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DOI
10.1007/s00792-018-1026-7

Publication date
2018

Document Version
Accepted author manuscript

Published in
Extremophiles: life under extreme conditions

Extremophiles (2018) 22:651–663
https://doi.org/10.1007/s00792-018-1026-7

ORIGINAL PAPER

Effect of salinity on diazotrophic activity and microbial composition of phototrophic communities from Bitter-1 soda lake (Kulunda Steppe, Russia)

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Received: 26 February 2018 / Accepted: 6 April 2018 / Published online: 16 April 2018

Abstract
Bitter-1 is a shallow hypersaline soda lake in Kulunda Steppe (Altai region, Russia). During a study period between 2005 and 2016, the salinity in the littoral area of the lake fluctuated within the range from 85 to 400 g/L (in July of each year). Light-dependent nitrogen fixation occurred in this lake up to the salt-saturating conditions. The rates increased with a decrease in salinity, both under environmental conditions and in laboratory simulations. The salinities below 100 g/L were favorable for light-dependent nitrogen fixation, while the process was dramatically inhibited above 200 g/L salts. The analysis of *nifH* genes in environmental samples and in enrichment cultures of diazotrophic phototrophs suggested that anaerobic fermenting and sulfate-reducing bacteria could participate in the dark nitrogen fixation process up to soda-saturating conditions. However, we cannot exclude the possibility that haloalkaliphilic nonheterocystous cyanobacteria (*Euhalothece* sp. and *Geitlerinema* sp.) and anoxygenic purple sulfur bacteria (*Ectothiorhodospira* sp.) might also play a role in the process at light conditions. The heterocystous cyanobacterium *Nodularia* sp. develops at low salinity (below 80 g/L) that is not characteristic for Bitter-1 Lake and thus does not make a significant contribution to the nitrogen fixation in this lake.

Keywords Nitrogen fixation · Phototrophic bacteria · Sulfate-reducing bacteria · Hypersaline · Soda lakes

Introduction

The equatorial soda lakes are considered to be the most productive types of an ecosystem on the Earth showing primary production rates in excess of 10 g/(m^2 day) (Grant 2006; Melack 1981). The primary production of organic matter in the soda lakes of the cryoarid climate during summer time is not so high and is up to 3.86 g/(m^2 day) according to Namsaraev et al. (2015). Nitrogen availability is one of the most important limiting factors for primary production (Axler et al. 1978). Detection of high concentrations of ammonium in many soda lakes, which easily escapes to atmosphere at high pH (Jellison et al. 1993), as well as the functioning of nitrifying/denitrifying bacteria (Sorokin and Kuenen 2005; Boltyanskaya et al. 2007; Shapovalova et al. 2008) indicates the operation of an active nitrogen cycle in these extreme ecosystems. Moderately haloalkaliphilic heterocystous cyanobacteria capable of fixing nitrogen under aerobic light conditions are abundant in soda lakes at salt concentrations below 70 g/L. Various species of *Anabaenopsis*, *Cyanospira*, and *Nodularia* are abundant in the plankton of the moderately saline soda lakes of the East African Rift and South Siberia (Krienitz and Schagerl 2016; Tsyrenova et al. 2011), while a dominance of heterocystous cyanobacteria (primarily *Anabaenopsis* spp.) in shallow soda lakes of the Kulunda Steppe (Southern Siberia, Russia) was noted as

Communicated by A. Oren.

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early as in the 1920s–1930s at salinities up to 50 g/L (Voronikhin 1929; Isachenko 1934).

At the salinities higher than 70 g/L, the heterocystous cyanobacteria are not usually detected (Oren 2011a). At these conditions, the diazotrophic bacteria are represented by heterotrophic microaerobes and anaerobes (Sorokin et al. 2014a, b, 2015a; Sorokin 2017). A study of the *nifH* gene diversity in the highly mineralized lakes of the Kulunda steppe and the Wadi Natrun (Egypt) showed the presence of genes belonging to representatives of various phyla, including anoxygenic purple sulfur and nonsulfur proteobacteria, green nonsulfur bacteria, heliobacteria, and oxygenic cyanobacteria, as well as anaerobic heterotrophic bacteria (Tourova et al. 2014). The activity of light-stimulated and light-independent nitrogen fixation in aerobic and anaerobic conditions was detected at salinity 90–100 g/L in bottom sediments and floating phototrophic biomass in the alkaline Mono Lake (USA) (Oremland 1990; Herbst 1998). Further increase of salinity inhibited the activity of light-dependent fixation of nitrogen in Mono Lake (Herbst 1998). Yet, it is still unclear which groups of prokaryotes are responsible for nitrogen fixation in hypersaline alkaline lakes, including lakes of Kulunda Steppe.

Unlike bigger and more stable Mono Lake, the shallow Kulunda lakes have significant annual fluctuations of salinity. We assumed that the haloalkaliphilic microbial communities in the Kulunda Steppe lakes should be diazotrophically active in a wide range of salinity to support the functioning of a nitrogen cycle. To test this hypothesis, we studied a hypersaline soda lake Bitter-1 located in the southern part of the Kulunda Steppe (Altai region, Russia). During the period of our studies (2005–2016), the total salt concentration of the lake brines fluctuated between 85 and 400 g/L, the total soluble alkalinity from 1 to 4 M, and the pH from 9.6 to 10.5 (all measurements were made in July of each year). It gave us an opportunity to study the influence of salinity on the in situ nitrogen-fixing activity and the composition of diazotrophic phototrophic communities developing in this unique hypersaline and hyperalkaline habitat.

Materials and methods

Study site

Lake Bitter-1 belongs to the Mikhailovsky group of soda lakes located 20 km east of the Russian-Kazakhstan border in the Kulunda Steppe (Altai Region, south-western Siberia, Russia) (Fig. 1). It has the following coordinates: 51°40′19.1″N 79°54′20.4″E. Four small shallow soda lakes under the general name of Bitter (or "Gorchiny" in Russian pronunciation) are located about 7 km to the east from the village of Malinovoe Ozero and Tanatar soda lake system

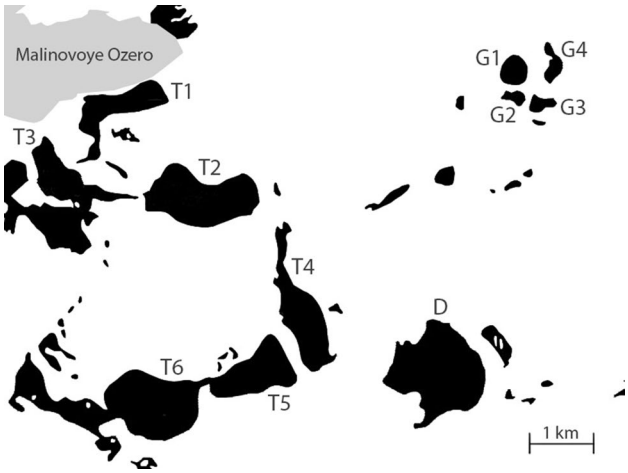

Fig. 1 Map showing a location of the lakes in the area of the Malinovoye Ozero village. *T1–T6* Tanatar 1 to Tanatar 6 soda lakes, *G1–G4* Bitter-1 to Bitter-4 lakes, *D* Demkino lake

and 4 km to the north from the freshwater lake Demkino. The Bitter lake system is formed in a drainless trona-cemented sand basin and surrounded by a pine forest. During the period of our studies, the Bitter-1 and Bitter-3 were lakes with soda brines, Bitter-2 was a dry soda pan, and Bitter-4 was a hyposaline soda lake. The microbiological study of these lakes have started in 2003 (Foti et al. 2007, 2008; Sorokin et al. 2008a, b, 2014a, b, 2015a; Kompantseva et al. 2010; Samylina et al. 2014; Tourova et al. 2014). Earlier research is not known to us; however, it was exploited by the local residents, since the 1940s as a trona mineral resource.

