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RESEARCH ARTICLE

Trophic state and geographic gradients influence planktonic cyanobacterial diversity and distribution in New Zealand lakes

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One sentence summary: Molecular analysis of samples from 143 New Zealand lakes indicated that latitude, longitude and altitude were significant in driving cyanobacterial community structure; however, their effects varied among trophic categories.

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ABSTRACT

Cyanobacteria are commonly associated with eutrophic lakes, where they often form blooms and produce toxins. However, they are a ubiquitous component of phytoplankton in lakes of widely varying trophic status. We hypothesised that cyanobacterial diversity would vary among lakes of differing trophic status, but that the relative importance of geographical and hydromorphological characteristics driving these patterns would differ across trophic groups. DNA from 143 New Zealand lakes that spanned a range of geographic, hydromorphological and trophic gradients was analysed using automated rRNA intergenic spacer analysis and screened for genes involved in cyanotoxin production. Statistical analysis revealed significant delineation among cyanobacterial communities from different trophic classes. Multivariate regression indicated that geographical features (latitude, longitude and altitude) were significant in driving cyanobacterial community structure; however, partitioning of their effects varied among trophic categories. High-throughput sequencing was undertaken on selected samples to investigate their taxonomic composition. The most abundant and diverse (71 operational taxonomic units) taxon across all lake types was the picocyanobacteria genus *Synechococcus*. Cyanotoxins (microcystins n = 23, anatoxins n = 1) were only detected in eutrophic lowland lakes. Collectively, these data infer that increasing eutrophication of lakes will have broad-scale impacts on planktonic cyanobacteria diversity and the prevalence of cyanotoxins.

Keywords: altitude; anatoxin; biodiversity gradient; cyanotoxins; high-throughput sequencing; latitude; longitude; liquid chromatography-mass spectrometry; microcystin; operational taxonomic unit; saxitoxin

INTRODUCTION

Cyanobacteria are photosynthetic prokaryotes that are common in almost all aquatic ecosystems. In these habitats, they can play a major role in nitrogen, carbon and oxygen dynamics, and form the base of food webs (Whitton 2012). In the pelagic zone of lakes, physiological adaptations, such as the ability to store essential nutrients and metabolites (Fay and Van Baalen 1987), fix dinitrogen gas (Stal 2008) and adjust their vertical position in the water column (Walsby 1994), have facilitated bloom formation by some species. Cyanobacterial blooms are usually associated with highly eutrophic lakes and are being reported with increasing frequency globally (Paerl and Huisman 2008; Harke *et al.* 2016). While cyanobacteria are most conspicuous in eutrophic lakes, particularly when they form blooms, they are present in lakes that span a wide range of water qualities. For example, picocyanobacteria (<3 μm in diameter) can contribute up to 80% of the primary production in oligotrophic lakes (Stockner, Callieri and Cronberg 2000). Their surface area to volume ratios and ability to hydrolyse dissolved organic phosphorus make them very efficient at nutrient uptake in these environments (Moore *et al.* 2005).

Few studies have investigated the spatial distributions of planktonic cyanobacteria over latitudinal, longitudinal and altitudinal gradients encompassing lakes of different trophic status. Most have focused on total biomass rather than taxa-resolved relationships (Catherine, Troussellier and Bernard 2008; Taranu *et al.* 2012). Traditionally, studies on pelagic cyanobacteria have used microscopy or flow cytometry (for picocyanobacteria) to assess diversity and abundance (e.g. Reynolds and Petersen 2000). These methods can be time consuming and therefore limit large-scale assessments. Touzet, McCarthy and Fleming (2013) used a molecular fingerprinting method (16S rRNA denaturing gradient gel electrophoresis, DGGE) to undertake a national scale survey of Irish lakes. They identified distinct distribution patterns, with filamentous cyanobacteria genotypes occurring in larger lakes where the catchment land use was predominantly forest cover. In contrast, lakes with a low trophic state contained a higher diversity of Chroococcales. Although DGGE enables some sequence-based taxonomy, its resolution is limited. The recent development of high-throughput sequencing (HTS) technologies has greatly advanced sequence-based characterisation of diverse natural microbial communities (e.g. Eiler *et al.* 2013; Wood *et al.* 2013; Brasell *et al.* 2015, Cahill *et al.* 2016). It affords many advantages over morphological or other genetic-based identification methods, including little reliance on taxonomic expertise, the ability to analyse hundreds of samples in a single run, standardised sample preparation/analysis that is well suited to automation, the ability to distinguish species such as picocyanobacteria that cannot be identified based on morphology and the potential to reanalyse data sets as taxonomic reference databases are extended or refined.

Cyanobacteria in lakes are the subject of intense interest, not only because of the water quality issues that cyanobacterial blooms create across the globe, but also because of their ability to produce natural toxins (cyanotoxins). These cyanotoxins have a range of modes of actions including neurotoxicity (anatoxins and saxitoxins), hepatotoxicity (microcystins, nodularins and cylindrospermopsins) and dermatoxicity (lipopolysaccharides and aplysiatoxins). As such, they pose chronic and acute health risks to humans and animals when contaminated water is used for recreational activities or as a drinking supply (Codd, Morrison and Metcalf 2005; Kouzminov, Ruck and Wood 2007). Previous field studies have highlighted strong relationships between trophic status and the presence/concentration of cyanotoxins (in particular microcystins; Mariani *et al.* 2015). These relationships are also apparent for nutrient concentrations (Horst *et al.* 2014; Sinang, Reichwaldt and Ghadouani 2015). Most of these studies have only targeted eutrophic water bodies or those experiencing conspicuous blooms, and knowledge of toxin distribution across lakes of widely varying trophic status is limited.

The island nation of New Zealand provides a unique opportunity to evaluate the effect of different factors on cyanobacterial diversity and toxin distribution, as there are many lakes of varying trophic status across a latitudinal gradient of 12° (between 35° and 47° south) and an altitudinal range of ~2000 m. In this study, we collected samples from 143 New Zealand lakes that spanned a range of geographical (altitude, longitudinal and latitude), hydromorphological (area and depth) and trophic gradients. Cyanobacterial diversity was assessed using a combined DNA fingerprinting and HTS approach, and cyanotoxins were analysed using a two-tiered approach involving PCR and chemical analysis. We hypothesised that (1) cyanobacterial diversity would shift with lake trophic status and that the relative importance of geographical and hydromorphological parameters driving these patterns would differ, and (2) that the presence and concentrations of cyanotoxins would be inherently linked to species composition and abundance.

MATERIALS AND METHODS

Study sites and sample collection

Water samples (n = 189) were collected from 143 lakes across New Zealand between 11 December 2012 and 27 April 2013 (Fig. 1). Samples were collected at either the edge or mid-lake from the water surface or depth integrated over the top 0.3 m. All samples were placed in sterile bottles (400 mL) without biomass concentration, stored chilled and in the dark, and were received at the laboratory within 48 h of collection. Samples were well mixed before two subsamples were taken: (1) 50 to 100 mL (in triplicate) was filtered on to GF/C glass microfibre filters (Whatman, Kent, UK) and stored frozen (–20°C) for DNA extraction, and (2) 45 mL was frozen (–20°C) for cyanotoxin analysis. Some picocyanobacteria may be lost when using the GF/C glass fibre filters. In the future, we recommend the use of a sequential filtering system to ensure that all picocyanobacteria are retained. The remaining sample was stored at 18 ± 1°C and 42 ± 7 μmol photons m^{-2} s^{-1} for strain isolation (data not presented).

