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Application of ancient DNA to the reconstruction of past microbial assemblages and for the detection of toxic cyanobacteria in subtropical freshwater ecosystems

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Abstract

Ancient DNA (aDNA) analysis of lake sediments is a promising tool for detecting shifts in past microbial assemblages in response to changing environmental conditions. We examined sediment core samples from subtropical, freshwater Laguna Blanca (Uruguay), which has been severely affected by cultural eutrophication since 1960 and where cyanobacterial blooms, particularly those of the saxitoxin-producer *Cylindrospermopsis raciborskii*, have been reported since the 1990s. Samples corresponding to ~1846, 1852, 2000 and 2007 AD were selected to perform denaturing gradient gel electrophoresis (DGGE) analysis of the 16S–23S rRNA intergenic transcribed spacer (ribosomal ITS) to compare their prokaryotic assemblage composition. Each stratum showed different ITS patterns, but the composition of 21st century samples was clearly different than those of mid-19th century. This compositional change was correlated with shifts in sediment organic matter and chlorophyll *a* content, which were significantly higher in recent samples. The presence of saxitoxin-producing cyanobacteria was addressed by quantitative real-time PCR of the *sxtU* gene involved in toxin biosynthesis. This gene was present only in recent samples, for which clone libraries and ITS sequencing indicated the presence of Cyanobacteria. Phylogenetic analyses identified *C. raciborskii* only in the 2000 sample, shortly after several years when blooms were recorded in the lake. These data suggest the utility of aDNA for the reconstruction of microbial assemblage shifts in subtropical lakes, at least on centennial scales. The application of aDNA analysis to genes involved in cyanotoxin synthesis extends the applicability of molecular techniques in palaeolimnological studies to include key microbial community characteristics of great scientific and social interest.

Keywords: ancient DNA, bacterial community composition, lake sediment, ribosomal ITS, saxitoxin-encoding genes

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Introduction

Shifts in microbial communities as a consequence of anthropogenic activities have been widely reported in limnological studies (Pesce *et al.* 2008; Naeher *et al.* 2012). The sedimentary records of lakes and lagoons hold potential for the detection of such shifts in the past, given that they contain a wealth of information about lake histories (Smol 2008; Antoniades *et al.* 2011). The analysis of DNA from sediment samples is a new approach that has been used to analyse changes in species composition in response to environmental variation, not only in terrestrial but also in marine and

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freshwater ecosystems (Willerslev *et al.* 2003; Coolen *et al.* 2004; Coolen & Overmann 2007). While chlorophyll *a* and carotenoids can be used as biomarkers for the taxonomic differentiation of algae and cyanobacteria (McGowan *et al.* 1999; Antoniades *et al.* 2011; Boere *et al.* 2011), DNA analysis can also provide information about the whole microbial community living in a given ecosystem. DNA preserved in lake sediments (ancient DNA, aDNA) has recently been used as a biomarker to infer the presence and ecology of green sulphur bacteria (Coolen & Overmann 2007; Manske *et al.* 2008) and to reconstruct palaeo-microbial community dynamics in lakes (Ravasi *et al.* 2012; Fernandez-Carazo *et al.* 2013). To date, most studies involving aDNA have focused on cold ecosystems; for example, aDNA-based techniques were applied to the detection of changes in the biodiversity of eukaryotic phytoplankton (Coolen *et al.* 2004) or cyanobacterial communities (Fernandez-Carazo *et al.* 2013) during the Holocene in Antarctic lakes, from fossilized cyanobacteria (Panieri *et al.* 2010) or hypersaline marine sediments (Danovaro *et al.* 2005). However, these studies were based on ribosomal genes that have low taxonomic resolution due to the relatively conserved 16S rRNA gene, which impedes the detection of microevolutionary changes that lead, for example, to nontoxic cyanobacteria populations or genotypes (Kurmayer & Gumpenberger 2006; Christiansen *et al.* 2008). The development of techniques that allow the detection of microbes at the population level in subtropical and tropical lakes thus remains a challenge in environmental studies.

It is well known that human impacts often promote the appearance of cyanobacterial blooms in both lotic and lentic freshwater ecosystems. While these impacts likely also affect general microbial community composition, few studies have specifically addressed this topic (Lindström 2001; Edlund *et al.* 2005). In the case of cyanobacteria species, there is controversy regarding their origin, evolution and dispersion, especially in the case of taxa capable of toxin production. Blooms of *Cylindrospermopsis raciborskii* are becoming frequent in lakes from tropical to temperate latitudes (Hamilton *et al.* 2005; Vidal & Kruk 2008; Figueredo & Giani 2009). Because this species has the potential to produce two kinds of toxins, saxitoxins and cylindrospermopsins (Chorus & Bartram 1999), its expansion and increased bloom frequency has generated widespread concern. Furthermore, the relationship between phylogeny and toxicity in this species has not yet been elucidated, but it is known that *C. raciborskii* populations from the Americas produce only saxitoxin and analogues (Lagos *et al.* 1999; Haande *et al.* 2008; Piccini *et al.* 2011).