Samples of the surface layer of sediments, microbial mats, and overlaying brines were collected from the littoral area of the Bitter-1 lake. The depth of the lake was less than 15 cm at the sampling site. Acetylene-reduction activity assays were conducted upon collection in the field and, in addition, in the laboratory experiments with different salinities. All collected samples were stored at ambient temperature until their analysis in the laboratory (max. storage time—5 days). Salinity and pH of the brines were measured using a WTW field potentiometer–conductometer. For the hypersaline conditions, the average pH values between the native brine and its 1:5 dilution was used. The total salinity values were additionally verified gravimetrically in the laboratory after the brine filtration through syringe 0.45 µm filters, and the average of the values obtained by the two methods was used. The soluble carbonate alkalinity was determined in the field by a two-step titration with 1 M HCl: (1) down to pH 8.0 (carbonate alkalinity) and (2) further down to pH 4.0 (the bicarbonate formed from carbonate + native bicarbonate alkalinity) (Reznikov et al. 1970). Chlorophyll *a* concentration in samples was analyzed spectrophotometrically according to Namsaraev (2009). Cyanobacterial morphology

was examined in wet mounts under a Jenaval microscope equipped with a Zeiss Bundle Canon PS G9 digital camera. The images were obtained using the AxioVision Rel. 4.7 software package.

C_2H_2 reduction assay

The C_2H_2 reduction assay for potential nitrogenase activity was conducted according to Hardy et al. (1968). For this, 5 cm^3 of phototrophic biomass slurry in the native brines (to maintain the natural density of biomass) was added in a 25 mL flask stoppered with gray rubber and flushed with argon gas and 15% v/v final concentration of C_2H_2 was added to the head space and incubated for 1, 3, 6, or 24 h. Dark-incubated samples were wrapped in two layers of aluminium foil. To some of the incubations, sulfide was added at concentration of 1 mg/L. 0.5 mL gas samples were taken for analysis of C_2H_4 by a Chromatek Krystall 5000.1 gas chromatograph with a flame ionization detector as a measure of nitrogenase activity.

To investigate the influence of salinity on nitrogenase activity, we used both the analysis of acetylene-reduction rates in the environmental samples collected during several seasons (data in Table 1; Fig. 3a) and the results of the laboratory experiment with a dilution of the environmental sample collected in 2014 with a salinity 200 g/L (data presented in Fig. 3b). We diluted it with distilled water down to 100 and 50 g/L. The samples were left overnight at low light intensity for adaptation of the microbial community to the decreased salinity levels before addition of C_2H_2 in the laboratory experiment. Incubation time was 72 h at 22 °C and a light intensity 100 μmol/m^2 s. Results were obtained from two biological replicates. All results are presented as means ± range of values.

Enrichment cultures

The enrichment cultures of haloalkaliphilic phototrophic bacteria were obtained using the Zarrouk medium (Vonshak 1997) (total salt concentration 22 g/L) with additional NaCl resulting in the salinity levels of 80, 150, and 200 g/L, respectively, and were incubated under aerobic conditions. At the salinity of 200 g/L, the second type of medium was also used. It contained an inhibitor of oxygenic photosynthesis DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) at a concentration of 7 μM, 0.5 g/L NH_4Cl, 0.5 g/L KH_2PO_4, 0.2 g/L $MgCl_2$, 18 g/L $NaHCO_3$, 180 g/L NaCl, 0.1 g/L yeast extract, 0.5 g/L sodium acetate, 0.5 g/L $Na_2S{\cdot}9H_2O$, and 0.5 g/L $Na_2S_2O_3{\cdot}5H_2O$, and were incubated anaerobically under N_2. For each type of medium, two versions of media were prepared: with nitrogen and without nitrogen source. In the aerobic variants with nitrogen at salinities of 22, 80, 150, and 200 g/L, 2.5 g/L sodium nitrate was used; in the anaerobic variant with a salinity of 200 g/L, 0.5 g/L ammonium chloride was used. Samples of surface layers of the sediments collected from the littoral area of the Bitter-1 lake in 2011 and 2012 were added at 5% v/v concentration.

Table 1 Environmental conditions, the rate of acetylene reduction, and the dominant groups of phototrophic microorganisms in Bitter-1 lake between 2005 and 2016 (in July of each year)

Year	pH	Salinity (g/L)	Alkalinity (M)		Acetylene reduction (nmol C_2H_4/mL h)		Chlorophyll *a* (μg/mL)	Dominant morphotypes of phototrophic bacteria
			CO_3^{2-}	Total	Dark	Light		
2005	10.3	130	0.6	1.1	n.d.	n.d.	n.d.	Biofilms dominated by cyanobacteria and purple bacteria *Ectothiorhodospira* sp.
2006	10.2	200	2.2	2.8	n.d.	n.d.	n.d.	
2007	10.5	170	1.1	2.9	n.d.	n.d.	n.d.	
2008	10.4	400	5.1	5.8	0	0	0	Endoevaporitic development of unicellular green alga *Dunaliella* sp. and cyanobacterium *Euhalothece* sp., as well as purple bacteria of the genus *Ectothiorhodospira*
2009	10.2	300	4.5	4.7	n.d.	n.d.	n.d.	
2010	10.1	400	2.0	4.4	n.d.	n.d.	n.d.	
2011	9.9	350	3.8	4.4	0	1.24 ± 0.17	32.0	
2012	10.2	400	4.4	4.9	0.59 ± 0.07	0.56 ± 0.005	19.3	
2013	9.6	125	1.9	2.0	n.d.	n.d.	n.d.	Ephemeral biofilms with the dominance of cyanobacteria *Geitlerinema* sp., *Euhalothece* sp., as well as purple bacteria of the genus *Ectothiorhodospira*. Diatoms are also present
2014	10.3	200	1.7	2.1	0.009 ± 0.003	0.27 ± 0.14	2.6	
2015	10.4	120	1.1	1.2	n.d.	n.d.	n.d.	
2016	10.2	85	1.0	1.3	0.47 ± 0.38	11.19 ± 0.46	17.2	Loose biomass of the filamentous green alga *C. circinnatus* and cyanobacteria *Geitlerinema* sp., *Nodosilinea* sp., *Euhalothece* sp., as well as individual cyanobacterial films of the same composition

n.d. not determined

Flasks were incubated during 1 month at a room temperature and a light intensity 100 μM/m^2 s.