Data on lake trophic status (i.e. eutrophic, mesotrophic or oligotrophic), maximum depth, area, altitude and geographic location were obtained from a number of sources: Livingston, Biggs and Gifford (1986a,b); Verburg *et al.* (2010); Wood *et al.* (2010); Perrie and Milne (2012); Schallenberg and Kelly (2013); Kelly, Robertson and Allen (2014); Duggan, Wood and West (2015) and various unpublished resources held at Regional Councils. For a small number of lakes where surface areas data were missing, estimates were made using Google Earth.

Molecular and bioinformatic analysis

DNA was extracted from one of the triplicate GF/C filters from each lake using a PowerBiofilm® DNA Isolation Kit (MO BIO Laboratories, Inc., California, USA) according to the manufacturer's protocol. Automatic ribosomal intergenic spacer analysis (ARISA) of PCR products obtained with cyanobacteria-specific primers CY-ARISA-F and 23S30R was undertaken as described previously (Wood *et al.* 2008b), with the only modification that

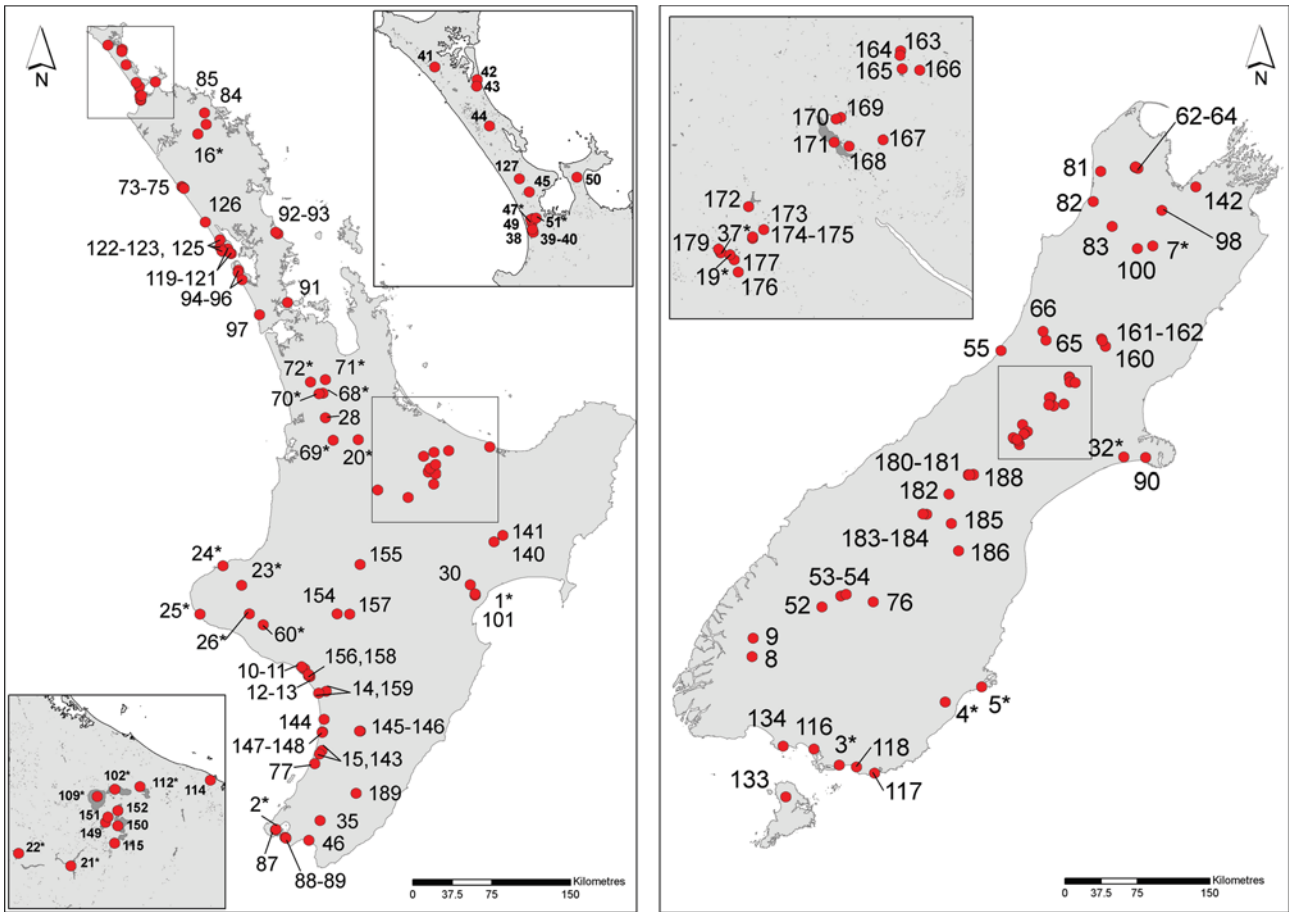

Figure 1. Locations of sampling sites (left North Island, right South and Stewart Island). Lake names and other sampling information are provided in Table S1. * = sampled on multiple dates. Boxes on primary figure are expanded in corner.

amplified products were diluted (1 in 20) with Milli-Q water. This PCR-based method exploits the length heterogeneity of the intergenic spacer region between the 16S and 23S ribosomal RNA (rRNA) genes. Total community DNA was amplified with a fluorescently labelled forward oligonucleotide, allowing the electrophoretic step to be performed with an automated system in which a laser detects the fluorescent PCR fragments. Amplicon lengths were resolved on an ABI 3130-xI sequencer (PE Applied Biosystems, Foster City, USA) and run under GeneScan mode at 15 kV for 45 min according to the manufacturer's manual. Each sample contained 0.25 μL of internal GS1200LIZ ZyStandard (PE Applied Biosystems) to determine the size of fluorescently labelled fragments. Electropherograms were processed using the PeakScanner Software v1.0 (Applied Biosystems). Fluorescence threshold was set to 50 relative fluorescence units, and ARISA fragment lengths (AFLs) ranging between 300 and 1200 base pairs (bp) were used for analysis. AFLs were binned to the nearest one base pair and data transformed to presence/absence.

The taxonomic composition of a selection of samples was assessed using HTS. We aimed to select samples that varied in taxonomic composition. The samples were selected based on the ARISA data. Samples were grouped together if they shared greater than 60% similarity (based on CLUSTER analysis using Bray–Curtis similarities (PRIMER 6 software package; PRIMER-E Ltd. Plymouth, UK)). Among each group, the sample with the highest number of AFLs was selected for HTS (49 samples; Table S1, Supporting Information). A region of the 16S rRNA gene was amplified by PCR (iCycler; Biorad, Hercules, USA) using cyanobacteria-specific primers MiCyanoF1 (5′-GTGCCAGCAGCCGCGGTAAK-3′; Giménez Papiol and Wood, unpublished) and 783R (5′- GACTACWGGGGTATCTAATCC-3′; Janse *et al.* 2003) modified with an Illumina adapter. PCR reactions were performed in 50 μL volumes with the reaction mixture containing 45 μL of Platinum® PCR SuperMix High Fidelity (Invitrogen, CA, USA), 10 μM of each primer and 10–20 ng of template DNA. The reaction mixture was held at 94°C for 2 min followed by 27 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, with a final extension step at 72°C for 5 min. Amplicons of the correct size were purified using Agencourt® AMPure® XP PCR purification beads (Beckman Coulter, IN, USA) following the manufacturer's instructions. Purified products were quantified using a Qubit (Invitrogen) diluted to 1 ng μL^{-1} using Milli-Q water and sent to New Zealand Genomics Ltd (Auckland, New Zealand) for library preparation. The libraries were sequenced on a MiSeq Illumina platform using a 2 × 250 bp paired-end protocol.