Hypotheses advanced to explain the expansion of *C. raciborskii* include climate change-associated water temperature increases (Wiedner *et al.* 2007), ecophysiological plasticity (Briand *et al.* 2004; Bonilla *et al.* 2012) and the existence of ecotypes with different environmental preferences and tolerances (Chonudomkul *et al.* 2004; Piccini *et al.* 2011). Blooms of this cyanobacterium have been detected in several water systems in Uruguay, including those that are commonly used as potable water source, such as the shallow lake Laguna Blanca (Maldonado, Uruguay).

In this study, we aimed to (i) study past shifts in the prokaryotic community of Laguna Blanca using molecular techniques; (ii) determine whether a gene (*sxtU*) belonging to the saxitoxin-encoding cluster could be detected and applied as a marker of the presence of toxic cyanobacteria in the past; and (iii) identify the cyanobacteria containing the *sxtU* gene. To this end, we examined a sediment core from Laguna Blanca from which we extracted aDNA and applied molecular techniques to analyse prokaryotic assemblages. Shifts in assemblages over the past 200 years were addressed by denaturing gradient gel electrophoresis (DGGE) of the ribosomal intergenic transcribed spacer (ITS) region. In addition, aDNA samples were used to detect genes encoding for saxitoxin biosynthesis (*sxtU*) by quantitative real-time PCR (qPCR) as an indicator of the presence of saxitoxin-producing cyanobacterial populations. In samples where *sxtU* was detected and quantified, ribosomal ITS clone libraries were constructed and sequenced to infer the presence of saxitoxin-producing cyanobacterial species.

Materials and methods

Sampling site

Laguna Blanca is a subtropical lagoon located along the eastern coast of Uruguay (34°54′S; 54°50′W) that serves as drinking water supply. Water temperature varies from 10 to 28 °C (annual mean temperature 19.7 °C) (Mazzeo *et al.* 2003; Pacheco *et al.* 2010). The lagoon's depth ranges from 2.1 to 3.2 m, and it has a total area of 28.7 ha; catchment land-use types include cattle grazing, forestation with *Eucalyptus* spp. and tourism. Previous sediment core studies in Laguna Blanca have reconstructed past changes related to anthropogenic processes in its catchment (García-Rodríguez *et al.* 2002, 2004). Fossil diatom community analysis indicated that the lagoon was mesotrophic due to intensive cattle ranching and soil removal until the late 1960s, but that it became eutrophic after ~1966 as a consequence of forestry activities in its catchment (García-Rodríguez *et al.* 2002). During the early 1990s, the lagoon's phytoplankton biomass was dominated by cyanobacteria, which were replaced by increased macrophyte growth (*Egeria*

densa) after a severe drought in 1997, and later by periodic blooms of *Cylindrospermopsis raciborskii* and *Microcystis* spp. that imply a risk to the potabilization of water (Mazzeo *et al.* 2003; Vidal & Kruk 2008; Pacheco *et al.* 2010). In 2006, the lagoon became eutrophic to hypereutrophic (total phosphorus: 107.5 ± 20.7 μg/L, phytoplankton chlorophyll *a*: 36.4 ± 7.6 μg/L) with *C. raciborskii* as one of the major phytoplankton species (Pacheco *et al.* 2010). The *C. raciborskii* strain MVCC14, isolated from Laguna Blanca in 2007, produces high concentrations of saxitoxin, a paralytic shellfish poisoning (PSP) cyanotoxin present in South American populations of the species (Piccini *et al.* 2011). In March 2011, *C. raciborskii* (6 mm^3/L) and saxitoxin (0.5 μg/L, ELISA test) were recorded in Laguna Blanca phytoplankton samples (S. Bonilla & A. Fabre, personal communication).

Organic matter and chlorophyll a determinations

A 65-cm sediment core was obtained in June of 2009 using an Aquatic Research Instruments (Hope, Idaho) gravity corer and a new tenite butyrate tube and sealed hermetically for transport to the laboratory. In the laboratory, the core tube was split and opened lengthwise, and the exterior sediments of the core were removed to preclude contamination resulting from contact with the tube. Metal discs were then used to cut 1-cm sections of the core that were removed and immediately placed into sealed bags; these samples were stored frozen at −20 °C until analysis. All working surfaces and sampling instruments were thoroughly cleaned with 70% ethanol before sampling and between each subsample. Analysis of organic matter (OM) content by loss-on-ignition (Dean 1974) was performed on 48 samples. Chlorophyll *a* concentrations were determined with a spectrophotometer (Thermo Evolution 6.0, USA) after extraction with acetone (90%) of lyophilized samples (28 selected sections from 0 to 65 cm) according to the methods of Jeffrey *et al.* (1997). Sediment pigment and DNA concentrations are expressed relative to the OM content of sediments to preclude any effects on concentrations of changing allochthonous sedimentation that likely accompanied the development of the catchment.

Sampling

Based on the OM and chlorophyll *a* profiles, four strata were selected for aDNA extraction that followed the protocol described by Zhou *et al.* (1996) using 10 g of sediment (wet weight) per sample. Samples were selected from horizons that suggested that they represented different stages in Laguna Blanca's evolution: two recent samples during the period of human disturbance including one with high concentrations of OM and chlorophyll *a* (i.e. 3–4 cm) (Fig. 1) and one with lower concentrations of OM and chlorophyll *a* but still elevated relative to older samples (12–13 cm); and two samples that preceded human disturbance of the lagoon and its catchment, one with low concentrations of OM and chlorophyll *a* typical of the lower section of the core (60–61 cm) and one from sediments near the base of the core where the chlorophyll *a* profile showed a local maximum suggesting higher biomass (61–62 cm; Fig. 2). We cross-correlated key horizons in our sedimentary OM and chlorophyll *a* profiles with those from the dated record of García-Rodríguez *et al.* (2002), and based on their ^{210}Pb age model, we assigned approximate ages of 2007, 2000, 1852 and 1846, respectively, to these strata.