Isolation of cyanobacterial cultures

The cyanobacterial culture *Nodularia* sp. 10KLT1 was isolated in 2008 from Tanatar 1 soda lake sample using solid Zarrouk medium without sodium nitrate (Rippka 1988; Vonshak 1997). The cyanobacterial cultures *Geitlerinema* sp. T6-1124, *Nodosilinea* sp. T6-1119, and *Euhalothece* sp. T2-1114 were isolated in 2011 from samples of phototrophic biomass collected in Tanatar 2 and Tanatar 6 soda lakes. The enrichment cultures were obtained at three different salt combinations: 4 M Na^+ (1.3 M $NaHCO_3 + Na_2CO_3$, pH 9.7), 2 M Na^+ (0.58 M $NaHCO_3 + Na_2CO_3$, pH 10.2), and 0.75 M Na^+ (0.2 M $NaHCO_3 + Na_2CO_3$, pH 9). The filamentous cyanobacteria *Geitlerinema* sp. T6-1124 and *Nodosilinea* sp. T6-1119 dominated in the media with 2 M and 0.75 M Na^+, while the unicellular *Euhalothece* sp. T2-1114—in the 4 M Na^+ medium, but it was also obtained in 2 M Na^+ medium after prolong incubation. The isolation of strains from enrichment cultures was performed on solid media of the same composition.

Nodosilinea sp. T6-1119 was maintained in the medium "S" with the following composition (g/L): $NaHCO_3$—16.8, NaCl—30.0, KCl—1.0, $K_2HPO_4 \cdot 3H_2O$—0.5, $NaNO_3$—2.5, K_2SO_4—1.0, $MgSO_4 \cdot 7H_2O$—0.1, $CaCl_2$—0.04, $FeSO_4$—0.01, EDTA—0.08, trace elements solution (TES) A5—1 mL/L, pH 9.0–9.5. *Geitlerinema* sp. T6-1124, and *Euhalothece* sp. T2-1114 were maintained in the medium "M" with the following composition (g/L): Na_2CO_3—79.5, $NaHCO_3$—21.0, KCl—2.0, $K_2HPO_4 \cdot 3H_2O$—0.5, KNO_3—2.0, Na_2SO_4—1.4, $FeCl_3$—0.0003, EDTA—0.0005, TES A5—1 mL/L, pH 10.0. The composition of TES A5 is (g/L): H_3BO_3—2.86, $MgCl_2 \cdot 4H2O$—1.81, $ZnSO_4 \cdot 7H_2O$—0.222, $Na_2MoO_4 \cdot 2H_2O$—0.39, $CuSO_4 \cdot 5H_2O$—0.079, and $Co(NO_3)_2 \cdot 6H_2O$—0.0494. All cultures were incubated at room temperature and natural light–dark cycles. The identification of the cultures and microscopic analysis of environmental samples were performed according to Samylina et al. (2014).

DNA isolation

DNA isolation from sediments of Bitter-1 Lake was carried out with the MoBio Power Soil DNA Isolation kit (MoBio Laboratories). Approximately 10 g of sediment was washed from alkalinity with 1 M NaCl; the suspension was centrifuged at 2000 rpm for 2 min to remove the coarse fraction, and the remaining fine colloidal fraction was centrifuged further 2–3 times at 5000 rpm, depending on the sediment type. From the colloidal suspension thus obtained, a 22 mL portion was finally collected at maximum speed and used for DNA isolation after adjusting the pH to 7.5 with 0.5 M HCl.

Amplification, cloning, and sequencing

For amplification and sequencing of the 16S rRNA gene, the universal bacterial primers were used (Edwards et al. 1989). For amplification and sequencing of the *nifH* and *cbbL* genes, we used primers and protocols described earlier (Marusina et al. 2001; Spiridonova et al. 2004; Tourova et al. 2010). PCR fragments of the expected size were excised from the gel, purified using the Wizard PCR Preps kit (Promega, United States), and sequenced immediately (for pure cultures) or after cloning (for soda lake sediment samples and enrichment cultures).

Cloning was performed using the CloneJet PCR Cloning kit (Fermentas) including plasmid pJET1.2/blunt according to the manufacturer's protocol. From each clone library, 20–50 clones were tested for the presence of the insert by PCR with specific primers. Clones containing the target insert were sequenced. Sequencing of amplification products and obtained clones was performed according to Sanger, using the Big Dye Terminator v.3.1 kit on an automatic ABI 3730 sequencer (Applied Biosystems Inc., United States), according to the instructions of the manufacturer.

Phylogenetic analysis

The sequences were edited with the use of BioEdit (http://jwbrown.mbio.ncsu.edu/BioEdit/bioediTca.html). Primary comparison of the de novo determined sequences with sequences available in the GenBank database was performed by using BLAST at the NCBI site (http://www.ncbi.nlm.nih.gov/blast). For further comparative analysis 16S rRNA, *nifH* and *cbbL* genes sequences were retrieved from GenBank. Nucleotide sequences of the genes studied and amino acid sequences deduced were aligned with relevant sequences of the closest bacterial species with the use of CLUSTALX 2.0 (http://bips.uustrasbg.fr/fr/Documentation/ClustalX/). Phylogenetic trees were constructed using the TREECONW (http://bioccwww.uia.ac.be/u/yvdp/treeconw.html) and MEGA 6.0 (http://www.megasoftware.net/) software packages. The statistical significance of the branching order was determined by bootstrap analysis of 1000 alternative trees and expressed in percent. The nucleotide sequences of the 16S rRNA, *nifH,* and *cbbL* genes determined in this work were deposited in GenBank with accession numbers MG197455- MG197477.

Results

Phototrophic community of the Bitter-1 soda lake

Bitter-1 soda lake has stable alkaline conditions with pH values of 9.6–10.5 due to its extremely high soluble carbonate alkalinity (Table 1). The fluctuations in salinity had a long interannual cycle with periods of relatively "high" and "low" salinity. The separation into periods was done by us depending on the type of phototrophic community that developed in the lake. During 11 years of observation, the change of such periods occurred twice. In 2005–2007, a period of relatively low salinity below 200 g/L was observed, followed by a hypersaline period of 2008–2012 (300–400 g/L), and in 2013–2016, the low salinity period (85–200 g/L) has started again. At the low salinity periods, the lake water was dominated by a loose biomass of a filamentous green alga *Ctenocladus circinnatus* or ephemeral cyanobacterial films (the same as described in Samylina et al. (2014). Potentially diazotrophic phototrophic prokaryotes in these types of communities were represented by haloalkaliphilic cyanobacteria *Geitlerinema* sp., *Nodosilinea* sp., *Euhalothece* sp., and anoxygenic purple sulfur bacteria of the genus *Ectothiorhodospira* (Fig. 2). The high salinity period was characterized by the endoevaporite phototrophic communities, which developed between the trona crystals under the salt crust. Potential diazotrophs, in this case, were extremely salt-tolerant unicellular cyanobacteria *Euhalothece* sp. and purple sulfur bacteria of the genera *Halorhodospira* and *Ectothiorhodospira.* The heterocystous cyanobacteria were not observed in the lake.