The Illumina data sets were demultiplexed using MiSeq Reporter v2.0. All further analyses were performed using MOTHUR (Schloss *et al.* 2009). The sequences corresponding to the forward and reverse primers were trimmed and merged into single contigs (max. length 292 nucleotides). Contigs were aligned to the SILVA bacteria reference database (Pruesse *et al.* 2007) and chimera removal performed using the UCHIME algorithm (Edgar *et al.* 2011). To reduce computational analysis time, the sequences were first grouped at the order level before

operational taxonomic units (OTUs) were created independently in each of the corresponding bins using a 0.02 pairwise sequence distance cut-off value. OTUs represented by less than 10 reads across all samples were removed. Sequences of unknown, archaeal or eukaryotic origin were removed. OTUs were then classified using Greengenes to identify taxonomic annotation (DeSantis *et al.* 2006; McDonald *et al.* 2011). Despite the utilisation of cyanobacteria-specific primers, non-cyanobacterial 16S rRNA genes were also retrieved in the sequencing. All OTUs not belonging to the phylum 'Cyanobacteria' along with those classified as 'Chloroplast' at class level were removed from all further analyses. Raw sequences were submitted to the National Center for Biotechnology Information Sequence Read Archive under the accession number PRJNA347490.

Statistical analysis

The majority of the multivariate statistical analyses were undertaken using the ARISA data set as this contained 158 samples compared with only 46 for the HTS analysis. Using the ARISA data, canonical discriminant analysis of principal coordinates (CAP; PRIMER 7) with permutation test (9999 permutations) was applied to identify differences in cyanobacterial community structure between eutrophic, mesotrophic and oligotrophic lakes. The Jaccard index was used for similarity matrix construction based on AFL data (presence/absence transformed). The results were visualised by 2D CAP ordination plot.

Using HTS OTU data that had been classified to the lowest possible taxonomic assignment (standardised by total number of sequences per sample), distribution patterns among the 11 most important taxa were visualised using a shade plot. Because of the high abundance and diversity (71 distinct OTUs) within the genus *Synechococcus*, a shade plot was also used to visualise differences within this taxon among trophic categories.

The relationship between cyanobacterial community structure and geographical or hydromorphological characteristics (altitude, depth, area, latitude and longitude) was analysed using a distance-based linear model (DistLM) implemented in PRIMER 7 (McArdle and Anderson 2001; Anderson and Gorley 2007). Given that CAP analysis showed distinct groups of cyanobacterial communities among different lake trophic categories, the DistLM on the ARISA data set (using presence/absence data and a Jaccard index similarity matrix) was undertaken separately for eutrophic, mesotrophic and oligotrophic data sets, applying Bonferroni correction of alpha level for multiple comparisons. For the HTS data set (number of sequences per OTU, standardized by total number per sample), the multivariate regression model was fitted based on the Bray–Curtis similarity matrix. Prior to the analysis, the standardised OTU data were square-root transformed. A marginal test was initially used, which involved individual variables that had been fitted separately to test their relationship with the ARISA or OTU data (ignoring other variables). This was followed by a conditional test (using the R^2 selection criteria) that considers variables already included in the model. The conditional test identifies the subset of variables that best predict the observed pattern in cyanobacteria ARISA or OTU data. The results were visualised by the distance-based redundancy analysis (dbRDA) plots (Legendre and Anderson 1999).

Analysis of cyanotoxin production potential and concentrations

To determine if cyanobacteria with the potential to produce cyanotoxins were present in samples, amplification of the following genes was undertaken: *mcyE* (for microcystins and nodularins using the HEPF/HEPR primers; Jungblut and Neilan 2006), *sxtA* (for saxitoxins using the sxtaf/sxtar primers; Ballot, Fastner and Wiedner 2010), *cyrJ* (for cylindrospermopsin using the CylsulfF/CylnamR primers; Mihali *et al.* 2008) and *anaC* (for anatoxins using the Ana-c-F/Ana-c-R primers; Rantala-Ylinen *et al.* 2011). Reactions were carried out using 25 μL of i-Taq 2× PCR master mix (Intron, Kyungki-Do, Korea), 0.4 μM of each primer and template DNA (30–50 ng). The PCR conditions for the *mcyE* primers were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 60 s with a final extension of 72°C for 5 min. Reactions for the *cyrJ*, *sxtA* and *anaC* primers were held at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s and a final extension of 72°C for 5 min. PCR products were visualised by 1% agarose gel electrophoresis with ethidium bromide staining and UV illumination. Amplicons of the correct size were purified using the AxyPrep PCR Clean-up Kit (Axygen Biosciences, CA, USA) and sequenced bidirectionally using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

All samples that yielded a positive result in the toxin-gene PCRs were analysed using analytical methods. Samples (45 mL) were defrosted, gently mixed and a subsample (10 mL) was lyophilised. The lyophilised samples were resuspended in 1 mL of either 80% methanol acidified with 0.1% acetic acid (microcystin/nodularin), Milli-Q water containing 0.1% formic acid (anatoxins) or 100% methanol acidified with 0.1% acetic acid (saxitoxins; Smith *et al.* 2011, 2012). Samples were sonicated (30 min, on ice) and clarified by centrifugation (4000 × *g*, 10 min). The supernatant for microcystin/nodularin and anatoxin was analysed by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) as described in Wood *et al.* (2012; for microcystin/nodularin) and Wood, Holland and MacKenzie (2011; for anatoxins) with a limit of quantitation of 0.015 μg L^{-1}.

The supernatant for saxitoxins analysis (1 mL) was reduced to dryness under nitrogen (35°C) and redissolved in 0.1 mM acetic acid (1 mL). The samples were vortexed and filtered using spin columns (3000 × *g*, 1 min) and analysed using high-performance liquid chromatography with fluorescence detection (HPLC-FD) as described in Smith *et al.* (2011).

RESULTS

Cyanobacterial community structure as determined by automatic ribosomal intergenic spacer analysis

Positive ARISA amplicons were obtained for 158 of the 189 samples. A total of 124 distinct AFLs were identified. The average number of AFLs per sample was six. Twenty-four samples contained only one AFL, while the maximum number (23) was detected in Lake Mokeno (sample #123; Table S1, Supporting Information).

The CAP ordination showed a clear delineation among AFLs from lakes of different trophic status (Fig. 2). Samples from eutrophic lakes were positioned on the positive-value side of the canonical axis 1. Samples from oligotrophic lakes grouped on the negative-value side of both axes and those from mesotrophic lakes at a somewhat intermediate position, demonstrating a highest positive correlation with the canonical axis 2 (Fig. 2). The permutation test confirmed the significance of the CAP results ($p < 0.001$; no randomly permuted data set had more extreme assemblage than the original data set). The overall group allocation success was 69%, with higher percentage of correct classification for eutrophic lakes (78%).