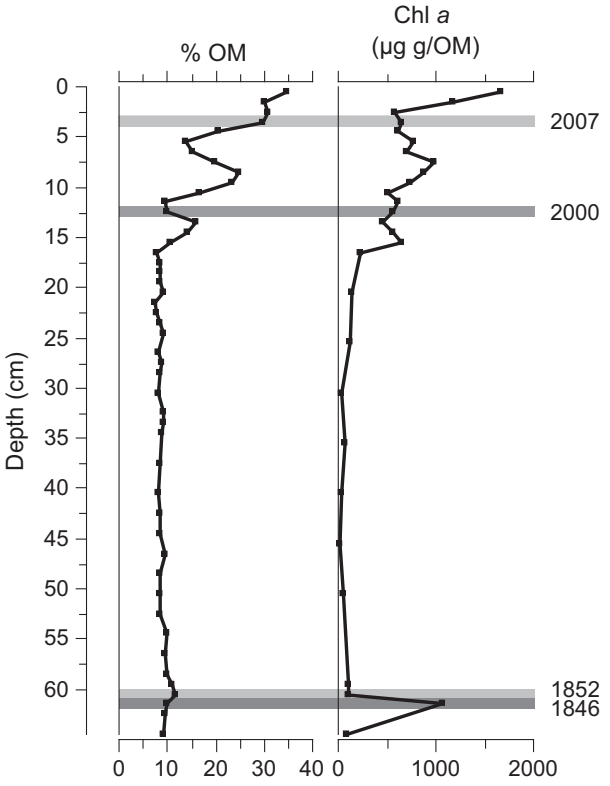

Fig. 1 Organic matter (OM) profile of the sediment core as a percentage (%) of dry weight (left panel), and chlorophyll *a* concentrations (right panel) in 28 selected samples of the sediment core, expressed per μg/g of OM. Grey bars indicate strata from which samples for DNA extraction and molecular analyses were taken.

Laboratory controls to prevent contamination

Precautions were taken to avoid sample contamination during all aDNA extraction, PCR amplification, ribosomal ITS sequencing and qPCR steps. All aDNA extraction steps were carried out under a vertical lami-

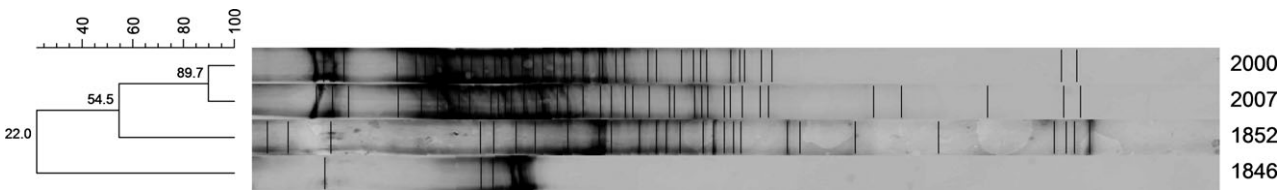

Fig. 2 Representative denaturing gradient gel electrophoresis (DGGE) image (right) and cluster analysis (left) showing band profiles obtained in each sediment section. UPGMA similarities between samples are shown at the nodes. The black lines show the band positions assigned by GelCompar II software.

nar flow cabinet (Holten HB2436) located in a separate room and exclusively used for DNA- and RNA-based studies, and whose surface was previously irradiated for 30 min using the built-in UV lamp. Sediment subsamples were taken with autoclaved stainless steel spoons in new, sterile plastic tubes. PCRs for DGGE and ribosomal ITS analyses were prepared in a separate room using a vertical laminar flow cabinet (ESCO Class II, Type A2) whose surface was previously irradiated using the built-in UV lamp for 30 min. This cabinet is only used to prepare PCRs, and the micropipettes, tips and other materials used are stored inside the cabinet and were also UV-irradiated each time PCRs were prepared. Negative blank controls using bidistilled pyrogen-free PCR water instead of aDNA were included in each DGGE, qPCR and PCR run.

aDNA extraction

For the aDNA extraction, sediment samples were transferred into new 50-mL Falcon tubes. In brief, the procedure involved the addition of extraction buffer containing 1% CTAB (Hexadecyltrimethylammonium bromide), EDTA and proteinase K to each sample tube and incubation at 37 °C during 30 min on a shaker. After that, sodium dodecyl sulphate (SDS) was added, and the mix was incubated at 65 °C for 2 h. Once the incubation finished, nucleic acids were separated using chloroform/isoamylalcohol extraction (three times). After collecting the aqueous upper phase, DNA was precipitated with isopropanol at room temperature. After centrifugation, the obtained pellet was washed with cold 70% (v/v) ethanol, dried and suspended in 1× TE overnight at 4 °C. The concentration and purity of aDNA were determined spectrophotometrically at 260 and 280 nm (NanoDrop), and aDNA samples were stored at −20 °C.