Acetylene reduction by phototrophic communities

At the period of high salinity from 2008 to 2012, phototrophic communities did not form mats on the surface of sediments, but developed among the crystals within the trona crust forming on the surface of sediments. This was collected to measure the acetylene-reduction activity. The acetylene-reduction activity was not detected in the sample collected in 2008 at salinity of 400 g/L (Table 1). However, in the 2011–2012 samples with total salt concentrations of 350–400 g/L a low, but detectable, level of acetylene reduction was observed (Fig. 3a). The addition of sulfide to the 2012 samples inhibited the light-dependent activity 4.8-fold (from 0.58 ± 0.08 to 0.12 ± 0.11 nmol C_2H_4/mL h). In contrast, sulfide did not influence the light-independent activity which maximum level was 0.18 ± 0.08 nmol C_2H_4/(mL h).

The decrease of the lake's salinity started in 2013 favored the formation of biofilms of the filamentous cyanobacteria on the bottom of the lake which diazotrophic potential was tested in 2014 at a salinity of 200 g/L. The rates of acetylene reduction were comparable to those measured at a higher salinity, and the light-dependent acetylene reduction exceeded the activity in the dark. Further decrease in salinity in 2016 down to a minimal 85 g/L caused a mass development of green algae and cyanobacteria that exhibited a much higher diazotrophic potential (Table 1).

A simulated decrease in salinity in a laboratory experiment with the samples collected in 2014 and diluted from 200 g/L to 100 and 50 g/L also stimulated nitrogen fixation. On the third day of the light incubation, the growth of cyanobacteria and anoxygenic purple bacteria on the bottom and walls of vials was observed accompanied with a statistically significant increase in the ethylene concentration in the salt-diluted variants (Fig. 3b). In contrast, the decrease in salinity did not stimulate nitrogen fixation in the dark incubations. The rates of acetylene reduction observed in this experiment cannot represent the in situ rates because of the prolonged incubation (3 days) and the obvious growth of phototrophic bacteria, but it certainly confirms data obtained with environmental samples.

Analysis of *nifH* and *cbbL* genes in the reference cultures of haloalkaliphilic cyanobacteria

In earlier studies of the Kulunda Steppe soda lakes, the *nifH* and *cbbL* gene sequences of haloalkaliphilic cyanobacteria isolated from those lakes were not available. Therefore, for a more accurate identification of these organisms in environmental samples and enrichment cultures, we isolated four cultures of haloalkaliphilic cyanobacteria. Based on the morphological features and analysis of the 16S rRNA gene, these cultures were assigned to the genera *Geitlerinema*, *Nodosilinea*, *Euhalothece,* and *Nodularia*. Amplification products with primers for *nifH* genes F1-R6 (Marusina et al. 2001) were obtained for all studied strains. Amplification with the primers for *cbbL* genes *cbbL*G1f-*cbbL*1106r (Tourova et al. 2010) was successful for strains *Geitlerinema* sp. T6-1124 and *Nodosilinea* sp. T6-1119. For *Euhalothece* sp. T2-1114, only the primers RubIgF–RubIgR (Spiridonova et al. 2004) were efficient. For *Nodularia* sp 10KLT1, amplification was unsuccessful with all primer pares used.

The position of the studied strains is shown in the phylogenetic trees (Figs. 4, 5, 6). In general, the functional gene phylogeny was consistent with the ribosomal gene phylogeny, with one exception. The T6-1124 culture according to analysis of the 16S rRNA and the *cbbL* genes belonged to the genus of *Geitlerinema*, while sequence of the *nifH* gene of this organism belonged to the cluster of *Deltaproteobacteria*. There, it formed a subcluster including sequences of the *nifH* gene from *Geitlerinema* (former *Microcoleus*) sp. IPPAS B353 and *Coleofasciculus* (former *Microcoleus*)

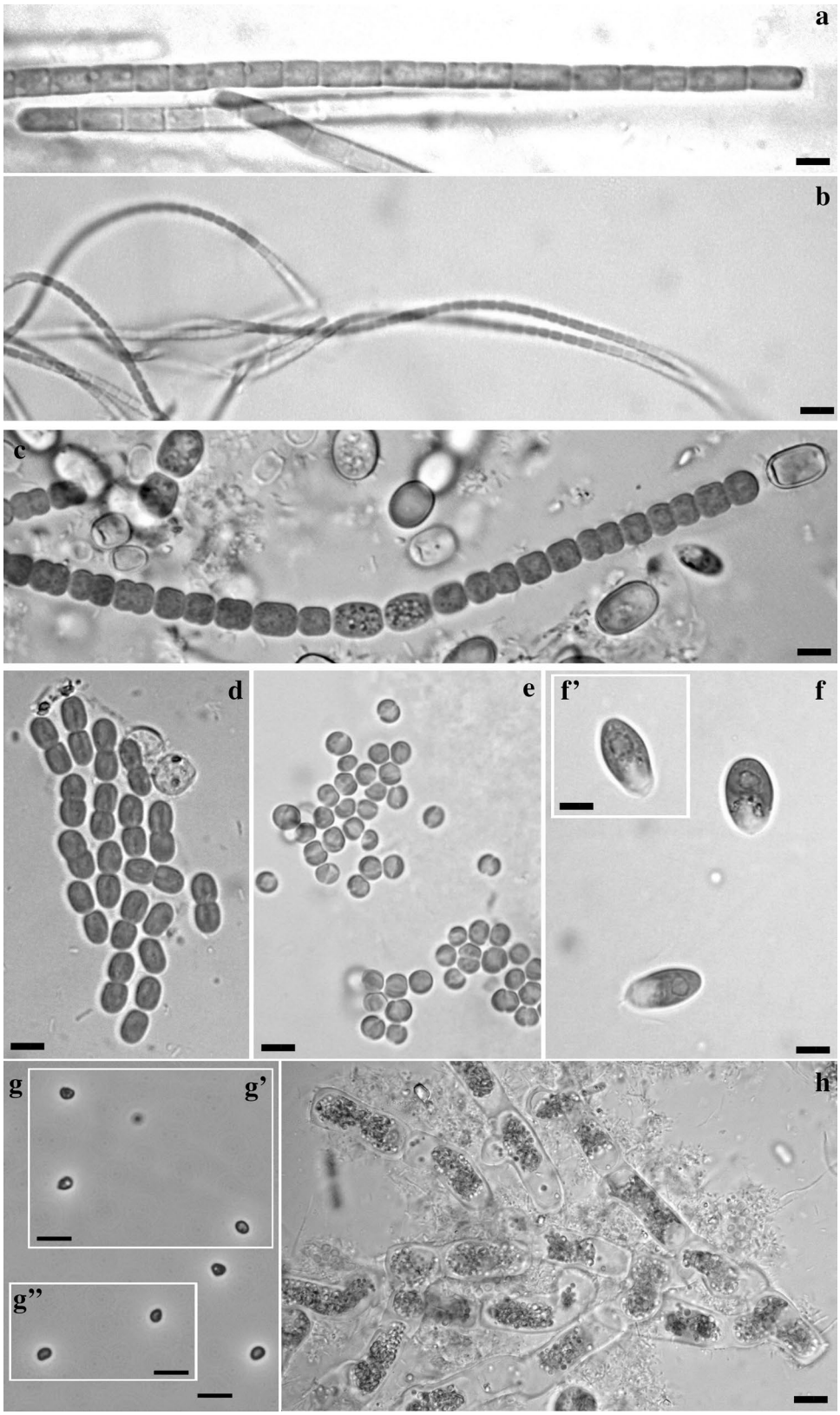

Fig. 2 Phototrophic microorganisms from Bitter-1 soda lake. **a** *Geitlerinema* sp., **b** *Nodosilinea* sp., **c** *Nodularia* sp., **d** *Euhalothece* sp., **e** *Picocystis salinarum*, **f–f′** *Dunaliella* sp., **g–g″** *Ectothiorhodospira* sp., **h** *C. circinnatus*. Bar: **a–g″** 5 µm, **h** 10 µm