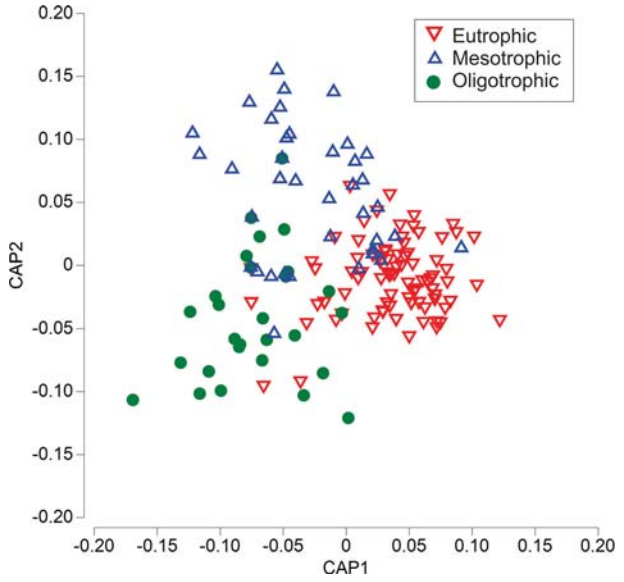

Figure 2. Two-dimensional ordination plot of the canonical discriminant analysis of principal coordinates (CAP; the first two canonical axes), based on Jaccard index similarity matrix cyanobacterial ARISA fingerprints (transformed to presence/absence) from 158 New Zealand lake samples.

Cyanobacterial community structure as determined by HTS

Three samples (Lake Kohangapiripiri [#88], Lake Pupuke [#91] and Lake Tomarata [#92]) failed to sequence and were removed from all subsequent analyses. Quality filtering and removal of chimera and primer errors generated 4 320 997 usable reads (avg. 93 935 reads per sample; rps). When all reads not classified as cyanobacteria were removed, 964 239 reads remained (avg. 20 962 rps). The percentage of sequences in each sample that were classified as cyanobacteria ranged between 1 and 87. These sequences clustered into 365 OTUs.

The highest number of OTUs (richness) occurred in mesotrophic lakes, Lake Rotoiti (Bay of Plenty)-122 [#153], Lake Sarah-131 [#163] and oligotrophic Lake Benmore 132 [#185], and the lowest number of OTUs in eutrophic Sullivan's Lake-17 [#114] and Lake Whangape-18 ([#69]; Table S1, Supporting Information).

When the OTU data were classified to the lowest possible taxonomic assignment, 11 dominant groups/taxa were identified (Fig. 3a). The most abundant and diverse taxon (containing 71 OTUs) across all samples was *Synechococcus* spp. (Fig. 3a and b). A comparison of these OTUs showed a core group that was common across all trophic groupings, with a lesser number of OTUs confined to samples from only one trophic category. This was particularity apparent for the samples from eutrophic lakes (Fig. 3b).

In a number of the eutrophic lakes assessed, a single taxon dominated, e.g. *Dolichospermum* spp. (Lake Roundabout [#36]), *Microcystis* spp. (Lake Horowhenua [#15]), Gomphosphaeriaceae (Lake Whangape [#69]) and *Aphanizomenon* spp. (Lake Sullivans [#114]; Fig. 3a). In mesotrophic lakes, *Crocosphaera* spp. (Lake Hawdon [#166], Lake Georgina [#168] and Lake Middleton [#184]), *Phormidium* spp. (Lake Sarah [#163]) and *Dolichospermum* spp. (Upper Karori [#87]) were highly abundant (Fig. 3a). The mesotrophic lakes also tended to have a higher number of taxa present in low abundance (captured in the 'Others' category of Fig. 3).

Crocosphaera spp. was present in high abundance in three of the oligotrophic lakes: Lake Matiri [#83], Lake Pearson [#165] and Lake Rotoiti (Tasman) [#7] (Fig. 3a). The following taxa were also relatively abundant in some oligotrophic lakes: *Gloeobacter violaceus* (Lake Matiri [#83], Lake Aviemore [#186]), *Pseudanabaena* spp. (Lake Matiri [#83], Little Sylvester [#62]) and *Dolichospermum* spp. (Cobb Reservoir [#63]; Fig. 3a). A large percentage of the Little Sylvester [#62] sequence abundance was made up by the 'Others' category, and this was comprised mostly of *Nostoc* spp. sequences (data not shown).

Multivariate multiple regression analysis

Analysis of the ARISA data using DistLM resulted in only 10% of the variance for eutrophic lakes, and 19% and 21% for mesotrophic and oligotrophic lakes, respectively, being explained by linear models fitted to the environmental parameters (Table 1). Marginal tests in DistLM showed that all of the considered environmental factors and geographical features (in particular latitude and altitude) were significant in determining the cyanobacterial community. However, partitioning of their effects was not consistent with lake trophic status. For example, among mesotrophic lakes, although all predictor variables were significant in isolation, the sequential tests indicated that only addition of altitude and depth variables contributed significantly to the variation explained by the model. In contrast, latitude was the only factor identified by the DistLM sequential tests to be important for oligotrophic lakes and latitude and altitude for eutrophic lakes.

The dbRDA based on ARISA data (Fig. 4) showed that for eutrophic and oligotrophic lakes, latitude correlated closely with the first dbRDA axis ($r = 0.88$ and 0.86, respectively), while in mesotrophic lakes the strongest correlation was with depth ($r = -0.89$). The second axis in all types of lakes correlated strongly with altitude ($r = 0.80$, -0.96 and -0.65 for eutrophic, mesotrophic and oligotrophic lakes, respectively), with high collinearity of longitude in oligotrophic lakes ($r = -0.70$).

The dbRDA based on HTS data (Fig. 5) also resulted in the highest correlations of altitude ($r = -0.79$) and latitude ($r = 0.88$) with the first two axes, supporting the importance of these two factors in shaping cyanobacterial diversity.

Cyanotoxin production potential and concentrations

Of the cyanotoxin production genes assessed, *mcyE* (involved in the production of microcystin) was the most prolific with 66 positive amplicons being obtained (Table 2). Two samples (Lake Mokeno [#123] and Lake Karapiro [#20]) were positive for both the *mcyE* and *anaC* genes, and a single sample (Lake Opunake [#25]) was positive for both the *anaC* and *sxtA* genes. No positive amplicons were obtained for the *cynJ* gene.

Sequencing of the positive PCR amplicons resulted in 50 gene sequences of *mcyE* (355 bp) and 3 *anaC* sequences (310 bp; Table 3). The majority of the *mcyE* sequences shared a high similarity (99%) with the same gene in *Dolichospermum* (basionym *Anabaena*) *lemmermannii* (Table 2). With the exception of two samples (Lake Virginia [#10] and Lake Rotoehu [#112]), the remainder contained no microcystin when analysed by LC-MS/MS. There were four distinct groups of *mcyE* sequences with high homology to this gene form *Microcystis* strains (Table 3). These differed by less than 14 bp. Nine of these sequences came from samples where no microcystins were detected using LC-MS/MS (Table 2). A single sequence from Lake Forsyth [#90] was identical to the *ndaF* gene from *Nodularia spumigena* and nodularin was detected