Denaturing gradient gel electrophoresis of the internal transcribed spacer (DGGE-ITS)

Bacterial composition and comparison of the selected strata was performed using DGGE of the ribosomal ITS (Muyzer & Smalla 1998; Janse *et al.* 2003; Kan *et al.* 2011). The primers used for PCR amplifications and DGGE were those described by Iteman *et al.* (2000) with a GC clamp attached to the forward primer, 5′-TGT ACA CAC CGC CCG TC CGC CCG CCG CGC GCG GCG GGC GGG-3′. Amplifications were performed in a 2720 Applied Biosystems thermal cycler. PCR conditions were an initial step of 5 min at 94 °C and then 33 cycles consisting of 30 s at 94 °C, 30 s at 48 °C and 1 min at 72 °C. A final extension step of 10 min at 72 °C was also performed. Amplification products, ranging from 500 to 600 bp, were applied to a 40% polyacrylamide gel in a denaturing urea gradient from 70% to 40% and run at 70 V and 60 °C for 12 h. Gels were stained with SYBR green, and images were taken using a FujiFilm Starion FLA 9000 Image Scanner. DGGE using the same samples was performed with different urea gradients and, after selecting the optimum, PCRs and their corresponding gels were repeated and one of them was chosen as a representative for community analyses. Images were analysed by GelCompar II software (Applied Maths, version 6.5). Jaccard's similarity relationships (Jaccard 1908) based on cluster analysis (UPGMA, Sneath & Sokal 1973) were calculated using the Past software package (Hammer *et al.* 2001). As the ribosomal ITS sequences reflect changes at the intraspecific level (Fisher & Triplett 1999; Kataoka *et al.* 2013), we considered DGGE bands as bacterial operational taxonomic units (OTUs) representative of predominant bacterial populations or 'strains' (Reche *et al.* 2005). The resulting profiles were normalized to the ladder (1 kb Plus; Fermentas).

Real-time quantitative PCR (qPCR)

aDNA from the same strata selected for DGGE sediment samples was used in qPCR for the specific detection and quantification of the *sxtU* gene involved in saxitoxin synthesis (Kellmann *et al.* 2008). Two microlitres of aDNA from each sample were applied to the Power SYBR Green PCR (Invitrogen) using primers sxtUF—ACTCCCAGAACATTCACATCG and sxtUR—GGAATTGGTGTGTTTGGTGC, which give an amplicon of 79 bp, designed and optimized in our laboratory (Vico *et al.* in prep.). Final reaction volume was 20 μL,

and the 96 FLX Touch™ thermal cycler (Bio-Rad) was used. Cycling conditions were 2 min at 50 °C, 15 min at 95 °C and 40 cycles of 15 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C. Melting curves were obtained after a melting step from 65 to 95 °C, at increases of 1 °C each 4 s. To quantify the *sxtU* gene in aDNA from the four sediment samples, we cloned the amplicon obtained with the described primers into the TOPO TA 2.1 vector (Invitrogen) (see below), and the plasmids containing the cloned *sxtU* were used as calibrators. To quantify the copy number of *sxtU* in the sample, five serial dilutions (from −10 to −100 000) of the cloned *sxtU* amplicon were performed and applied to qPCR using the sxtUF and sxtUR primers. Each dilution was amplified in duplicate, and their threshold cycle (TC) values were used to perform a standard curve, where the TC values were plotted against the dilutions. Data were fitted to a straight line, achieving a correlation coefficient (R^2) of 0.992. This plot was used as standard or calibration curve for extrapolating the number of *sxtU* gene copies in the samples. Amplification efficiency obtained for the standard curve was always ≥0.85.

Cloning of the sxtU gene

To clone the *stxU* gene, a culture strain of *C. raciborskii*, isolated from Laguna Blanca, was used. The *C. raciborskii* strain MVCC14 was grown as described in Piccini *et al.* (2011) until reaching stationary phase, when cyanobacteria filaments were collected by centrifugation and DNA was extracted as described above (see Sampling and aDNA extraction). The *sxtU* gene was amplified by PCR using sxtUF and sxtUR primers as described above, and amplicons were run in a 1% agarose gel electrophoresis and gel-extracted using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Purified amplicons were cloned using the TOPO 2.1 vector (TOPO-TA cloning kit; Invitrogen) and competent TOP10 *Escherichia coli* cells delivered with the kit as described by the manufacturer. White-blue screening of clones was used to select those clones having an insert. Clones having right-sized inserts (79 bp) were selected by PCR and agarose gel electrophoresis and sequenced at the Institut Pasteur de Montevideo. Quantification of clones used as calibrators in qPCR was performed according to Rinta-Kanto *et al.* (2005).