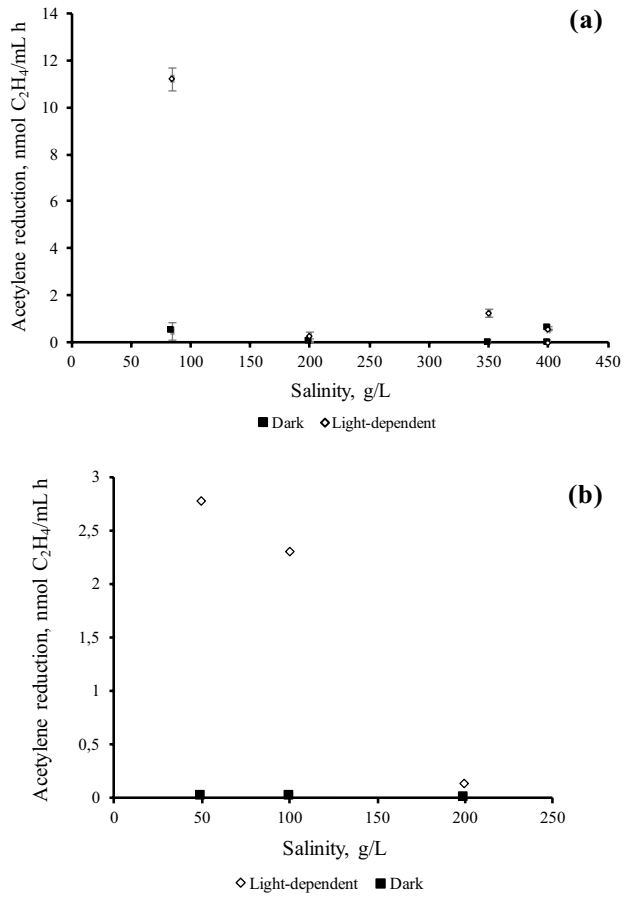

Fig. 3 Acetylene reduction **a** in the environmental samples and **b** in the laboratory experiment

chthonoplastes PCC7420. For the latter, it was previously shown that its *nif*-operon (*nifHDKENB*) was most likely obtained during horizontal gene transfer from sulfate-reducing bacteria (Bolhuis et al. 2010).

Diversity of *nif*H genes in hypersaline soda lake Bitter-1

From the environmental samples of the surface layers of the sediments collected in Bitter-1 soda lake in 2011 and 2012 (at the salinity level of 350 and 400 g/L, respectively), the clone libraries were created using primers specific for *nifH* genes. The libraries with 43 and 47 clones contained 2 unique *nifH* phylotypes, pairwise identical in both libraries (Fig. 5). The dominant phylotypes in both libraries (34 and 39 clones) formed a separate subcluster within the cluster of sulfate-reducing *Deltaproteobacteria*. This phylotype was not identical to the *Geitlerinema* sp. T6-1124 that also possessed the *nifH* gene related to *Deltaproteobacteria*. The second phylotype found in environmental samples was distantly related to members of *Clostridia*. Thus, in the diazotrophic community of environmental samples collected during the salt-saturated period, the phototrophic diazotrophs, including cyanobacteria, were undetectable.

Enrichment cultures of phototrophic microorganisms

We tried to model the changes of salinity in the lake and obtained the enrichment cultures of phototrophic microorganisms at different salinities on media without combined nitrogen, as well as on media with the introduction of nitrogen compounds ($NaNO_3$ or NH_4Cl). We used surface layers of the sediments collected in 2011 and 2012 as inoculum for this experiment. An overview of the variants of enrichment cultures is presented in Table 2.

In the absence of nitrogen sources, a stable growth was observed only in two variants: first, at low levels of salinity (22 g/L) and aerobic conditions, in which filamentous heterocystous cyanobacterium *Nodularia* sp. dominated in the sample; second, at high salinity (200 g/L) and anaerobic conditions (gas phase with N_2), in which extremely haloalkaliphilic anoxygenic phototrophic bacteria of the genus *Ectothiorhodospira* were the dominant diazotrophs. At the salinity of 200 g/L, aerobic conditions, and absence of combined nitrogen, steady growth of microorganisms was not observed except a weak initial development of *Picocystis salinarum* (previously identified as *Chlorella minutissima*; Gerasimenko et al. 1999), and at the salinity of 80–150 g/L—of the unicellular microalgae *Dunaliella* sp., *Picocystis salinarum* (Fig. 2) and filamentous cyanobacterium *Geitlerinema* sp., probably due to the traces of combined nitrogen introduced into the medium with the inoculum.

In the presence of combined nitrogen, growth was observed at all salinity values. At 22 and 80 g/L, the unicellular microalgae *Dunaliella* sp., *Picocystis salinarum,* and filamentous cyanobacterium *Geitlerinema* sp. were dominating the cultures. At salinity of 150 g/L, the filamentous cyanobacterium *Geitlerinema* sp. was replaced by the unicellular cyanobacterium *Euhalothece* sp. capable of growth at salt-saturating conditions (Garcia-Pichel et al. 1998; Mikhodyuk et al. 2008). At salinity of 200 g/L in the aerobic conditions, the weak growth of *Picocystis salinarum* was observed. In the anaerobic light conditions and salinity of 200 g/L with combined nitrogen, the purple anoxygenic bacteria of the genus *Ectothiorhodospira* were dominant.

Thus, despite the absence of phototrophic *nifH* sequences in the clone libraries obtained from the sediments at salinity 350–400 g/L, several groups of phototrophic diazotrophic microorganisms were present in sediments. These are filamentous heterocystous cyanobacteria *Nodularia* sp. and the anoxygenic phototrophic bacteria of the genus *Ectothiorhodospira*.