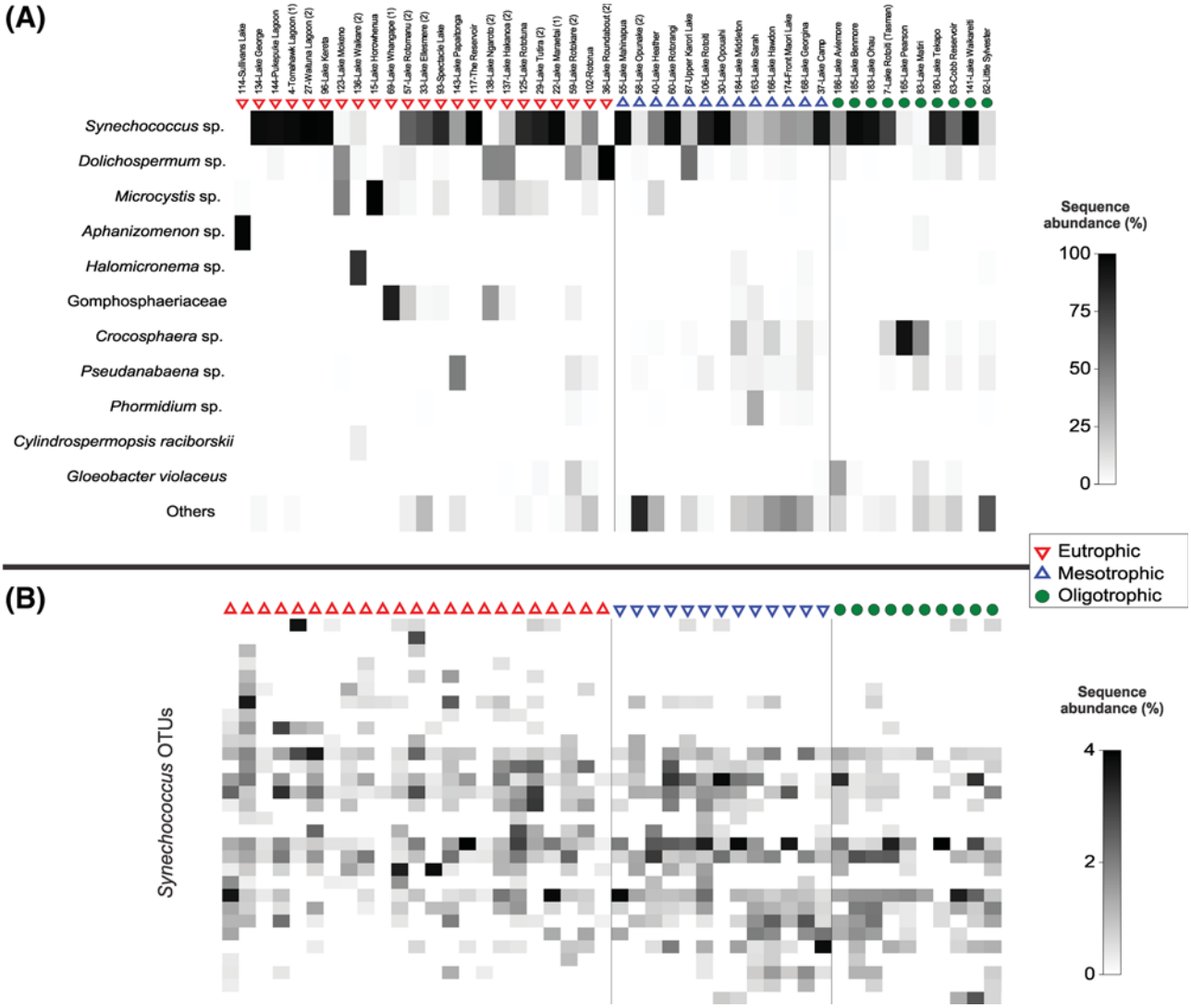

Figure 3. (**A**) Shade plot of the 11 most prevalent taxa generated from standardised OTU data classified to the lowest possible taxonomic assignment. Taxa that accounted for <0.3% of the sequences across all samples were combined into 'Other'. (**B**). Shade plot of the 30 most prevalent OTUs within *Synechococcus*. Data are fourth-root transformed to aid visualisation. Samples are ordered from left to right by increasing altitude within trophic groupings.

in this sample using LC-MS/MS (Tables 2 and 4). Two distinct *anaC* sequences were obtained, the first with only 75% similarity to this gene in *Cuspidothrix issatschenkoi* and the second with 96% similarity to *Oscillatoria* sp. (Table 3).

Microcystins were detected in 21 of the samples using LC-MS/MS (Table 2) and 12 different structural congeners were observed (Table 4). The highest microcystin concentration measured was in Lake Horowhenua (55.4 μg/L); however, the majority of the samples contained low microcystin concentrations (<1 μg/L; Tables 2 and 4). Anatoxins (anatoxin-a and dihydroanatoxin-a) were only detected in one sample at low concentration (Table 4). No saxitoxins were detected in the samples tested.

DISCUSSION

Cyanobacterial diversity

Phytoplankton at various different taxonomic levels or in functional trait groupings have been used for many decades as indicators of lakes trophic status, with cyanobacteria commonly associated with eutrophic systems (Interlandi and Kilham 2001; Paul *et al.* 2012; Yang *et al.* 2012). Such studies generally examine the entire phytoplankton community and rarely use species or taxa-resolved data. In this study, two molecular techniques (ARISA and HTS) were used to investigate cyanobacterial diversity in New Zealand lakes of varying trophic status during the summer of 2012 to 2013. Samples were collected across geographical and hydromorphological gradients. The catchment land use of the lakes sampled spanned a range of land-cover classes including alpine, native forest/scrub, exotic forest and pasture. Although not directly investigated in this study, previous analysis of New Zealand lakes has shown a strong relationship between trophic status and land cover, with eutrophic lakes strongly correlated with pastoral land use (Sorrell *et al.* 2006). We used trophic status as an integrated measure of long-term nutrient concentrations (i.e. as a proxy for total nitrogen and total phosphorus) in a lake. Other parameters known to influence phytoplankton communities were not measured such as temperature and mixing regime (Ryan *et al.* 2006). Nonetheless,

Table 1. DistLM analysis showing results of a marginal test followed by sequential tests based on adjusted R^2 and 9999 permutations. ARISA data were analysed using each trophic grouping separately, whereas HTS data were applied to all trophic classes combined. Statistically significant effects are in bold, marginal effects are in italics (Bonferroni correction, $\alpha = 0.01$).

	ARISA: Eutrophic lakes			ARISA: Mesotrophic lakes			ARISA: Oligotrophic lakes			HTS data		
Environ. variable	Pseudo-F	P*	Prop.	Pseudo-F	P*	Prop.	Pseudo-F	P*	Prop.	Pseudo-F	P*	Prop.
Marginal tests												
Altitude	1.85	0.011	0.02	2.01	**0.001**	0.05	1.18	0.225	0.04	2.55	**0.001**	0.05
Depth	1.57	0.035	0.02	2.12	**0.002**	0.06	0.80	0.792	0.03	1.10	0.298	0.02
Area	1.54	0.031	0.02	1.84	**0.008**	0.05	0.78	0.914	0.03	0.86	0.713	0.02
Latitude	2.53	**0.001**	0.03	1.73	**0.006**	0.05	1.77	0.019	0.06	2.54	**0.000**	0.05
Longitude	1.48	**0.001**	0.02	1.77	**0.005**	0.05	1.57	0.044	0.05	1.98	**0.004**	0.04
R^2		0.10			0.19			0.21			0.16	
Sequential tests												
Altitude	1.85	**0.005**	0.02	2.01	**0.001**	0.05	1.18	0.241	0.04	2.54	**<0.001**	0.05
Depth	1.46	0.066	0.02	2.19	**0.001**	0.06	0.69	0.898	0.02	0.89	0.621	0.02
Area	1.50	0.044	0.02	0.93	0.606	0.02	1.11	0.318	0.04	0.93	0.589	0.02
Latitude	2.59	**0.001**	0.03	1.34	0.069	0.03	2.27	**0.001**	0.07	1.91	**0.006**	0.04
Longitude	1.32	0.023	0.01	1.06	0.359	0.03	1.29	0.137	0.04	1.25	0.156	0.03

