They are co-produced with known cyanotoxins by bloom-forming cyanobacteria [21–25]. In addition to microcystins, these include cyanopeptides with characteristic moieties such as cyclic cyanopeptolins [ $\beta$ -lactone ring, Ahp moiety (3-amino-6-hydroxy-2-piperidone)] and anabaenopeptins (ureido bond connecting the primary amine of lysine with the primary amine of the neighboring amino acid) as well as linear cyanopeptides such as aeruginosins [Choi moiety (2-carboxy-6-hydroxyoctahydroindole)] and microginins [Ahda moiety (3-amino-2-hydroxy-decanoic or octanoic acid)]. Compounds from these classes have shown acute toxicity in planktonic grazers ( $LC_{50} < 10$  mg/L) and to inhibit various enzymes ( $IC_{50} < 30$  µg/L often  $< 8$  µg/L) [26]. While their mode of action and toxic potency is not sufficiently studied yet, their occurrence has been reported in several surface waters [27–32]. With the exception of some microcystin variants, no analytical reference standard materials exist for these other cyanopeptides. Only few studies used available bioreagents to perform absolute quantification of up to two anabaenopeptins, three cyanopeptolins, and one microginin in U.S. and Canadian lakes [27, 28, 32]. Globally, there is a knowledge gap regarding the entry of these cyanopeptides into drinking water treatment plants, their removal during treatment, along with cyanobacterial cells and known toxins. A recent study reported similar concentrations of cyanopeptolins and anabaenopeptins in raw drinking water compared to well-known microcystins [28]. Awareness of those cyanopeptides that are abundant in drinking water reservoirs can facilitate the prioritization of additional compounds that should be included in monitoring along with cyanotoxins and other indicator parameters such as chlorophyll-a and cell abundance. These measurements aim to improve the early identification of bloom formation and design of water management strategies. A better understanding of those cyanopeptides that are abundant in drinking water

reservoirs would also aid the prioritization of compounds for which their removal efficiency during water treatment should be assessed.

In this study, we analysed toxins in raw water from three reservoirs that supply the drinking water systems in the U.K. between August and October 2019. We used a combination of target- and suspect-screening approaches based on high-performance liquid chromatography–high-resolution tandem mass spectrometry (HPLC–HRMS/MS). We quantified the co-occurrence of toxins and cyanopeptides across reservoirs and sampling times and compared these profiles to chlorophyll-a concentrations and cyanobacterial cell counts. To the best of our knowledge, this is the first time that anabaenopeptins, cyanopeptolins, aeruginosins, and microginins have been quantified in U.K. waters along with microcystins and anatoxin-a.

## Materials and methods

### Study sites, sample collection, and storage

The water reservoirs sampled were Tophill Low (53°54′59.2416″ N, 000°22′20.4024″ W), Ingbirchworth (53°32′59.3808″ N, 001°40′40.9800″ W), and Embsay (53°59′13.9344″ N, 002°00′11.1492″ W). Tophill Low consists of 2 water reservoirs D and O (due to their shape), their volumes, maximal depth, and surface area are 900 million L (ML) and 773 ML, 3.8 m and 6.1 m, 0.239 km<sup>2</sup> and 0.141 km<sup>2</sup>, respectively. Both reservoirs provide 45 ML/d drinking water each. Ingbirchworth is the biggest reservoir with a volume of 1370 ML, maximal depth of 18.5 m, and a surface area of 0.235 km<sup>2</sup>. The Embsay reservoir has a volume of 797 ML, maximal depth of 15 m, and a surface area of 0.11 km<sup>2</sup>. Both Ingbirchworth and Embsay provide 20 ML/d of drinking water each. While these waters are used for recreational activities, including bird watching, game bird shooting, and sailing, their primary purpose is to serve as drinking water sources.

Samples were collected from three water treatment facilities connected to these reservoirs operated by Yorkshire Water. At Tophill Low, river water from the River Hull is pumped into two storage reservoirs, operating in series, and water is abstracted for treatment after about 30 days retention time via sub-surface draw-offs at around 4 m depth. Ingbirchworth reservoir impounds water from the Blackwater Dike, which enters the treatment plant under gravity via a draw-off tower at a depth of around 14 m. Embsay reservoir receives water from Lowburn Gill and Moor Beck and enters the treatment plant by gravity via a draw-off tower at a depth of around 12 m. Samples were collected on August 13th, September 3rd, and October 10th 2019, at the inlets to the respective water treatment facilities; in each case travel time of the raw water storage at the reservoir to the treatment plant

is only a few minutes, so that the samples represent water from the raw water storage. Samples were collected in green polyethylene terephthalate bottles and transported to the laboratory in coolers, in the dark. For biological analyses, 1 L water samples were kept at 4–8 °C in the dark. Taxonomic analysis by microscopy was performed on the day following sampling and chlorophyll-a concentrations were assessed by filtration and solvent extraction followed by spectrophotometric measurement (see details in Additional file 1: Text S1, S2). Analysis of chlorophyll-a, total ammonium, nitrate, and total phosphate was done spectrophotometrically (see details in Additional file 1: Text S3–S5, and results in Additional file 1: Tables S2, S3 for Ingbirchworth and Tophill Low reservoirs). For cyanotoxin and cyanopeptide analysis, 1 L water samples were frozen and shipped to Swiss Federal Institute of Aquatic Science and Technology (Eawag), where they were stored frozen until sample preparation.

### Chemicals

Reference standards (>95% purity) for cylindrospermopsin, anatoxin-a, MC-LR, MC-YR, MC-RR, MC-LF, MC-LA, MC-LW, MC-LY, and nodularin were purchased from Enzo Life Science (Lausen, Switzerland). Bioreagents (>90% purity) for cyanopeptolin A, anabaenopeptin A, anabaenopeptin NZ857, and oscillamide Y were bought from CyanoBiotech GmbH (Berlin, Germany). Aerucyclamide A was obtained as a purified bioreagent in dimethyl sulfoxide by Prof. Karl Gademann (University Zurich, Switzerland). Stock solutions of 5.5 mg/L were prepared for anatoxin-a and cylindrospermopsin in methanol/water (50/50, v/v%) and methanol, respectively. Stock solutions of cyanopeptide reference standards and bioreagents were prepared in ethanol at 50 mg/L (except for [D-Asp<sup>3</sup>,E-Dhb<sup>7</sup>]MC-RR with 10 mg/L). Stock solutions were aliquoted and kept at -20 °C. Formic acid (FA) (98–100%) was obtained from Sigma-Aldrich/Merck AG. Methanol (Optima<sup>®</sup>LC/MS 99.9%), and acetonitrile (Optima<sup>®</sup>LC/MS 99.9%) were purchased from Thermo Scientific. Nanopure water was obtained using a NANOpure<sup>®</sup>21 water purification system (Barnstead from Thermo Scientific). NH<sub>4</sub>OH (25%) was obtained from Sigma-Aldrich (Steinheim, Germany).