Ribosomal ITS clone libraries and sequencing

Clone libraries were constructed using the aDNA extracted from the 3- to 4-cm and 12- to 13-cm samples (see Sampling and aDNA extraction), which had approximate ages of 2007 and 2000, respectively, calculated based on the chronology in García-Rodríguez *et al.* (2002). aDNA was subjected to PCR amplification using the ITS bacterial primers described by Iteman *et al.* (2000) and the same cycling conditions described above for DGGE analyses. Negative controls using bidistilled pyrogen-free PCR water instead of aDNA were included in each PCR run. All PCRs were carried out using the same protocols and precautions for the vertical laminar flow cabinet described above (e.g. DGGE-ITS section). Amplifications were performed in a 2720 Applied Biosystems thermal cycler. The PCR products were checked by agarose (1%, wt/vol) gel electrophoresis. The ITS amplification from the 3- to 4-cm sample gave two amplicons having *c.* 500 and 600 bp, while the sample from 12 to 13 cm depth gave a unique band of *c.* 500 bp. All the obtained bands were excised from the gel and purified with a QIAquick Gel Extraction kit (QIAGEN). One amplicon from every sample was used to prepare each library; therefore, a library corresponded to a single PCR. The purified amplicons were inserted into the pGEM vector (PGEM-T cloning kit; Promega) and cloned into competent *E. coli* cells delivered with the kit as described by the manufacturer. White clones were picked, and plasmids were extracted with a QIAprep Spin Miniprep kit (QIAGEN) according to the manufacturer's specifications. Ninety-six clones from each library (a library corresponded to a cloned band) were analysed for correctly sized inserts by PCR using the vector primers (M13F and M13R) and screened by agarose gel electrophoresis of the amplicons (1%). Thirty-six clones from the 3- to 4-cm sample and 46 from the 12- to 13-cm sample produced high-quality sequences using the primer M13F and M13R (Macrogen Inc., Korea).

Sequencing and phylogenetic analysis

DNA sequences were assembled and edited using DNA Baser Sequence Assembly software (Heracle BioSoft). The multiple sequence alignment tool from CLUSTALW (Larkin *et al.* 2007) was used to align the sequences. Before the phylogenetic analyses, a ribosomal ITS database enriched in cyanobacterial sequences obtained from GenBank was generated. After that, the obtained sequences were aligned to the constructed database using MEGA version 5 (Tamura *et al.* 2011). The bioinformatics analysis of the sequences obtained from cloned ITS amplicons showed that a region of the sequences had larger differences when related to the ITS database, resulting in poor alignments. As these differences could be attributed to the propagation of damaged-induced errors during amplifications, we decided to exclude these portions of the sequences from phylogenetic analyses. By doing so, we expected that the differences observed in sequence

alignment would reflect phylogenetic differences. Alignments, which comprised 300 bp, were checked manually, and maximum-likelihood (ML) phylogenetic analyses were conducted using the software MEGA 5.

Results and discussion

Organic matter and chlorophyll a profiles

Sedimentary OM and chlorophyll *a* were used as general indicators of changes in ecosystem biomass, and their profiles were reflective of the productive nature of Laguna Blanca. OM ranged from 7.5% to 34.6% of sediments (mean: 12.6%), while chlorophyll *a* ranged from 16.5 to 1672.9 μg/g OM, with a mean of 506.8 μg/g OM. Concentrations of both variables were generally low below 16 cm sediment depth (inferred date: 1996), with a sharp increase above this horizon that continued to the modern sediment surface (Fig. 1). The two variables were significantly correlated (Spearman, $r_s = 0.69$; $P < 0.05$). The OM and chlorophyll *a* profiles reflected changes in the lagoon's history as recorded in both limnological and palaeolimnological studies (García-Rodríguez *et al.* 2002, 2004; Vidal & Kruk 2008; Pacheco *et al.* 2010). Laguna Blanca was mesotrophic until the mid-1960s when eutrophication began as a consequence of livestock grazing and fertilization during afforestation (García-Rodríguez *et al.* 2002). Oscillations in OM and chlorophyll *a* between ~1997 (15–16 cm) and ~2007 (Fig. 1) record the dramatic increase of macrophyte biomass following a severe drought in 1997–1998 (Mazzeo *et al.* 2003). High chlorophyll *a* and OM values then reflect the recovery of the water level in subsequent years and the consequent increase in phytoplankton biomass (Mazzeo *et al.* 2003; Pacheco *et al.* 2010). The highest values recorded near the surface suggest that the trend of increasing phytoplankton biomass found in 2006 by Pacheco *et al.* (2010) has continued. Pigment analyses, however, cannot provide species-specific information about community change (Leavitt & Hodgson 2001), and we thus investigated the potential of aDNA analyses for reconstructing microbial changes at finer taxonomic resolution.

Ancient DNA extraction and bacterial community analyses

The successful recovery of aDNA from samples spanning nearly two centuries suggests that the applied techniques are an appropriate tool examining past microbial communities in Laguna Blanca. The sample from *c.* 2007 contained the highest aDNA concentration (18 μg/g OM), followed by the sample from the year 2000 (14 μg/g OM), while the amounts of aDNA retrieved from the samples from ~1852 and ~1846 were similar, with 1.1 μg/g OM to 1.7 μg/g OM, respectively. We confirmed the recovery of genomic aDNA by agarose gel electrophoresis, which showed high molecular weight DNA bands with more than 12 000 bp in the case of the most recent samples and a smear between 250 and 3000 bp in the case of the two older samples (data not shown). The size of the aDNA fragments obtained from the different strata may reflect the longer time for which older sections of the core were exposed to degradation. These differences in aDNA degradation could be explained by the variability in the fragmentation of aDNA between algal groups observed by Boere *et al.* (2011). This can be overcome if relatively short gene fragments are analysed to minimize PCR bias, lower the detection limit and enable palaeogenomic studies. Studies have already shown the effectiveness of DGGE for the analysis of ribosomal genes (e.g. 16S or 18S rRNA; Coolen *et al.* 2006; Boere *et al.* 2011); however, due to the conserved nature of ribosomes and the need to amplify short sequences, it may be necessary to target more variable, faster-evolving phylogenetic marker genes, such as the ribosomal ITS.