**Bonferroni correction applied, $\alpha = 0.01$.*

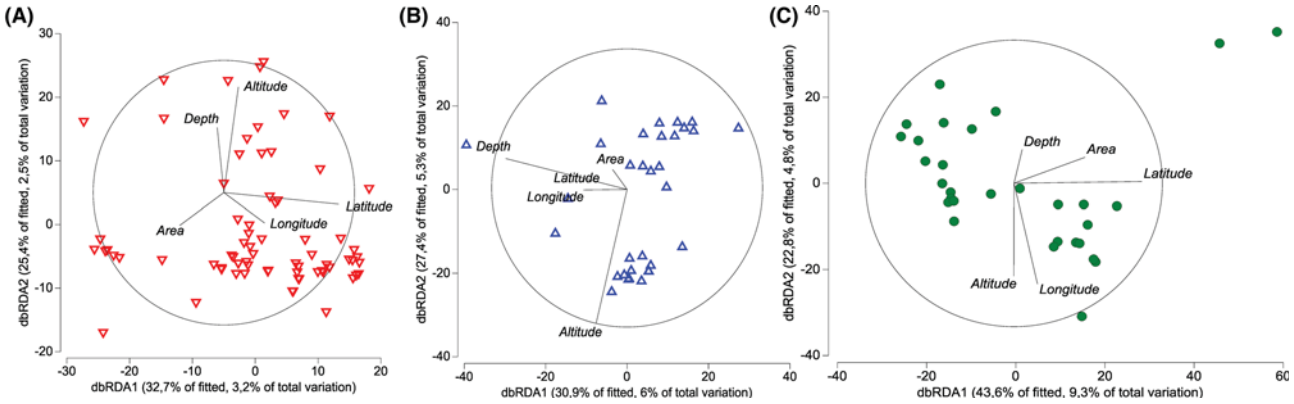

Figure 4. dbRDA biplots from the ARISA data set (presence/absence transformed, Jaccard similarity matrix). (**A**) Eutrophic lakes, (**B**) mesotrophic lakes and (**C**) oligotrophic lakes. The overlaid vectors represent environmental variables: hydromorphological features of a lake (depth and area) and geographical location (latitude, longitude and altitude). Length and direction of vectors indicate the strength and direction of the relationship.

both the ARISA and HTS data showed significant delineations in cyanobacterial communities among lakes of varying trophic status.

In this study, we used the data from the ARISA analysis to guide the selection of samples for HTS analysis for further in-depth taxonomic assessment. ARISA is based on length heterogeneity of the ITS region, while the HTS assay used in this study targets a region of 16S rRNA. Given the highly variable nature of the ITS region, a greater number of AFLs compared with OTUs might have been expected; however, this was not observed (OTU = 365, AFL = 124). The most likely reason is the high-sequencing depth of new HTS platforms. Despite these differences, previous studies have shown highly similar patterns when using ARISA and HTS to explore cyanobacteria diversity (Kleinteich *et al.* 2014). A similar result was evident in our study with the multivariate analysis of the ARISA and HTS data showing a clear delineation in cyanobacterial community structure among lakes of varying trophic status. A significant advantage of HTS data is that it can be used to infer taxonomy. An important consideration when interpreting the ARISA data is that no information on abundance is provided. HTS data provides some semiquantitative information and in this study analysis of data from both methods showed similar patterns. We recommend the use of HTS for future broad-scale cyanobacterial diversity studies.

Among eutrophic lakes, well-known bloom-forming taxa were identified (e.g. *Microcystis*, *Aphanizomenon* spp., *Dolichospermum* spp., *Cylindrospermopsis raciborskii* and *Nodularia*). *Nodularia* and the toxin nodularin (see the section on toxins below) were only detected from Lake Forsyth, which is a brackish water lake with a long history of *Nodularia* blooms (Carmichael *et al.* 1988). Of note was the detection of *Halomicronema* (particularly abundant in eutrophic Lake Waikare [#136]) and *Crocosphaera* (abundant in oligotrophic Lake Pearson [#165]). *Halomicronema* is a moderately halophilic/halotolerant, benthic, filamentous, non-heterocystous genus of cyanobacteria (Abed, Garcia-Pichel and Hernández–Mariné 2002), and *Crocosphaera* is a diazotropic marine cyanobacterium described previously from the tropical Atlantic and Pacific Oceans (Dyhrman and Haley 2006). Neither of these identifications seems plausible, highlighting some of the

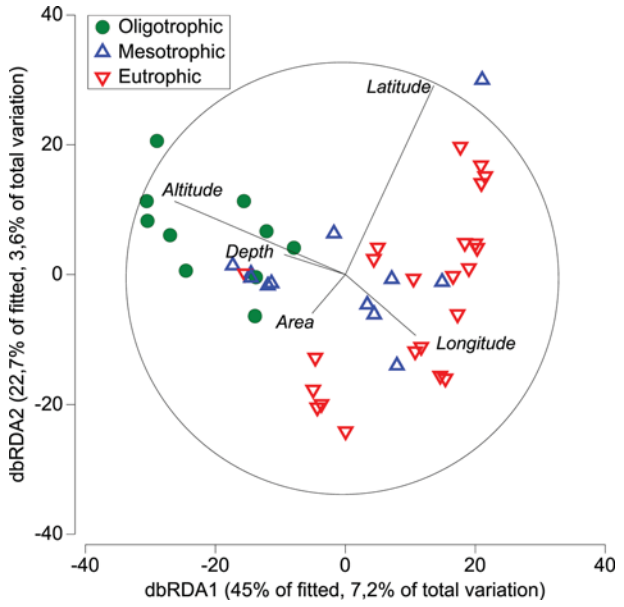

Figure 5. dbRDA biplot from the HTS data set (standardised, square root, Bray–Curtis similarity matrix). The overlaid vectors represent environmental variables: hydromorphological features of a lake (depth and area) and geographical location (latitude, longitude and altitude). Length and direction of vectors indicate the strength and direction of the relationship.

well-known limitations of HTS approaches. Voucher specimens with verified sequences are lacking for many species and even in groups with well-developed reference databases, >20% of entries may be incorrectly identified (Nilsson *et al.* 2006). Morphological analysis of the Lake Waikare samples (data not shown) identified a high prevalence of small *Planktolyngbya*-like species, which have not been well characterised via molecular methods.