### Sample preparation for chemical analysis

Cyanopeptides were extracted and pre-concentrated according to a recently validated method [33]. Briefly, 300 mL of each freshwater sample was sonicated in an ultrasonic bath (30 min, 200 W, 60 Hz) to lyse cells and release intracellular cyanopeptides. Next, samples were centrifuged at 3219.84 × g for 7 min. A 250 mL supernatant aliquot was collected and subjected to two sequential solid-phase extraction (SPE) procedures based,

respectively, on Oasis HLB (500 mg, 6 cc, Waters Corporation, Milford, MA, USA) and Supelclean™ ENVI-Carb™ (500 mg, 6 cc, Supelco, Sigma-Aldrich, St. Louis, MO, USA) cartridges. The HLB cartridges were conditioned with methanol and equilibrated with water (10 mL each). Then, the supernatant was loaded at 1 mL/min, and the elution of the cartridge was accomplished using 20 mL of methanol at 50 °C. The resulting extract was collected, basified up to 0.1% ammonia, and then transferred to a Supelclean™ ENVI-Carb™ cartridge, pre-conditioned with methanol, and equilibrated with water containing 0.1% ammonium hydroxide (10 mL each). After loading at 1 mL/min under vacuum, elution was carried out by back-flushing the cartridge with 20 mL of heated methanol (50 °C) containing 0.5% formic acid. Thus, the loading was sequential, and the extraction step was performed separately for each cartridge. Both Oasis HLB and Supelclean™ ENVI-Carb™ extracts were combined, concentrated in a Turbovap (Biotage) under a gentle stream of gaseous nitrogen at 25 °C, and before reaching dryness 500 µL methanol:water (1:9, v/v) was added and solutions were stored at -20 °C until analysis. All samples were analysed in triplicate (extraction step), except for Ingbirchworth October samples, which were analysed in duplicate.

#### Analysis of cyanopeptides

Cyanopeptides extracted from water samples were analysed using high-performance liquid chromatography coupled with high-resolution tandem mass spectrometry. Samples were injected into the HPLC system (Ultimate3000, Dionex, Thermo Scientific) using a CTC PAL autosampler fitted with a 20 µL stainless sample loop; the injection volume was 20 µL. Chromatographic separations were performed over a Kinetex C<sub>18</sub> column [2.1 × 100 mm (I.D. × L), 2.6 µm particle diameter] fitted with a SecurityGuard C<sub>18</sub> guard cartridge and in-line filter (aluminum frit, 0.7 µm pore diameter) at 40 °C. Mobile phase comprised 0.1% v/v formic acid in nanopure water (solvent A) (18.2 MΩ-cm-resistivity) and methanol (solvent B), which were used to generate the following binary gradient elution profile: 0/20/50/70/100/100/20/20% B at 0/1.5/5.5/21.4/21.5/26/26.1/30 min at a flow rate of 0.255 mL/min. HPLC eluates were introduced into a high-resolution tandem mass spectrometer (Exactive-Plus™ Orbitrap, Thermo Scientific) via an electrospray ionization (ESI) probe operated with the following conditions: +3.5 kV spray voltage, 325 °C capillary temperature, 35 arbitrary units (AU) sheath gas, 17 AU auxiliary gas, 1 AU spare gas, and 275 °C probe heater temperature. A data-dependent *top-N* MS<sup>2</sup> acquisition procedure was applied for analyte detection. Here, full-scan mass spectrometry data were acquired in profile mode from 450

to 1350 *m/z* at 70,000 FWHM (full-width at half maximum, at 200 *m/z*) resolution with automated gain control (AGC) of 5e5, 100 ms maximum ion injection time, 1 microscan, and 60% S-lens RF setting. Data-dependent MS<sup>2</sup> scans were triggered for the top-3 most-intense ions (with intensity > 2e4) from the preceding full scan, using the following acquisition parameters: profile acquisition mode, 17,500 FWHM resolution, 1 *m/z* isolation window (0 *m/z* offset), 1 microscan, 5e4 AGC target, 70 ms maximum ion injection time, 5 s dynamic exclusion, 'True' for 'pick others', and stepped normalized collision energies of 15%, 30%, and 45%. The scan range for MS<sup>2</sup> events was dynamically adjusted (by the instrument) based on the target ion's *m/z* value. The suspect-screening included 1219 cyanopeptides in total with 160 microcystins, 177 cyanopeptolins, 73 anabaenopeptins, 65 cyclamides, 78 microginins, 79 aeruginosins, and 587 other compounds, accounting for structural isomers and the mass window of 450–1350 *m/z*. External calibration was used to support cyanopeptide quantification. Here, matrix-matched calibrants were prepared in the range 5–125 µg/L for each sampled water reservoir, using water derived therefrom as calibrant matrix. For Aeruginosin 98B, external calibration in the mobile phase was used at 0.5–500 µg/L. Each calibrant was prepared using reference standards of 10 microcystins and nodularin as well as 6 bioaeruginosins of additional cyanopeptides. The dominant precursor ions and the limits of detection and quantification in nanopure water and lake matrices are listed in Additional file 1: Table S1. For nanopure water, the LODs were between 0.02 and 1.01 µg/L.

#### Analysis of anatoxin-a and cylindrospermopsin

Analysis of anatoxin-a and cylindrospermopsin was performed by HPLC (Dionex UltiMate3000 RS pump, Thermo Scientific) with an autosampler (CTC Analytics). Chromatographic separation was carried out on a reversed-phase C<sub>18</sub> Atlantis T3<sup>®</sup> column (3 mm × 150 mm, 3 µm particle size) fitted with precolumn (VanGuard<sup>®</sup> Cartridge, Waters) and in-line filter (BGB<sup>®</sup>). The mobile phase consisted of nanopure water (solvent A) and acetonitrile (solvent B) both acidified with formic acid (0.05%). Binary gradient elution was carried out at a constant flow rate of 300 µL min<sup>-1</sup> with the following gradient profile: 0/5/55/75/95/95/5% B at 0/1/10/20.5/24/26/26.1 min; and column was re-equilibrated for 3.4 min under the initial conditions. The injection volume was 20 µL. Detection of analytes was carried out by HRMS/MS (Q-Exactive-Plus™ Orbitrap, Thermo Scientific) with an ESI probe in positive mode operated at the following conditions: 4 kV spray voltage, 325 °C capillary temperature, 35 arbitrary units (AU) sheath gas, 17 AU auxiliary gas, 1 AU spare gas, 275 °C probe heater

temperature. Full-scan mass spectrometry data were acquired from 90 to 1100  $m/z$  with a nominal resolving power of 70,000 FWHM, AGC target of  $1e6$ , maximal injection time of 100 ms with 1 ppm mass accuracy. Data-dependent  $MS^2$  mode was acquired at a resolving power of 17,000 FWHM, AGC target of  $1e5$ , and maximal injection time of 50 ms. Data-dependent  $MS/MS$  acquisition was optimized for target compounds, and the applied collision energies for anatoxin-a and cylindrospermopsin were 10 eV and 25 eV, respectively. External calibration in the mobile phase was included in the analysis at 0.05–50  $\mu\text{g/L}$  (details in Additional file 1: Table S1).