While the analysis of ITS sequences has been widely used to explore shifts at the species or strain level in planktonic bacterial communities (Ferris *et al.* 2003; Brown & Fuhrman 2005; Kan *et al.* 2007; Alonso *et al.* 2010; Wu *et al.* 2010), its combination with DGGE separation has been used in only a few studies addressing, for example, differences at the strain level in Cyanobacteria (Janse *et al.* 2003; Kan *et al.* 2011; Kataoka *et al.* 2013). The combination of the variability in size displayed by the ribosomal ITS with sequence discrimination obtained by DGGE produces a high-resolution pattern allowing the detection of short-scale microdiversity, such as the existence of different strains and ecotypes. Thus, the methodology employed makes high-resolution determinations of the bacterial community possible. Using this approach in our study, we found that assemblages from the early 21st century were strikingly different from those of the 19th century (Fig. 2). OTU richness (assessed as band richness) was 39, 39, 27 and 5 for the samples from 2007, 2000, 1852 and 1846, respectively. Cluster analysis based on presence/absence of bands revealed that the composition of prokaryotic communities of samples from 2000 and 2007 was 89% identical. The composition of communities from *c.* 1852 had a 54% similarity to that of the samples from the most recent cluster; however, the band patterns from the *c.* 1846 sample had only a 22% similarity compared to the other three (Fig. 2).

Several possible hypotheses may explain the difference in richness observed between the oldest sample

Table 1 Percentage of organic matter (OM, %), chlorophyll *a* content (chl *a*), number of DGGE bands (OTU richness) and number of *sxtU* copies found in each assessed sample normalized by gram of OM; detection of sequences belonging to Cyanobacteria and to saxitoxin-producer species *Cylindrospermopsis raciborskii* in the ribosomal intergenic transcribed spacer (ITS) clone libraries constructed from the samples where *sxtU* was detected

Sample (cm)	Estimated year of sample midpoint	OM (%)	Chl *a* (μg/g OM)	OTU richness	Copies of *sxtU* per g OM (average ± SD)	Presence of Cyanobacteria sequences in clone libraries	Presence of *C. raciborskii*-related sequences in clone libraries	Cyanobacteria registered in the lagoon	Human activities
3–4	2007	29.7	644.8	39	5.6×10^5 ($\pm 3.3 \times 10^5$)	Yes	No	2004 to 2007—*C. raciborskii*[†‡] 2006—*Microcystis* and *C. raciborskii*§	Increased urban development. Increased forestation with *Eucaliptus* spp.
12–13	2000	10.1	554.6	39	5.3×10^6 ($\pm 2.1 \times 10^6$)	Yes	Yes	Cyanobacterial blooms for several years prior to 1997[¶]; No data from 1997 to 1999	Soil removal and watershed erosion Replacement of sand dunes by forestation with exotic species. Reduction of lake area due to overuse of drinking water facility[††]
60–61	1852	11.7	113.6	27	0	NA	NA	No information	Little to no human impact.
61–62	1846	10.1	1074.1	5	0	NA	NA	No information	Very scarce[‡‡]

NA, not assessed; OTU, operational taxonomic unit.
[†]Piccini *et al.* (2011),
[‡]Vidal & Kruk (2008),
[§]Pacheco *et al.* (2010),
[¶]Mazzeo *et al.* (2003),
[††]García-Rodríguez *et al.* (2002),
[‡‡]Country population in 1830: 74.000 inhabitants.