Many of the OTUs, regardless of lake trophic status, were classified as *Synechococcus* and among this genus, there was high genetic diversity, with 71 OTUs identified. Recent molecular ecology studies (e.g. Becker, Richl and Ernst 2007; Sánchez-Baracaldo, Handley and Hayes 2008) have highlight similarly diverse freshwater autotrophic picoplankton communities, and suggest a need for further characterisation with particular regard to their response to environmental change. Whether there are niche-adapted *Synechococcus* clades in freshwater systems remains uncertain (Callieri *et al.* 2012). Becker *et al.* (2012) found clade-specific difference between pelagic and littoral zone macrohabitats and between seasons and years in *Synechococcus* in Lake Constance (Germany). The data in this study indicate a core group of ca. 10–12 OTUs which are ubiquitous across lakes irrespective of trophic status, with other OTUs present in low abundance and restricted to a few lakes (Fig. 3B). This finding corroborates that of previous studies which have found correlations between picocyanobacteria (predominantly *Synechococcus*) composition and their habitat and its trophic status (Takamura and Nojiri 1994; Vörös *et al.* 1998; Ernst *et al.* 2003.). However, the exact nature of the relationship with nutrient concentrations and their role in planktonic productivity remains unclear. Even when taking into consideration the limitations of the HTS approach (e.g. PCR primer biases which could cause preferential amplification of this genus), data in this study suggest that further in-depth phylogenetic and functional studies are required to understand nutrient and trophic status dependences. This is particularly so for New Zealand lakes, where knowledge of this abundant and seemingly important component of the phytoplankton community is still restricted to only a few lakes (e.g. Burns and Stockner 1991; Schallenberg and Burns 2001).

Diversity distribution patterns

Latitudinal and altitudinal gradients and their interplay with temperature and productivity play major roles in structuring species diversity and richness among animals and plants (Allen, Brown and Gillooly 2002; Mittelbach *et al.* 2007; Rosenzweig 1995). Because of their small size, high levels of dispersal and rapid generation times, it has been disputed whether similar diversity patterns would be observed in microorganisms. However, significant latitudinal and altitudinal gradients in diversity are now well documented for a range of marine, freshwater and terrestrial taxa (Fuhrman *et al.* 2008; Bryant *et al.* 2008; Ptacnik *et al.* 2010).

Stomp *et al.* (2011) examined the species diversity of freshwater phytoplankton from 540 lakes and reservoirs distributed across the United States and demonstrated that large-scale biodiversity patterns were driven mostly by local environmental factors (e.g. chlorophyll-a, surface area and possibly temperature), with latitude, longitude and altitude contributing to diversity patterns to a lesser degree. The results of this study at least partly concur with these findings, with clear delineation in cyanobacterial community structure based on trophic status (a combined measure of chlorophyll-a and nutrients). Few studies have investigated only the cyanobacteria component of phytoplankton, but those that have reinforce our observations. For example, Touzet, McCarthy and Fleming (2013) found that filamentous genotypes were more prominent in larger lakes with substantial forest cover, while Chroococcales were more commonly observed in lakes with lesser trophic states.

Table 2. Summary of molecular and chemical analysis of cyanotoxins in 189 samples collected from 143 New Zealand lakes between 11 December 2012 and 27 April 2013.

		Toxin via LC-MS/MS or HPLC-FD (μg/L)				
	PCR positives	Below LOD	<0.1	0.1 ≥ 1.0	1.0 > 10	≥10
Microcystin/Nodularin	66	44	11	6*	3	2
Anatoxins	3	2	1	–	–	–
Saxitoxins	1	1	–	–	–	–

LC-MS/MS, liquid chromatography-tandem mass spectrometry; HPLC-FD, high-performance liquid chromatography with fluorescence detection, LOD, limit of detection, *toxin identified as nodularin in a single sample.

Table 3. Sample number and nearest GenBank (*mcyE, ndaF* or *anaC* gene sequence from a cultured organism) sequence match from positive PCR amplicons. Bold type indicates that toxins were detected using LC-MS/MS.

Sample no.	Highest GenBank match	Access. no.	Similarity (%)
mcyE/*ndaF			
1, 2, 3, 6, **10**, 16, 17, 18, 19, 28, 53, 54, 67, 87, 106, 107, 108, **112**, 144, 150, 152, 153, 156, 157, 158, 159, 177, 187, 188	*Dolichospermum lemmermannii*	EU916774	99
11, **14, 15**, 20, 21, **26, 57, 77,** 91, **95**, **114, 130**, 132	*Microcystis* sp.	FJ393327	100
45, 46, **69, 71**, 136	*Microcystis* sp.	KF219500	100
13, 78, 93, 123	*Microcystis aeruginosa*	JQ290095	99
138, 139, 147	*Microcystis* sp.	KF219502	99
90*	*Nodularia spumigena*	AY817170	100
anaC			
123	*Cuspidothrix issatschenkoi*	KM245024	75
20, 25	*Oscillatoria* sp.	JF803652	96

Table 4. Concentrations (μg/L) of microcystin variants, nodularin and anatoxin variants in New Zealand lake samples measured using liquid chromatography- tandem mass spectrometry.

		Microcystins												Anatoxins		Total
No.	Lake	-RR	dm-RR	didm-RR	Nodularin	-YR	-LR	dm-LR	didm-LR	-FR	-WR	-LA	-LY	-LF	ATX	dhATX	
15	Horowhenua	4.76	0.09	0.03		1.52	12.71	0.82	0.04	13.86	12.90	8.43		0.23			55.40
13	Pauri	4.86	0.03	0.04		6.71	5.89	0.40		0.30	0.24						18.47
11	Westmere	2.64	0.03	0.02		0.54	2.03	0.04		2.10	1.64	0.57		0.04			9.66
114	Sullivans	0.67		0.01		0.14	0.68			0.56	0.78	0.30	0.01				3.16
71	Waikare							2.23	0.03								2.26
14	Dudding	0.07	<0.01	<0.01		0.04	0.15	0.01		0.12	0.09	0.07		0.01			0.56
77	Waikere	0.12				0.06	0.16			0.06	0.08	0.03					0.52
130	Rotomanu	0.08					0.08	0.01		0.08	0.13	0.03					0.40
95	Kuwakatai	0.05					0.07			0.03	0.05	0.02					0.22
135	Whangape							0.01	0.10					0.06			0.17
10	Virginia	0.02	<0.01	<0.01			0.07			0.01	0.01						0.12
72	Whangape	0.01		<0.01				0.09			0.01						0.10
139	Waahi								0.09								0.09
45	Wairarapa							0.07		0.01							0.08
138	Ngaroto					0.06		0.02									0.07
69	Ngaroto	0.01					0.02				0.02			0.02			0.05
90	Forsyth				0.04												0.04
26	Rotokauri	<0.01	<0.01				0.02	<0.01			0.01						0.04
122	Karaka	0.02				0.02											0.04
116	Murihiku									0.03							0.03
57	Rotomanu	<0.01					0.01			<0.01							0.02
147	True Omanu							0.01									0.01
112	Rotoehu													0.01			0.01
20	Karapiro														0.22	0.03	0.25

dm, desmethyl; didm, didesmethyl; ATX, anatoxin-a; dh, dihydro.