### Identification and quantification

Compound Discoverer version 3.1.0.305 was used to process HPLC– $MS/MS$  data files. Feature detection (extracting mass-chromatographic peaks), grouping (grouping of features with correlated retention time profiles), deconvolution (assigning adduct and isotope annotations to grouped features), and compound annotation were effected using a customized non-targeted workflow based on CyanoMetDB (v01), a database of known cyanopeptides [5]. Compound annotations were assigned based on one or more of the following conditions: match occurred between a) an experimental  $MS^2$  spectrum and one or more mzCloud library spectrum/spectra; b) an experimental  $MS^2$  spectrum and mzVault library spectrum/spectra, c) base ion (i.e.  $[M+H]^+$  or  $[M+2H]^{+2}$ ) mass and one or more entries in the mass list, i.e., metabolites listed in CyanoMetDB or; d) predicted elemental composition and one or more formulae in the mass list. Next, the features (i.e., mass-chromatographic peaks of dominant adducts + H, + Na, +  $\text{NH}_4$ ) associated with each ‘compound’ annotation were inspected to ensure Gaussian-like peak integration and an isotopic ‘S-fit’ greater than 50%. Where no peak was identified, e.g., in the blank samples, the ‘Fill gaps’ workflow node was activated, retaining filled peak areas associated with ‘filled by redetected peak’ and ‘filled by matching ion’ flags. We used the confidence level scheme for mass spectrometry outlined by Schymanski et al. [34]. Herein, only those compounds that could be identified by one of the following criteria were reported: a cyanopeptide was defined as a *tentative candidate* (Level 3) based on exact mass (<5 ppm mass error), accurate isotopic pattern, and evidence from fragmentation data (which was evaluated manually); a cyanopeptide was defined as a *probable structure* (Level 2b) based on indicative fragmentation information supporting the connectivity of the building blocks of the peptide; and a cyanopeptide was defined as a *confirmed structure* (Level 1) when these parameters were in agreement with available reference standards or bioreagents.

For all reference standards and bioreagents, linear regression models of the calibration curves were determined. The limits of detection and quantification (LOQ, LOD) ranged in the high ng/L to low  $\mu\text{g/L}$  range, and were calculated from the regression models as three or ten times, respectively, the standard deviation of the response, divided by the slope parameter (Additional file 1: Table S1). For those compounds for which a reference standard or bioreagent was available, the concentrations were quantified using the data derived from matrix-matched external calibration. Since standard reference materials were not available for most cyanopeptides, we used the class-equivalent approach for quantification according to previous work [22]. In this class-equivalent approach, quantification of cyanopeptides is achieved using the regression models of the structurally most similar bioreagent or reference standard assigned for each compound (Additional file 1: Table S2). For those compounds for which no class equivalent could be assigned, the calibration parameters of MC-LR were used for quantification. Concentrations were only reported when the peak area was above the limits of quantification (LOQs) of the respective regression model. Recoveries from the sample preparation procedure were previously assessed for anatoxin-a, cylindrospermopsin, and several microcystins in the range of 50–87% [33]. The analysis sequence spanned 3 days, with a nanopure water-based calibration curve analysed midway through and a matrix-matched calibration curve analysed at the end of the sequence. Blank injections comprised nanopure water and were introduced after each set of triplicate for a given sample and 2–3 blanks after calibration curves. No retention time drift was observed across the sequence and matrices.

## Results and discussion

### Total metabolite concentrations

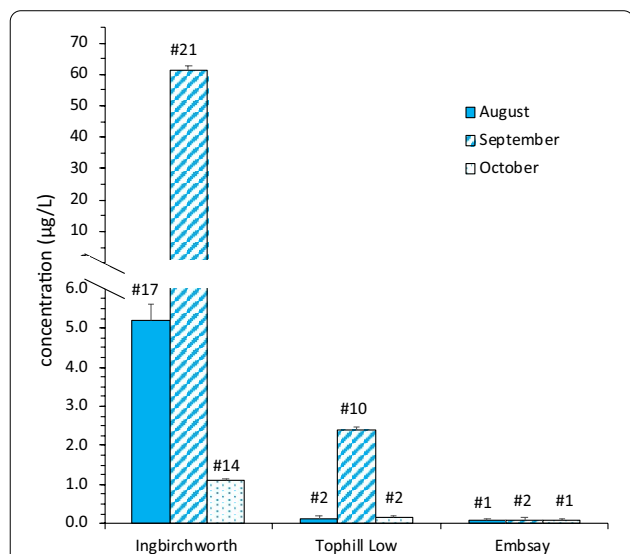
Water from three reservoirs was analysed for the presence of toxins and cyanopeptides. The analysed samples represent water-soluble concentrations after partial liberation of intracellular compounds (cell lysis during sonication) but no active extraction of the biomass by additional organic solvents. Overall, 8 cyanopeptides were identified by the comparison to their reference standards that were used for absolute quantification (Level 1, confirmed structures, Additional file 1: Figures S1–S8). A further 20 cyanobacterial metabolites were revealed through suspect-screening, with levels of confidence ranging from ‘tentatively identified’ to ‘probable structures’ based on the interpretation of the fragmentation spectra (Level 2–3, Additional file 1: Table S2, Figures S9–S11). Figure 1 summarises the total concentration and number of identified toxins

and cyanopeptides recorded for each individual reservoir and month. Overall, samples from Ingbirchworth reservoir had the highest concentration of cyanobacterial secondary metabolites, reaching  $5.2 \pm 0.4 \mu\text{g/L}$  in August,  $61.2 \pm 1.6 \mu\text{g/L}$  in September, and  $1.1 \pm 0.1 \mu\text{g/L}$  in October. Overall, 16 out of 21 metabolites showed the same concentration pattern, with lower concentrations occurring in August and October, and the highest concentration in September in Ingbirchworth samples. Among the compounds that did not follow this pattern was the target analyte anatoxin-a with overall lower concentrations in the ng/L range (26 ng/L in August, 12 ng/L in September, and 6 ng/L in October). Samples from Tophill Low reservoir also had the highest concentration of cyanobacterial metabolites during September, reaching  $2.4 \pm 0.1 \mu\text{g/L}$ . In August and October, concentrations were below  $0.2 \mu\text{g/L}$  for this reservoir. Only traces of single cyanobacterial metabolites were tentatively identified in samples from the Embsay reservoir with comparable concentrations across the sampling months ( $0.10 \pm 0.02 \mu\text{g/L}$ ).