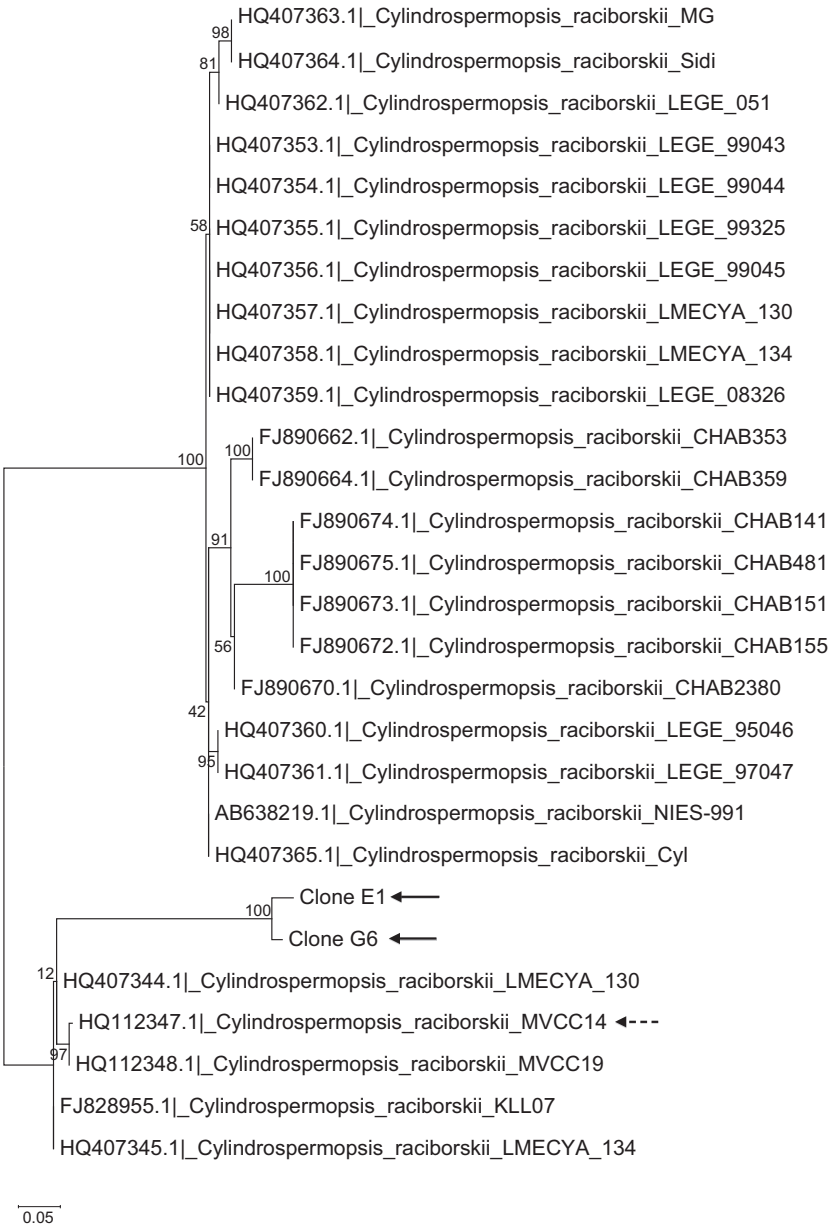

Fig. 3 Phylogenetic tree of the ribosomal intergenic transcribed spacer (ITS) sequences showing the identities to *Cylindrospermopsis raciborskii* strains found in Laguna Blanca in ~2000. The alignment was performed using 300 bp, and phylogenetic analysis was based on a maximum-likelihood test. Black filled arrows show the clones obtained in this study, and dotted arrow indicates the modern *C. raciborskii* strain (MVCC14) isolated from the same ecosystem (Piccini *et al.* 2011).

and the other three. It has been shown that ITS size in cyanobacteria ranges between *c.* 300 and 700 bp (Iteman *et al.* 2000; Janse *et al.* 2003). Poor aDNA preservation could therefore be invoked based on the fragment size of the aDNA obtained from older samples, which in turn would result in lower amounts of ribosomal ITS amplicons. The degradation of aDNA in lake sediments to shorter fragments usually occurs within the first several thousand years after deposition (Coolen & Overmann 1998), with cold, anoxic bottom waters with limited light penetration favouring preservation (Coolen *et al.* 2004; Coolen & Gibson 2009). It has thus been recommended that fragments of less than 500-bp aDNA be analysed in sediment aDNA (Coolen & Gibson 2009). Although our samples were less than two centuries old, Laguna Blanca's conditions (i.e. eutrophic to hypereutrophic, warm subtropical climate) imply less than ideal conditions for DNA preservation. Its depth (2–3 m maximum) also implies that the water column is mixed by wind and stable stratification and bottom oxygen depletion is unlikely. The lower number of bp in older samples may be due to nucleic acid degradation, which could hinder an exhaustive detection of bacterial ITS analysis and would give lower OTU richness in deeper samples. Moreover, the formation of resistant structures (bacterial spores, cyanobacterial akinetes, etc.) may favour the preservation of nucleic acids of certain species and potentially result in some of the observed

OTUs. Nevertheless, during the period of high density of rooted macrophyte growth (Mazzeo *et al.* 2003), or short-term stratification periods, Laguna Blanca's sediment–water interface may have been subject to anoxia, favouring DNA preservation even in warm climate conditions. The successful recovery in Laguna Blanca of aDNA suitable to produce ITS bands from all samples (ranging from 250 to 3000 bp in the case of ~1852 and ~1846 samples) underlines the potential of the sedimentary record of subtropical lakes to record differences between bacterial communities, at least on centennial scales.

Regardless, despite similarly low fragment sizes in the two 19th century samples, the sample from 1852 had 27 OTU versus only five in the 1846 sample, and each was likely subject to similar degradation processes during the last one and a half centuries. Therefore, while degradation may be responsible for the reduced fragment size observed between 21st and 19th century samples, it does not appear to account for the differences in richness. As an alternate hypothesis, the sharp peak in chlorophyll *a* in the 1846 sample may suggest high biomass under bloom conditions, leading to the dominance of a small number of species and resulting in the lower observed OTU richness. The difference in bacterial composition (as inferred from OTUs) between *c.* 1852 and 21st century sediments may be linked to the biological effects of recent human-driven eutrophication of the lagoon. During the first half of the 18th century, the population of Uruguay was ~74 000 people (Barrán 1995), and Laguna Blanca's catchment was not impacted by humans until ~1880, implying that both direct and indirect human impacts on the lagoon were negligible at the time of deposition of our older samples.