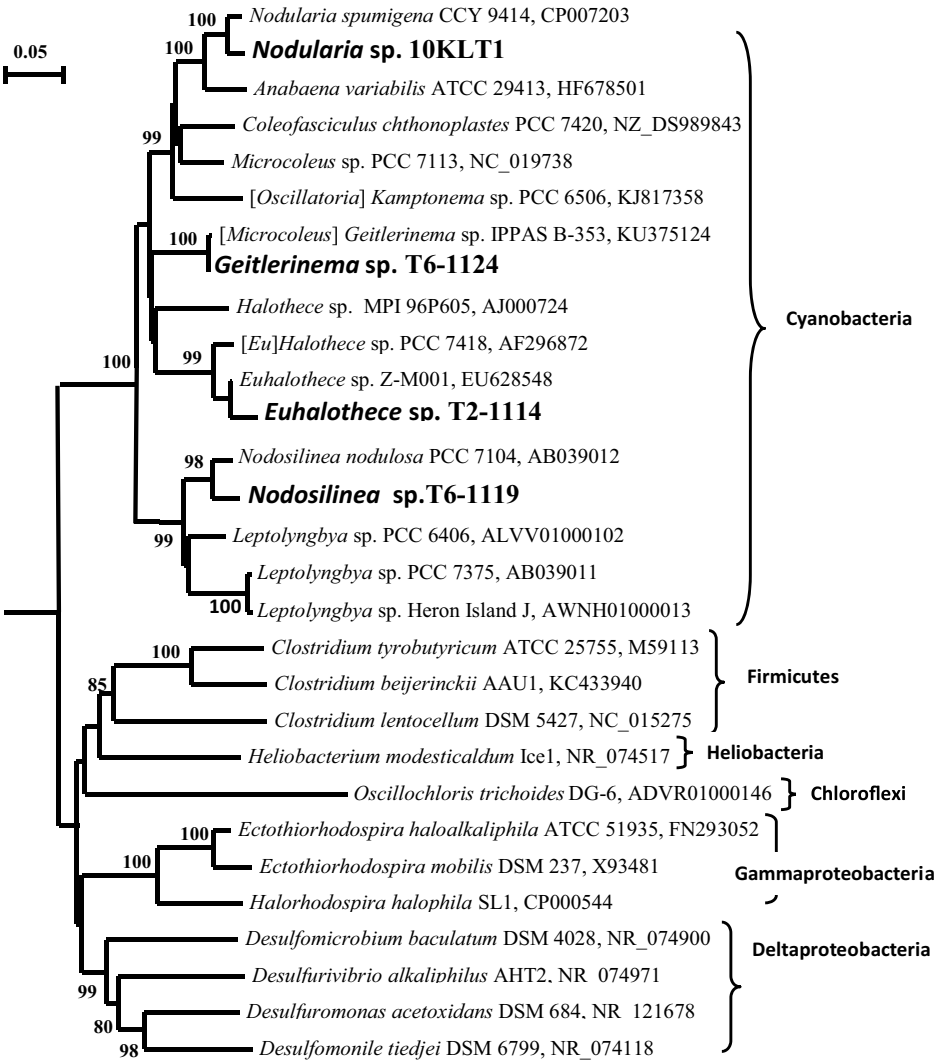

Fig. 4 16S rRNA gene-based phylogenetic tree showing the position of the studied new strains isolated from the Kulunda steppe soda lakes (marked in bold) among cyanobacteria. The tree was constructed with the use of the neighbor-joining algorithm. Scale bar shows evolutionary distance corresponding to 5 substitutions per 100 nucleotides. Numerals at the nodes show the statistical significance of the branching order as determined by bootstrap analysis of 1000 alternative trees (only values higher than 80% are shown). The cyanobacterial culture Nodularia sp. 10KLT1 was isolated from Tanatar 1 lake, *Geitlerinema* sp. T6-1124, *Nodosilinea* sp. T6-1119, and *Euhalothece* sp. T2-1114 which were isolated from Tanatar 2 and Tanatar 6 soda lakes

Diversity of *nifH* and *cbbL* genes in enrichment cultures

Three aerobic enrichment cultures, 12G1-23, 12G1-80, 11G1-150, and anaerobic enrichment culture 12G1-200 (Table 2), obtained from Bitter-1 at different salinity (22, 80, 150, and 200 g/L) were analyzed using the *nifH* gene-specific primers. The clone library from enrichment culture 12G1-23 (19 clones) yielded a single phylotype identical to the *nifH* gene of the alkaliphilic strain *Nodularia* sp. 10KLT1. A clone library 12G1-80 (30 clones) included 2 phylotypes. The dominant phylotype (27 clones) was identical to the *nifH* gene of the alkaliphilic cyanobacterium *Geitlerinema* sp. T6-1124, and a minor one—to the alkaliphilic cyanobacterium *Euhalothece* sp. T2-1114 and *nifH* phylotypes found in the samples from Bitter-1 lake collected in 2009 (Tourova et al. 2014). The clone library 11G1-150 (17 clones) contained a single phylotype also identical to *Euhalothece* sp. T2-1114. The clone library from the anaerobic enrichment culture 12G1-200 (21 clones) contained a single phylotype, closely related to the *nifH* gene of the purple sulfur bacterium *Ectothiorhodospira haloalkaliphila*.

In an additional analysis using specific primers *cbbL*G1f-*cbbL*1106r for the detection of *cbbL* genes, the amplification product was detected in three cultures out of four (except 11G1-150) (Fig. 6). Each clone library contained only a single *cbbL* phylotype. A clone library 12G1-23 (25 clones) was most similar to the *cbbL* gene of the green nonsulfur bacterium *Oscillochloris trichoides* DG-6, a clone library 12G1-80 (30 clones)—to the *cbbL* gene of the haloalkaliphilic cyanobacterium *Geitlerinema* sp. T6-1124, and the library 12G1-200 (15 clones)—to a *cbbL* gene sequence of a *gammaproteobacterium*, most probably a member of *Ectothiorhodospira*. These data, in general, do not contradict the data obtained during analysis of *nifH* genes in the same cultures. Some discrepancies, in