**Diversity of metabolites**

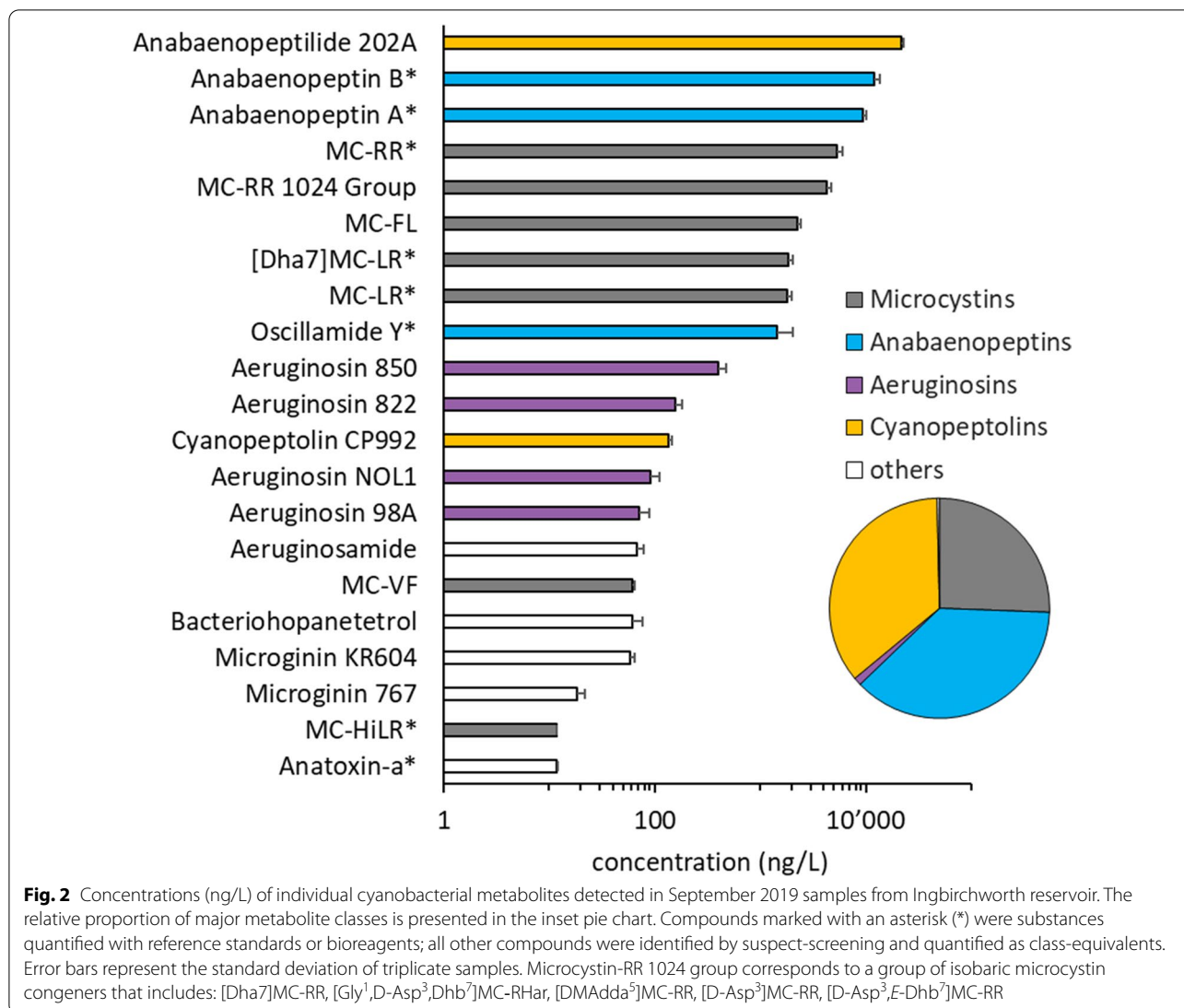
Compounds belonging to different cyanopeptide classes were identified, including microcystins, anabaenopeptins, aeruginosins, cyanopeptolins, microginins, and anatoxin-a. Figure 2 shows the concentrations of individual metabolites recorded in Ingbirchworth reservoir samples in

September, when the highest concentrations and greatest number of cyanobacterial metabolites were observed. We were able to quantify 59% of the total metabolite concentration through targeted analysis of microcystins and anabaenopeptins using available reference standards or bioreagents. Anabaenopeptins, cyanopeptolins, and microcystins were the three dominating classes representing 37%, 36%, and 26% of total number of identified compounds, respectively. Among the anabaenopeptins, the dominating compounds were anabaenopeptin B ( $12 \pm 2 \mu\text{g/L}$ ), anabaenopeptin A ( $9.3 \pm 0.7 \mu\text{g/L}$ ), and oscillamide Y ( $1.5 \pm 0.6 \mu\text{g/L}$ ), all of which were quantified with bioreagents. The cyanopeptolin anabaenopeptilide 202A (identification confidence level 3) was the most abundant compound with  $22 \pm 2 \mu\text{g/L}$ ; however, this compound was only quantified indirectly by the class-equivalent approach, because no bioreagent was available at the time of analysis. Among the detected microcystins, the most abundant were MC-RR, an MC-RR variant 1024 (group of isomeric microcystins, details see Additional file 1: Table S2), MC-LF or MC-FL, [Dha<sup>7</sup>]MC-LR, and MC-LR. The maximum MC-LR concentration recorded in Ingbirchworth reservoir was  $1.8 \pm 0.2 \mu\text{g/L}$ , while the maximum total microcystin concentration summing all variants was  $16 \pm 1 \mu\text{g/L}$ . Both of these values are below the water quality guideline value of  $20 \mu\text{g/L}$  suggested by the WHO for a moderate health alert in recreational waters [4]. Notably, individual anabaenopeptins occurred at slightly higher concentrations than the maximal total microcystins concentration. The risks posed by anabaenopeptins remains unclear. They are not classified as toxins, yet do have inhibitory effects on enzymes. Like some microcystin congeners, anabaenopeptins have been shown to inhibit protein phosphatases, albeit with lower potency [35]. Anabaenopeptins were also identified as potent inhibitors of carboxypeptidases and the concentrations recorded here ( $1.5\text{--}12 \mu\text{g/L}$  individual and  $>22 \mu\text{g/L}$  total), exceed the  $\text{IC}_{50}$  values for anabaenopeptin B ( $1 \mu\text{g/L}$ ) by tenfold ( $\text{IC}_{50}$  of  $371 \mu\text{g/L}$  for anabaenopeptin A) [36]. The only difference between these two anabaenopeptins is the C-terminal amino acid outside of the cyclic structure, where anabaenopeptin has an arginine while anabaenopeptin A has a tyrosine moiety. The dominating cyanopeptolin, anabaenopeptilide 202A, can be produced by genera *Anabaena*, and while toxicological studies have not been reported, other cyanopeptolins are known to inhibit proteases involved in metabolism and blood coagulation [26, 37].