Phylogenetic analysis and evidence of the presence of Cylindrospermopsis raciborskii

The most common cyanobacterial species found at Laguna Blanca after the drought that occurred in the 1990s was the saxitoxin-producer *C. raciborskii* (Vidal & Kruk 2008; Pacheco *et al.* 2010). However, most of the ribosomal ITS sequences obtained from sediments corresponding to *c.* 2000 and 2007 were affiliated with heterotrophic bacteria, and cyanobacterial sequences related to *C. raciborskii* were found only in the 2000 sample (Table 1). The low abundance of cyanobacteria sequences retrieved and the absence of *C. raciborskii* sequences from ~2007 samples may be a consequence of the number of clones sequenced, which may not have been enough to retrieve more cyanobacteria genes in this sample. Given that the OM content in the 2007 sample was almost three times that of the 2000 sample (i.e. 29.7% vs. 10.1%), it is likely that the amount of DNA belonging to heterotrophic and other autotrophic microbes diluted the cyanobacteria DNA, making the recovery of *C. raciborskii* clones more difficult. As such, the detection of DNA belonging to a particular cyanobacteria species may simply require a greater sequencing effort.

According to Blast analysis, two clones from the ~2000 sample shared ≥98% identity to the closest *C. raciborskii* relative (Accession nos KJ738307 for clone G6 and KJ738308 for clone E1). The sequences obtained in our study were closely related to sequences of modern populations previously isolated from Laguna Blanca (MVCC14) and from another Uruguayan lake (MVCC19; Lago Javier, Canelones) in 2007 (Fig. 3). The *C. raciborskii* ITS sequences from our study grouped together on the tree with modern strains from Uruguay, Asia and Europe.

In a previous study, we described a strain of *C. raciborskii* isolated from Laguna Blanca that, after comparison with isolates from other aquatic systems, led us to propose that phenotypic and genetic variability of *C. raciborskii* populations is linked to the existence of different ecotypes whose success is subject to the local environmental conditions (Piccini *et al.* 2011). The phylogenetic differences found between sequences cloned from our sediments and those from modern populations of *C. raciborskii* may therefore be related to the existence of different strains or ecotypes in the recent past of Laguna Blanca.

Use of the saxitoxin-encoding gene sxtU as a proxy of C. raciborskii presence

To detect and quantify potentially toxic *C. raciborskii* or other saxitoxin-producing Cyanobacteria (e.g. *Aphanizomenon)*, we performed a real-time qPCR assay based on the *sxtU* gene that encodes for saxitoxin synthesis (Kellmann *et al.* 2008). Because each cyanobacterial cell has one genomic copy of the saxitoxin cluster, the number of *sxtU* copies should be a proxy of the abundance of saxitoxin-producing cyanobacteria, and *sxtU* detection may therefore allow the reconstruction of historical trends of saxitoxin-producing species, including *C. raciborskii*. *sxtU* was detected in samples from ~2007 and ~2000, with $5.6 \pm 3.3 \times 10^5$ and $5.3 \pm 2.1 \times 10^6$ copies of *sxtU* per g OM, respectively (Table 1), but the gene was not detected in either of the two 19th century samples. While no historical data are available to corroborate the absence of *C. raciborskii* during the 19th century, blooms were observed in Laguna Blanca in the years preceding both 2007 and 2000 (Mazzeo *et al.* 2003; Vidal & Kruk 2008; Pacheco *et al.* 2010). The *sxtU* record therefore agrees qualitatively with the existing data

regarding *C. raciborskii* in Laguna Blanca and suggests its utility as a tool for reconstructing past *C. raciborskii* presence. However, the concentration of *C. raciborskii* cells was not determined prior to 2004 in limnological studies and so we are unable to evaluate the relationship between its abundance and the number of *sxtU* copies in the sediments. Further studies of well-dated, high-resolution cores in lakes with historical records of *C. raciborskii* abundance are needed to confirm the potential of *sxtU* as a tool for quantifying the dynamics of toxic cyanobacteria in the past.

As mentioned above, ribosomal ITS variability in prokaryotes is linked to differences between strains, subspecies or ecotypes (Ferris *et al.* 2003; Kan *et al.* 2007; Alonso *et al.* 2010; Wu *et al.* 2010). The identification of *C. raciborskii* with the potential for saxitoxin production in samples from the past and the determination of their association to present populations of the same ecosystem may help understand the environmental filters that select modern *C. raciborskii* populations. Moreover, these data may help to determine the influence of anthropogenic processes in driving microbial changes in subtropical freshwater ecosystems. These techniques, applied in palaeolimnological studies on broad spatial scales, may also provide data critical to resolving outstanding controversies about the evolution and global dispersal of toxic cyanobacterial species. Our study reinforces the usefulness of the aDNA-based methods to study past ecosystem changes and emphasizes the importance of prokaryotic communities as indicators of past trophic change in aquatic ecosystems.

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Data accessibility

Sampling location: 34°53′57″S, 54°50′15″W. DNA sequences: ribosomal ITS sequences are currently available at GenBank with Accession nos KJ738307 for clone G6 and KJ738308 for clone E1. Organic matter and chlorophyll *a* concentrations, data used for phylogenetic tree, DGGE matrix data and qPCR data: Dryad entry doi: 10.5061/dryad.q2b2 m.