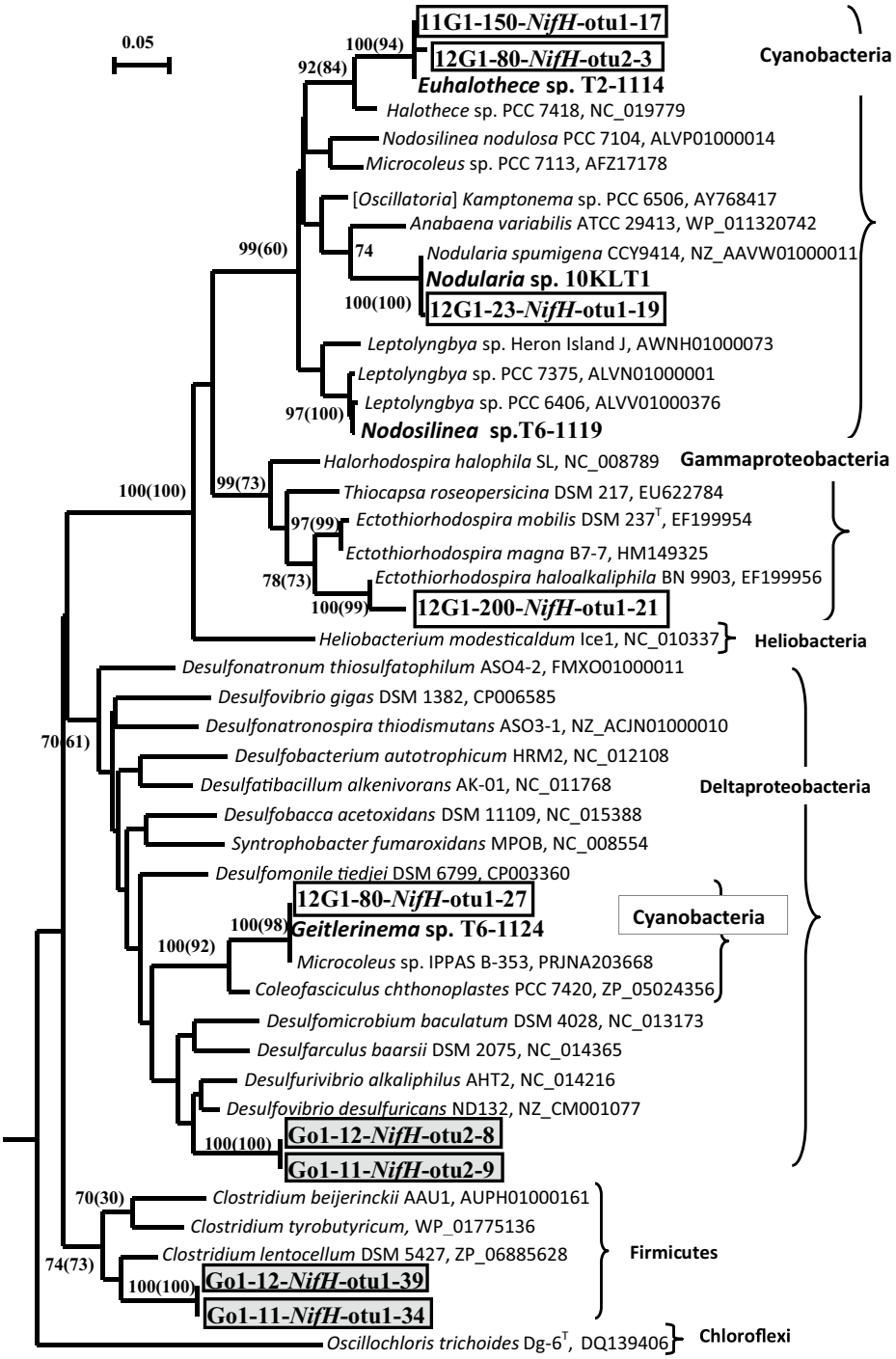

Fig. 5 Position of the studied strains and clones among diazotrophic bacteria in the NifH based phylogenetic tree. The new strains were marked in bold, the clones from enrichment cultures were marked in boxes, and the clones from environmental samples were marked in gray boxes. The cyanobacterial culture *Nodularia* sp. 10KLT1 was isolated from Tanatar 1 lake, *Geitlerinema* sp. T6-1124, *Nodosilinea* sp. T6-1119, and *Euhalothece* sp. T2-1114 were isolated from Tanatar 2 and Tanatar 6 soda lakes. The enrichment cultures and environmental clones originate from Bitter-1 soda lake. The tree was constructed with the use of the neighbor-joining algorithm. Scale bar shows evolutionary distance corresponding to 5 substitutions per 100 amino acid residues. Numerals at the nodes show the statistical significance of the branching order as determined by bootstrap analysis of 1000 alternative trees (only values higher than 70% are shown). In parentheses, bootstrap values for an analogous tree (not shown) constructed using maximum-likelihood algorithm are indicated

particular, a failure with the detection of *cbbL* phylotypes of *Euhalothece* sp. and *Nodularia* sp. could be explained by the insufficient universality of the degenerate primers used. In this case, a minor component of the community can be detected. This does not contradict the previously obtained results, according to which the *nifH* phylotype of *Oscillochloris* sp. was detected in sediments of soda lakes of the Bitter system at low salinity (Tourova et al. 2014). The contradiction between the phylogenetic position of the *nifH* and *cbbL* phylotypes found in the clone library 12G1-200 dominated by *Ectothiorhodospira* could be explained by the horizontal transfer of the *cbbL* gene. Earlier, the transfer of *cbbL* gene from Chromatiaceae purple sulfur bacteria was proposed in *Ectothiorhodospira magna* strains (Bryantseva et al., 2010).