**Fig. 1** Concentrations of cyanobacterial metabolites in  $\mu\text{g/L}$  of three UK water reservoirs sampled in August, September, and October 2019. # at the top of each bar denotes the number of individual compounds identified. Error bars represent the standard deviation of triplicate analysis except for duplicates in September at Ingbirchworth

Figure 3 shows the concentration of individual metabolites for the September sample from Tophill Low reservoir, when, analogous to Ingbirchworth, the highest concentrations and greatest number of cyanobacterial metabolites were observed. Anabaenopeptins accounted

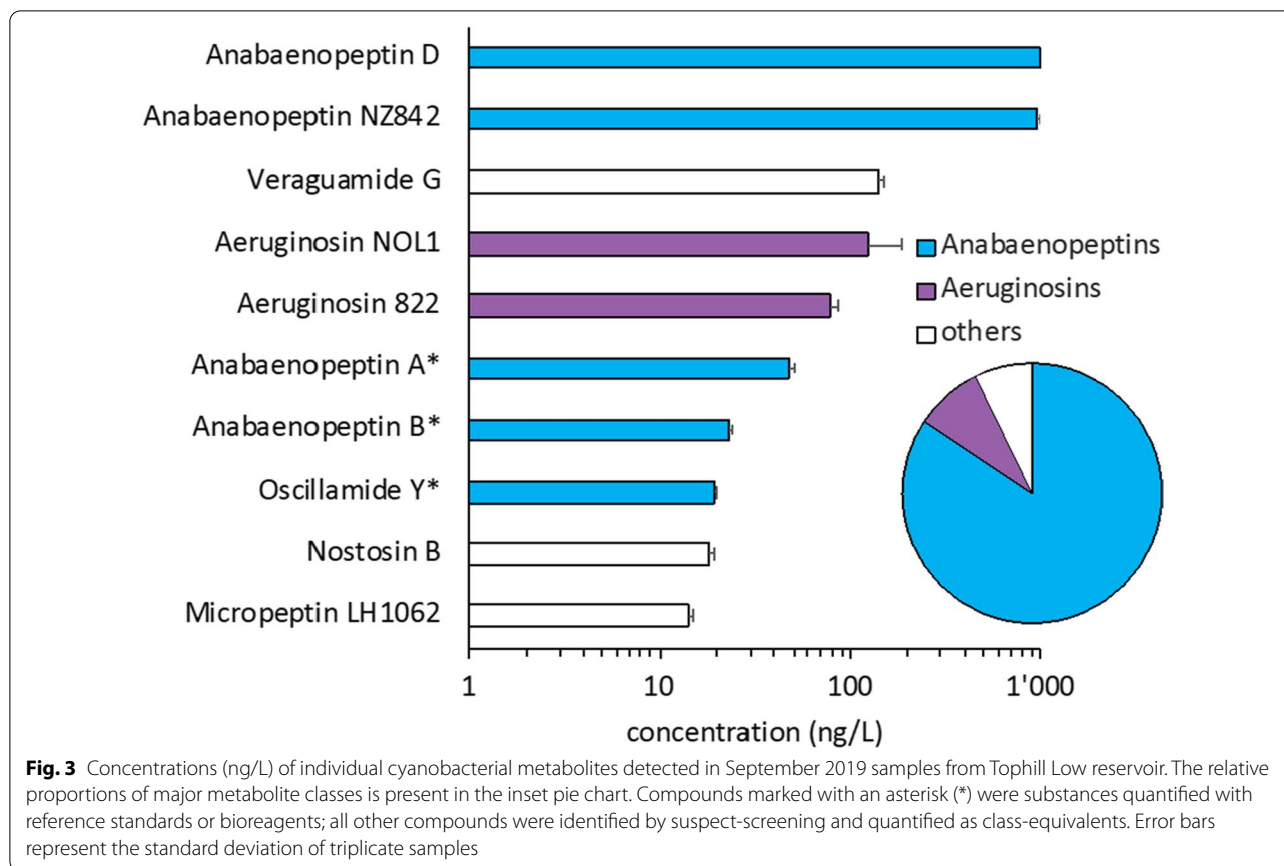


for 84% or  $2 \pm 0.05 \mu\text{g/L}$  of the total concentration, with anabaenopeptin D and anabaenopeptin NZ842 being the most abundant of the annotated anabaenopeptins. Anabaenopeptin A, anabaenopeptin B, and oscillamide Y accounted for only 4% of the total concentration. Compared to Ingbirchworth ( $23 \pm 2 \mu\text{g/L}$ ), the anabaenopeptin concentrations were one order of magnitude lower at Tophill Low, yet still in the range reported to have inhibitory effects, as discussed above. Aeruginosins accounted for 8% or  $0.20 \pm 0.06 \mu\text{g/L}$  at Tophill Low. Thus far, aeruginosins have only been shown to induce toxic effects at high (mg/L) concentrations for *T. platyurus* ( $LC_{50}$  values of 10–41 mg/L) [38]. Aeruginosins also inhibit human serine proteases involved in blood coagulation at  $IC_{50}$  values ranging from 4 to 93  $\mu\text{g/L}$  [39, 40]. No microcystins were detected in samples from Tophill Low reservoir.

Cylindrospermopsin was not identified in any reservoir sample.