Multivariate regression analysis in this study demonstrated that geographical locations (latitude, longitude and altitude) were significant drivers of cyanobacterial community composition; however, partitioning of their effects varied among trophic categories. Latitudinal and longitudinal gradients were evident for both eutrophic and oligotrophic lakes, with stronger patterns, and an additional influence of altitude was observed in the oligotrophic data category (Figs 4 and 5). The effect of altitudinal gradients appears even more pronounced for mesotrophic lakes (Fig. 4), regardless of geographic location. Although we did not measure water temperature in this study, Stomp *et al.* (2011) showed that it had a strong statistical relationship ($R^2 = 0.73$) with altitude, latitude and longitude across the continental United States. Temperature has been shown to be a good predictor of diversity patterns for many terrestrial plants and animals (Mittelbach *et al.* 2007) and marine organisms (Fuhrman *et al.* 2008). This potential link with temperature highlights the vulnerability of these systems to change under predicted climate change scenarios and demonstrates that studies such as the present one that use space for time substitutions may assist in understanding how cyanobacterial communities might shifts with climate change (sensu Jeppesen *et al.* 2014). The explanatory power of the model used in our study was relatively low, and this most likely reflects the role of other environmental parameters (chemical, physical and biological) determining cyanobacterial diversity in lakes and

further studies where these parameters are collected in parallel are recommended.

Interestingly, area and depth appear to have only a limited role in structuring cyanobacterial communities (i.e. the presence/absence or abundance of specific AFLs or OTUs). Area has previously been shown as important in structuring phytoplankton, with larger systems generally harbouring greater phytoplankton diversity (Smith *et al.* 2005; Stomp *et al.* 2011), although these studies have focused on species richness rather than community structure per se. The limited impact of lake depth on diversity in this study may reflect the sampling strategy used, which only involved collection of surface or surface-integrated (to 0.3 m depth) samples. If samples had been collected throughout the entire water column, it is likely that a wider diversity of cyanobacteria would have been obtained, including species known to form a deep chlorophyll maximum, even in hypereutrophic lakes (Simmonds *et al.* 2015).

Toxin production

Previous broad-scale studies have identified relationships between microcystin concentration and trophic status of lakes, with some studies suggesting relationships to nitrogen and phosphorus concentrations (Downing *et al.* 2001; Dolman *et al.* 2012). We found that microcystins were only present in eutrophic lakes. Although HTS is best interpreted as semiquantitative, the highest microcystin concentrations in our study aligned with samples which had a high percentage abundance of *Microcystis* sequences. Thus, we suggest that concentrations of toxins are more closely linked to the presence and abundance of toxin-producing species rather than to nutrient concentrations per se.

The use of a dual approach for detecting toxins provided a useful method for screening and confirming toxin production, and added new information on toxin distributions. Wood *et al.* (2006) undertook the first survey of cyanotoxins in New Zealand between 2001 and 2004. This study used ELISAs to screen samples for cyanotoxins and selected a subset of samples for further toxin analysis using LC-MS, HPLC or neuroblastoma assays. Microcystins were detected in 27 lakes, anatoxins in 3 lakes and saxitoxins in 16 lakes. The results of the current study reinforce that microcystins are the most widespread and commonly detected cyanotoxin in New Zealand lake water samples. The concentrations of microcystins detected in Wood *et al.* (2006) were markedly higher (max. 36 500 $\mu g\ L^{-1}$ in Lake Horowhenua) than those detected in this study (55.4 $\mu g\ L^{-1}$ also Lake Horowhenua). These differences likely reflect the respective sampling strategies. Wood *et al.* (2006) targeted surface scums, whereas we generally collected from a single fixed sampling point in this study. In general, the dominant microcystin variants detected in this study were -LR, -RR, -FR and -WR, and the toxin profiles were similar to those previously described in blooms (Wood *et al.* 2006, 2016), and culture-based studies (Puddick *et al.* 2014) in New Zealand. Although the PCR results from one sample were positive for a gene involved in saxitoxin production, we could not confirm the presence of this toxin through either sequencing of the gene or chemical methods. Given that we were unable to obtain a clean sequence, it is plausible that this PCR detection was a false positive. Wood *et al.* (2006) detected saxitoxins in 16 lakes; however, the ELISA used is known to give false positives, particularly when working with environmental cyanobacteria samples (Kleinteich *et al.* 2013). Although a number of lakes contained genera known to produce STX in other countries (i.e. *Aphanizomenon* and *Cylindrospermopsis*), the *sxtA* gene was not identified in these samples, and the only confirmed STX-producing species in New Zealand to date is the benthic species *Scytonema crispum* (Smith *et al.* 2011, 2012).

For logistical reasons, the samples collected in this study were mostly taken at one point in time. Given the patchy distribution of blooms, it is likely that this study has underestimated the number of waterbodies containing cyanotoxins. Fastner *et al.* (2001) found that the likelihood of detecting microcystins in a given lake depends to some extent on the frequency of sampling. They found that in lakes sampled only once, 33% contained microcystins compared with 94% for those sampled 2–10 times over 3 years.

Prior to our study, four planktonic cyanobacterial species were known toxin producers in New Zealand; *Cuspidothrix issatschenkoi* (anatoxin-a, Wood *et al.* 2007), *Cylindrospermopsis raciborskii* (cylindrospermopsin and deoxy-cylindrospermopsin, Wood and Stirling 2003), *Microcystis* spp. (microcystin, Wood *et al.* 2008a) and *Nodularia spumigena* (nodularin, Carmichael *et al.* 1988). Sequencing of toxin-specific PCR amplicons in this study confirmed in general these earlier findings. No genes involved in cylindrospermopsin were detected in any samples in this study and there remains some doubt about the presence of these toxins as they have not been detected in recently isolated strains of *C. raciborskii* nor in any environmental samples since their initial detection in 2003 (Wood *et al.* 2014). In this study, the majority of environmental samples containing the *mcyE* with a high-sequence similarity to *mcyE* from *Dolichospermum lemmermannii* did not contain microcystins when assessed via LC-MS. Subsequent isolation, culturing and toxin analysis of *D. lemmermannii* strains from these samples has shown that although these strains contain the *mcyE* gene they do not produce microcystins (data not presented). Previous studies have shown that the entire *mcy* gene cluster is required for toxin production (Kurmayer *et al.* 2004), and further sequencing is underway to investigate which *mcy* genes are absent in our *D. lemmermannii* strains. The sequence of the *anaC* gene from lakes Karapiro (#20) and Opunake (#25) was most similar to that of *Oscillatoria* sp. and this may represent a new toxin producing species in New Zealand. Attempts to isolate and culture Oscillatoriales from these samples were unsuccessful during this study.

CONCLUSIONS

Analysis of cyanobacterial-specific ARISA and HTS data from 143 New Zealand lakes demonstrated significant differences in communities among eutrophic, mesotrophic and oligotrophic lakes. The data provided compelling evidence that latitudinal, longitudinal and altitudinal gradients contribute to structuring these communities, but that the relative importance of these varies among trophic categories. These data confirm other studies that indicate that ongoing environmental change will have far-reaching impacts on cyanobacteria abundance and diversity. OTUs assigned to *Synechococcus*-dominated samples across all lake types and high levels of diversity (71 OTUs) were identified, suggesting that further studies on the phylogenetics and ecological role of this genus are warranted. Cyanotoxins were only detected in eutrophic lowland lakes using LC-MS; however, the PCR-based methods detected genes involved in microcystin production in many other lakes. Sequencing of these identified that this was due to the presence of the *mcyE* gene in *Dolichospermum lemmermannii*. Similar results have been obtained by other researchers and demonstrate that the presence of one gene does not mean that the entire operon is present, or that the toxin is being produced. This highlights the need

for molecular techniques to be aligned with other confirmatory analytical tools. Molecular methods have many promising prospects for assessing aquatic microbial community diversity across varying scales of space and time. In addition to providing broad-scale data on diversity, they have huge potential to complement current monitoring programmes aimed at measuring water quality or protecting human health.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflict of interest. None declared.

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