#### Seasonal trends of chlorophyll-a and cyanobacterial cell counts

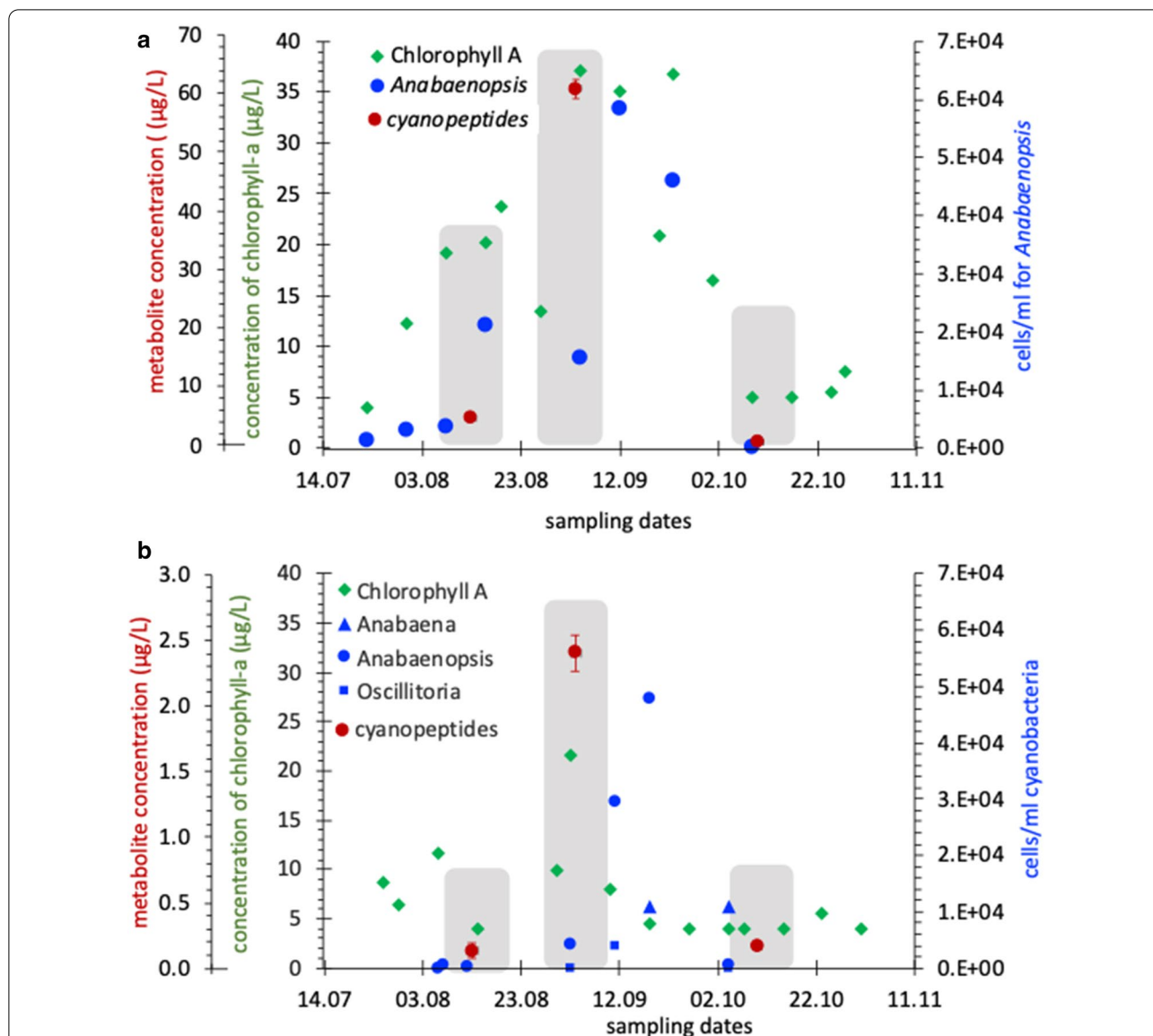
Cyanobacteria are known to be present in the reservoirs analysed herein, which is supported by the identification of cyanotoxins and other cyanobacterial metabolites discussed above. Ingbirchworth reservoir has experienced periphyton blooms in recent years, which may be related to increased farming activity in the catchment area. Authorities monitor water quality and cyanobacteria in these reservoirs to manage possible taste and odour development and to prevent issues related to cyanotoxins. Figure 4 summarises the chlorophyll-a concentrations and cyanobacterial cell counts recorded at Ingbirchworth and Tophill Low, between July and



October 2019. In both reservoirs, the maximum chlorophyll-a concentrations of 37 µg/L and 22 µg/L, respectively, coincided with the maximum concentration of cyanopeptides, which occurred during September 2019. Both values exceed the WHO guideline level for relatively low probability of adverse health effects, which is currently defined as 10 µg/L chlorophyll-a [4]. For Ingbirchworth and Tophill Low reservoirs, this was the case most of the year, for 9 and 6 months in 2019, respectively (Additional file 1: Tables S3, S4). While a general trend of chlorophyll-a concentration with cyanobacterial abundance is expected [41–43], an absolute relationship across water bodies cannot be inferred. Based on the samples evaluated in this study, the peak cyanopeptide concentration at Ingbirchworth was almost 30-fold higher compared to Tophill Low, while the maximum chlorophyll-a concentrations were more comparable. The cyanobacterial cell counts peaked in both reservoirs about 2 weeks after the peak cyanopeptide concentration, reaching  $6 \times 10^4$  cells/mL with being *Anabaenopsis* the most abundant genus. Similar to the chlorophyll-a concentrations, the relationship of absolute cyanopeptide concentrations with cyanobacterial cell count differs between reservoirs. Compared to other bloom-forming

cyanobacteria, such as *Microcystis*, *Dolichospermum/Anabaena*, and *Oscillatoria/Planktothrix*, less information is available about secondary metabolites produced by *Anabaenopsis*, though it has been reported that they produce microcystins [44, 45]. At Tophill Low, *Anabaena* ( $1.1 \times 10^4$  cells/mL) and *Oscillatoria* ( $0.4 \times 10^4$  cells/mL) also contributed to the cyanobacterial abundance in September. At Ingbirchworth, meanwhile, only a low co-abundance of *Microcystis* ( $0.09 \times 10^4$  cells/mL) and *Snowella* ( $0.05 \times 10^4$  cells/mL) was detected alongside the dominant *Anabaenopsis* ( $5.8 \times 10^4$  cells/mL). Despite *Anabaenopsis* being the major cyanobacterial genus at both Ingbirchworth and Tophill Low, the cyanopeptide profiles of these two reservoirs were not identical. Both had high concentrations of anabaenopeptin A, anabaenopeptin B, oscillamide Y, and aeruginosin 822, though only Ingbirchworth samples were rich in the cyanopeptolin anabaenopeptilide 202A as well as various microcystins. Tophill Low samples contained no detectable microcystins, though did contain additional anabaenopeptins and aeruginosins. This discrepancy suggests that other cyanobacterial species may have contributed to the observed metabolite profiles, and/or that the production dynamics of metabolites from *Anabaenopsis* differed between





**Fig. 4** Chlorophyll-a concentrations and cyanobacteria cell counts (secondary y-axis) measured in **a** Ingbirchworth and **b** Tophill Low reservoir samples. Total cyanopeptide concentrations are plotted for comparison (additional y-axis on the left). Shaded areas highlight the biological data closest to the cyanopeptide data sampled on August 13th, September 3rd, and October 10th 2019

reservoirs. No cyanobacteria were identified in Emsay reservoir, which agrees with only traces of cyanotoxins detected herein. We also did not observe a correlation of total ammonium, nitrate, total phosphate, and temperature with cyanobacterial abundance or toxin concentrations in Ingbirchworth or Tophill Low reservoirs (data in Additional file 1: Tables S3, S4).

**Conclusions**

In this study, 28 cyanopeptides were identified in three freshwater reservoirs in the U.K, including microcystins, anabaenopeptins, aeruginosins, cyanopeptolins,

microginins, and anatoxin-a. Of these, 8 were directly quantified using reference standards or bioreagents, while the remaining 20 cyanopeptides were identified by suspect-screening and quantified using a class-equivalents approach. Total concentrations of microcystins did not exceed WHO guideline level of relatively low probability of adverse health effects in recreational water. However, levels of chlorophyll-a and cyanobacterial cell count were higher than the guideline values. These two parameters had a similar pattern as cyanobacterial metabolites (peaking in September).

There is currently a sparsity of data addressing the occurrence and concentrations of microcystins and anatoxin-a in U.K. water bodies. To the best of our knowledge, this is the first time that anabaenopeptins, cyanopeptolins, aeruginosins, and microginins have been quantified in U.K. waters along with microcystins and anatoxin-a. Globally, there is a knowledge gap regarding entry of these cyanopeptides into drinking water treatment plants and the effectiveness of their abatement during water treatment, along with cyanobacterial cells and other known toxins. A better understanding of those cyanopeptides that are abundant in drinking water reservoirs would help to guide monitoring strategies. Furthermore, abundant cyanopeptides should be prioritized to study their abatement during water treatment.

Herein, we successfully selected three sampling dates that captured a summer peak of toxin concentrations at the intake to the drinking water treatment plants of two reservoirs. However, to improve the understanding of seasonal variation of cyanotoxins in these reservoirs, a more-detailed temporal resolution is desired, for example with weekly frequency. As cell count and particularly toxin analysis are resource intensive, monitoring can switch to increase sampling and analysis for cyanobacterial toxins once the WHO guidelines' value of 10 µg/L is reached. For Ingbirchworth and Tophill Low reservoirs, this was the case most of the year, for 9 and 6 months in 2019, respectively (Additional file 1: Tables S3, S4). With limited resources, increased toxin analysis may be at least considered during the summer/fall peak period.

#### Abbreviations

U.K.: United Kingdom; WHO: World Health Organization; MC: Microcystin; HPLC–HRMS/MS: High-performance liquid chromatography coupled with high-resolution tandem mass spectrometry.

#### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12302-021-00472-4>.

#### Additional file 1.

#### Acknowledgements

We thank Regiane Natumi for discussion and advice on the mass spectrometry identification of cyanopeptides; Catherine Keedy and Gemma Nicholson of Yorkshire Water for organising sampling and provision of routine water quality data.

#### Authors' contributions

DF: conceptualization, investigation, experimental analysis, data evaluation and visualization, writing (original draft), and editing. MJ: experimental analysis, data evaluation, reviewing, and editing writing (final draft). JH: sampling, sample analysis for biological endpoints, reviewing, and editing writing (final draft). MF: reviewing (final draft); ON: reviewing and editing writing (final draft); EJ: supervision, conceptualization, data evaluation, reviewing, and editing writing. All authors read and approved the final manuscript.

#### Funding

This study has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant Agreement No. 722493.

#### Availability of data and materials

The datasets obtained and analysed in the current study are available from the corresponding author on reasonable request.

#### Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interest.

#### Author details

<sup>1</sup> Department of Environmental Chemistry, IDAEA-CSIC, 08034 Barcelona, Spain. <sup>2</sup> Department of Environmental Chemistry, Swiss Federal Institute of Aquatic Science and Technology (Eawag), 8600 Dübendorf, Switzerland. <sup>3</sup> Water Quality Strategy, Yorkshire Water, Leeds, UK. <sup>4</sup> Department of Chemical Engineering and Analytical Chemistry, University of Barcelona, 08028 Barcelona, Spain. <sup>5</sup> Serra Hünter Professor, Generalitat de Catalunya, 08007 Barcelona, Spain.

Received: 17 December 2020 Accepted: 26 February 2021

Published online: 09 March 2021

#### References

- Chorus I, Bartram J (1999) Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management / edited by Ingrid Chorus and Jamie Bertram. World Health Organization, Geneva
- Mantzouki E, Visser PM, Bormans M, Ibelings BW (2016) Understanding the key ecological traits of cyanobacteria as a basis for their management and control in changing lakes. *Aquat Ecol* 50(3):333–350
- Dokulil MT, Teubner K (2000) Cyanobacterial dominance in lakes. *Hydrobiologia* 438(1):1–12
- World Health, O., *Guidelines for safe recreational water environments. Volume 1, Coastal and fresh waters*. 2003, World Health Organization: Geneva.
- Jones MR, Pinto E, Torres MA, Dörr F, Mazur-Marzec H, Szubert K, Tartaglione L, Dell'Aversano C, Miles CO, Beach DG, McCarron P, Sivonen K, Fewer DP, Jokela J, Janssen EML (2020) Comprehensive database of secondary metabolites from cyanobacteria. *bioRxiv* 78:7
- Fawell JK, Mitchell Re FauHill RE, Hill Re Fau – Everett DJ, Everett DJ. The toxicity of cyanobacterial toxins in the mouse: II anatoxin-a. (0960–3271 (Print))
- Puschner B, Humbert J-F (2007) CHAPTER 59 - Cyanobacterial (blue-green algae) toxins. In: Gupta RC (ed) *Veterinary Toxicology*. Academic Press, Oxford, pp 714–724
- Sivonen K (2009) Cyanobacterial Toxins. In: Schaechter M (ed) *Encyclopedia of Microbiology* (Third Edition). Academic Press, Oxford, pp 290–307
- Rzymiski P, Poniedziałek B (2014) In search of environmental role of cylindrospermopsin: A review on global distribution and ecology of its producers. *Water Res* 66:320–337
- Picardo M, Filatova D, Nuñez O, Farré M (2019) Recent advances in the detection of natural toxins in freshwater environments. *TrAC, Trends Anal Chem* 112:75–86
- Ibelings BW, Backer LC, Kardinaal WEA, Chorus I (2015) Current approaches to cyanotoxin risk assessment and risk management around the globe. *Harmful algae* 49:63–74
- WHO (2020) Background document for development of WHO Guidelines for drinking-water quality and Guidelines for safe recreational water environments. Cyanobacterial toxins: microcystins. World Health Organization, Geneva. <https://apps.who.int/iris/handle/10665/338066>

13. WHO (2020) Background document for development of WHO Guidelines for drinking-water quality and Guidelines for safe recreational water environments. Cyanobacterial toxins: cylindrospermopsins. World Health Organization, Geneva. <https://apps.who.int/iris/handle/10665/338063>
14. WHO (2020) Background document for development of WHO Guidelines for drinking-water quality and Guidelines for safe recreational water environments. Cyanobacterial toxins: anatoxin-a and analogues. World Health Organization, Geneva. <https://apps.who.int/iris/handle/10665/338060>
15. WHO (2020) Background document for development of WHO Guidelines for drinking-water quality and Guidelines for safe recreational water environments. Cyanobacterial toxins: saxitoxins. World Health Organization, Geneva. <https://apps.who.int/iris/handle/10665/338069>
16. Filatova D, Picardo M, Núñez O, Farré M (2020) Analysis, levels and seasonal variation of cyanotoxins in freshwater ecosystems. *Trends Environ Anal Chem* 26:e00091
17. Hartnell DM, Chapman IJ, Taylor NGH, Esteban GF, Turner AD, Franklin DJ (2020) Cyanobacterial abundance and microcystin profiles in two southern british lakes: the importance of abiotic and biotic interactions. *Toxins* 12(8):503
18. Tyler AN, Hunter PD, Carvalho L, Codd GA, Elliott JA, Ferguson CA, Hanley ND, Hopkins DW, Maberly SC, Mearns KJ, Scott EM (2009) Strategies for monitoring and managing mass populations of toxic cyanobacteria in recreational waters: a multi-interdisciplinary approach. *Environ Health* 8(1):12
19. Turner AD, Dhanji-Rapkova M, O'Neill A, Coates L, Lewis A, Lewis K (2018) Analysis of Microcystins in Cyanobacterial Blooms from Freshwater Bodies in England. *Toxins* 10(1):39
20. James KJ, Sherlock IR, Stack MA (1997) Anatoxin-a in Irish freshwater and cyanobacteria, determined using a new fluorimetric liquid chromatographic method. *Toxicol* 35(6):963–971
21. Pawlik-Skowrońska B, Toporowska M, Mazur-Marzec H (2019) Effects of secondary metabolites produced by different cyanobacterial populations on the freshwater zooplankters *Brachionus calyciflorus* and *Daphnia pulex*. *Environ Sci Pollut Res* 26(12):11793–11804
22. Natumi R, Janssen EML (2020) Cyanopeptide co-production dynamics beyond microcystins and effects of growth stages and nutrient availability. *Environ Sci Technol* 54(10):6063–6072
23. Tonk L, Welker M, Huisman J, Visser PM (2009) Production of cyanopeptolins, anabaenopeptins, and microcystins by the harmful cyanobacteria *Anabaena* 90 and *Microcystis* PCC 7806. *Harmful Algae* 8(2):219–224
24. Zafirir-Ilan E, Carmeli S (2010) Eight novel serine proteases inhibitors from a water bloom of the cyanobacterium *Microcystis* sp. *Tetrahedron* 66(47):9194–9202
25. Elkobi-Peer S, Carmeli S (2015) New prenylated aeruginosin, microphycin, anabaenopeptin and micropeptin analogues from a *Microcystis* bloom material collected in Kibbutz Kfar Blum Israel. *Mar Drugs* 13(4):2347–2375
26. Janssen EML (2019) Cyanobacterial peptides beyond microcystins – A review on co-occurrence, toxicity, and challenges for risk assessment. *Water Res* 151:488–499
27. Beversdorf LJ, Weirich CA, Bartlett SL, Miller TR (2017) Variable Cyanobacterial Toxin and Metabolite Profiles across Six Eutrophic Lakes of Differing Physiochemical Characteristics. *Toxins* 9(2):62
28. Beversdorf LJ, Rude K, Weirich CA, Bartlett SL, Seaman M, Kozik C, Biese P, Gosz T, Suha M, Stempa C, Shaw C, Hedman C, Piatt JJ, Miller TR (2018) Analysis of cyanobacterial metabolites in surface and raw drinking waters reveals more than microcystin. *Water Res* 140:280–290
29. Ferranti P, Fabbrocino S, Chiaravalle E, Bruno M, Basile A, Serpe L, Gallo P (2013) Profiling microcystin contamination in a water reservoir by MALDI-TOF and liquid chromatography coupled to Q/TOF tandem mass spectrometry. *Food Res Int* 54(1):1321–1330
30. Gkelis S, Lanaras T, Sivonen K (2015) Cyanobacterial Toxic and Bioactive Peptides in Freshwater Bodies of Greece: Concentrations, Occurrence Patterns, and Implications for Human Health. *Mar Drugs* 13:10
31. Grabowska M, Kobos J, Toruńska-Sitarz A, Mazur-Marzec H (2014) Non-ribosomal peptides produced by *Planktothrix agardhii* from Siemianówka Dam Reservoir SDR (northeast Poland). *Arch Microbiol* 196(10):697–707
32. Roy-Lachapelle A, Duy S, Munoz G, Dinh QT, Bahl E, Simon D, Sauvé S (2019) Analysis of multiclass cyanotoxins (microcystins, anabaenopeptins, cylindrospermopsin and anatoxins) in lake waters using on-line SPE liquid chromatography high-resolution Orbitrap mass spectrometry. *Anal Methods* 56:9
33. Filatova D, Núñez O, Farré M (2020) Ultra-Trace Analysis of Cyanotoxins by Liquid Chromatography Coupled to High-Resolution Mass Spectrometry. *Toxins* 12:4
34. Schymanski EL, Jeon J, Gulde R, Fenner K, Ruff M, Singer HP, Hollender J (2014) Identifying small molecules via high resolution mass spectrometry: communicating confidence. *Environ Sci Technol* 48(4):2097–2098
35. Sano T, Usui T, Ueda K, Osada H, Kaya K (2001) Isolation of New Protein Phosphatase Inhibitors from Two Cyanobacteria Species, *Planktothrix* spp. *J Nat Prod* 64(8):1052–1055
36. Schreuder H, Liesum A, Lonze P, Stump H, Hoffmann H, Schiell M, Kurz M, Toti L, Bauer A, Kallus C, Klemke-Jahn C, Czech J, Kramer D, Enke H, Niedermeyer THJ, Morrison V, Kumar V, Bronstrup M (2016) Isolation, Co-Crystallization and Structure-Based Characterization of Anabaenopeptins as Highly Potent Inhibitors of Activated Thrombin Activatable Fibrinolysis Inhibitor (TAFI). *Scientific Reports* 6:8
37. Fujii K, Sivonen K, Nakano T, Harada K (2002) Structural elucidation of cyanobacterial peptides encoded by peptide synthetase gene in *Anabaena* species. *Tetrahedron* 58(34):6863–6871
38. Scherer M, Bezold D, Gademann K (2016) Investigating the toxicity of the aeruginosin chlorosulfopeptides by chemical synthesis. *Angew Chem Int Ed* 55(32):9427–9431
39. Hanessian S, Del Valle JR, Xue Y, Blomberg N (2006) Total synthesis and structural confirmation of chlorodysynosin A. *J Am Chem Soc* 128(32):10491–10495
40. Kohler E, Grundler V, Haussinger D, Kurmayer R, Gademann K, Perntaler J, Blom JF (2014) The toxicity and enzyme activity of a chlorine and sulfate containing aeruginosin isolated from a non-microcystin-producing *Planktothrix* strain. *Harmful Algae* 39:154–160
41. Desortová B (1981) Relationship between Chlorophyll-a concentration and phytoplankton biomass in several reservoirs in czechoslovakia. *Int Revue Gesamten Hydrobiologie Hydrographie* 66:153–169
42. Kotak BG, Lam AKY, Prepas EE, Kenefick SL, Hrudey SE (1995) Variability of the hepatotoxin microcystin-Lr in hypereutrophic drinking water lakes I. *J Phycol* 31(2):248–263
43. Rantala A, Rajaniemi-Wacklin P, Lyra C, Lepistö L, Rintala J, Mankiewicz-Boczek J, Sivonen K (2006) Detection of Microcystin-Producing Cyanobacteria in Finnish Lakes with Genus-Specific Microcystin Synthetase Gene E (<em>mcyE</em>) PCR and Associations with Environmental Factors. *Appl Environ Microbiol* 72(9):6101
44. Lanaras T, Cook CM (1994) Toxin extraction from an *Anabaenopsis milleri* — dominated bloom. *Sci Total Environ* 142(3):163–169
45. Mohamed ZA, Al-Shehri AM (2009) Microcystin-producing blooms of *Anabaenopsis arnoldi* in a potable mountain lake in Saudi Arabia: Research article. *FEMS Microbiol Ecol* 69(1):98–105

## Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.