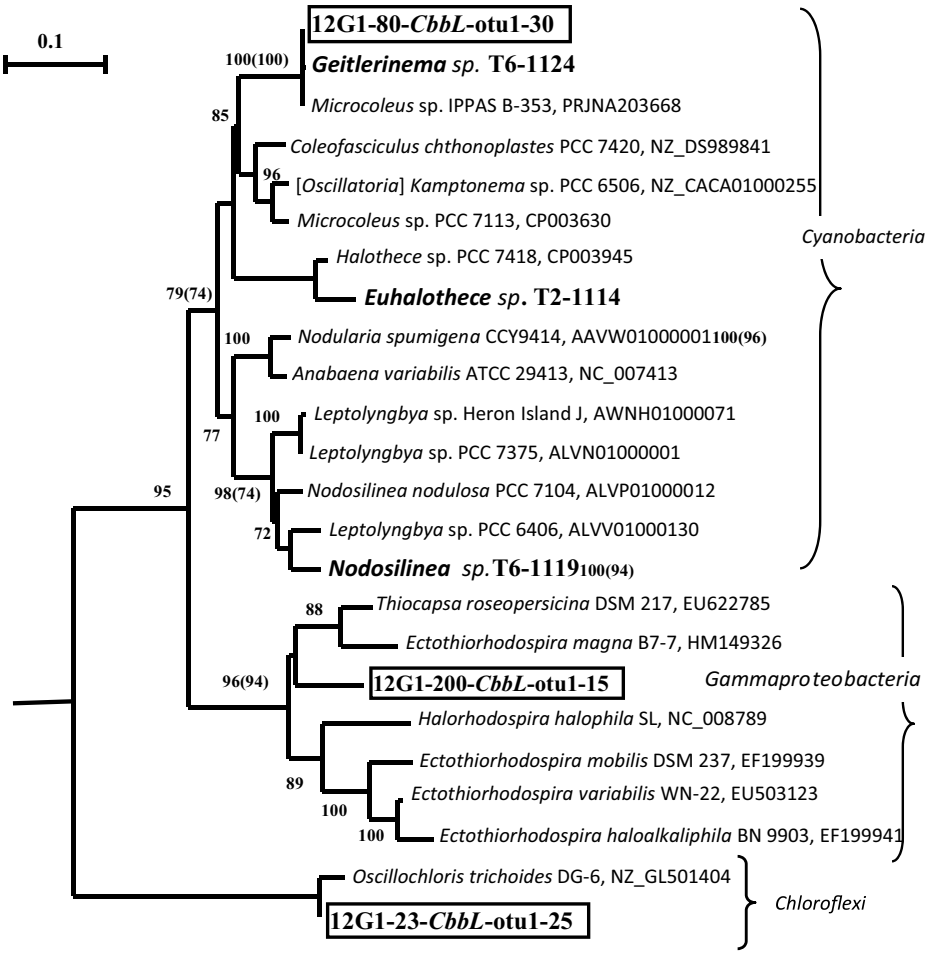

Fig. 6 Position of the studied strains and clones among autotrophic bacteria in the phylogenetic tree constructed based on analysis of conceptual amino acid translations of cbbL genes. The new strains were marked in bold and the clones from enrichment cultures were marked in boxes. The cyanobacterial culture *Nodularia* sp. 10KLT1 was isolated from Tanatar 1 lake, *Geitlerinema* sp. T6-1124, *Nodosilinea* sp. T6-1119, and *Euhalothece* sp. T2-1114 were isolated from Tanatar 2 and Tanatar 6 soda lakes. The enrichment cultures were obtained from Bitter-1 soda lake. The tree was constructed with the use of the neighbor-joining algorithm. Scale bar shows evolutionary distance corresponding to 10 substitutions per 100 amino acid residues. Numerals at the nodes show the statistical significance of the branching order as determined by bootstrap analysis of 1000 alternative trees (only values higher than 70% are shown). In parentheses, bootstrap values for an analogous tree (not shown) constructed using maximum-likelihood algorithm are indicated

Table 2 Enrichment cultures of phototrophic microorganisms at different salinity levels

Salinity (g/L)	Without nitrogen	With nitrogen
22	Growth of heterocystous cyanobacterium *Nodularia* sp. (12G1-30)	Eukaryotic microalgae *Picocystis salinarum* and *Dunaliella* sp., filamentous cyanobacterium *Geitlerinema* sp.
80	No growth	Eukaryotic microalgae *Picocystis salinarum* and *Dunaliella* sp., filamentous cyanobacterium *Geitlerinema* sp. (12G1-80)
150	No growth	Eukaryotic microalgae *Picocystis salinarum* and *Dunaliella* sp., unicellular cyanobacterium *Euhalothece* sp. (11G1-150)
200 (aerobic)	No growth	Weak growth of the eukaryotic microalgae *Picocystis salinarum*
200 (anaerobic)	Purple bacteria *Ectothiorhodospira* sp. (12G1-200)	Purple bacteria *Ectothiorhodospira* sp.

Discussion

At the salinity level below 70 g/L, heterocystous cyanobacteria are one of the dominant groups of diazotrophic phototrophs in soda lakes (Oren 2011a). Due to the presence of heterocysts, protecting nitrogenase from the oxidative stress, these cyanobacteria are capable of fixing nitrogen in fully aerobic environment. As a result, the rate of light-dependent nitrogen fixation significantly exceeds the fixation in the dark as was shown by Oremland (1983) during a study of Big Soda Lake. At hypersaline conditions above 70 g/L, the situation becomes more complicated. The heterocystous cyanobacteria become inactive, but oxygenic photosynthesis still occurs (Namsaraev et al. 2015). Which groups of bacteria are capable of fixing nitrogen under these conditions remained unclear. In addition to the formation of heterocysts, the phototrophic microorganisms have various mechanisms of nitrogen fixation in the presence of oxygen (Berman-Frank et al. 2003). First, some cyanobacteria, for example, *Plectonema boryanum*, are capable of nitrogen fixation under microaerobic conditions with an oxygen content of less than 1.5% in the dark and less than 0.5% in the light. Second, temporal separation is possible, when nitrogen fixation occurs at night and oxygenic photosynthesis during the day time. Third, a combination of these strategies is possible, as in the marine nonheterocystous filamentous cyanobacterium *Trichodesmium*, in which a part of the cells in the cyanobacterial trichome (up to 10–20% of the total number of cells) can fix nitrogen, and the ability to achieve nitrogen can be activated when necessary. The diversity of these strategies in cultures isolated from hypersaline soda lakes has not been studied in detail. Nevertheless, it cannot be ruled out that phototrophic cyanobacteria from soda lakes can implement these strategies. Soda lakes also have a number of features that potentially affect the nitrogen fixation process. For example, the presence of microbial mats with a sharp gradient of conditions creates a wide variety of ecological niches. Thus, stratified mats were described in soda lakes of Cariboo Plateau, British Columbia, Canada (Schultze-Lam et al. 1996), but this type of mats is not widely distributed in hypersaline soda lakes. Furthermore, high salinity results in lower solubility of oxygen that can be beneficial for diazotrophic bacteria (Medová et al. 2011). In addition, many representatives of anoxygenic phototrophic bacteria living on the surface sediment layer of soda lakes are also capable of nitrogen fixation (Tourova et al. 2014).

The nitrogen fixation in hypersaline soda lakes attracted the attention of researchers, although the total number of publications is not high. The main evidences were obtained in Mono Lake (California) at a salinity of about 90–100 g/L. In experiments with microcosms, it was shown that a decrease in mineralization leads to an increase in the rate of nitrogen fixation. At the same time, the rate of light-dependent acetylene reduction exceeded the rate in the dark approximately threefold (Herbst 1998). Oremland (1990) further showed that the nitrogen fixation in Mono Lake was performed by different types of communities and occurred in different samples in anaerobic dark conditions or in the light with stimulation by sulfide.

To our knowledge, no such data exist in the literature for hypersaline soda lakes with salinity above 100 g/L, such as, for example, the Bitter-1 lake in the Kulunda Steppe. Most interestingly, also definitely decreasing, the light-dependent acetylene reduction occurred there up to nearly soda-saturating concentrations of 350–400 g/L with the highest activity at the lowest margin during the study period (85 g/L). The maximum level of acetylene-reduction values recorded in Bitter-1 (11.19 ± 0.46 nmol C_2H_4/(mL h) was comparable to those registered in Mono Lake [75 ± 28 nmol C_2H_4/(mL h) (Oremland 1990) and 13.85–14.06 nmol C_2H_4/(mL h) (Herbst 1998)].

The minimal rates of acetylene reduction (0.009–1.24 nmol C_2H_4/(mL h) observed at salinities above 200 g/L were comparable with the rates recorded in marine sediments with low rates of nitrogen fixation (0.144 nmol C_2H_4/(g(dr w) h) (Herbert 1975).

One of the most interesting results of this study is a very limited diversity of *nifH* gene during the salt-saturating period of 2011–2012 in Bitter-1 with the only detectable sequences belonging to anaerobic sulfate-reducing *Deltaproteobacteria* and *Clostridia*. In the samples from the surface layers of the sediments, we could not detect gene sequences belonging to known representatives of phototrophic microorganisms, including possibly obtained by cyanobacteria as the result of horizontal transfer from deltaproteobacteria (Bolhuis et al. 2010). The high concentration of chlorophyll *a* (Table 1) in these samples was possibly due to the dominance of eukaryotic alga *Dunaliella* sp. (Fig. 2f–f′) which is not capable of nitrogen fixation. On the other hand, an earlier analysis of *nifH* gene in the samples from Bitter-1 lake collected at a salinity of 300 g/L in 2009 (Tourova et al. 2014) detected a presence of extremely salt-tolerant diazotrophic cyanobacteria of the genus *Euhalothece* and anoxygenic purple sulfur bacteria of the genus *Halorhodospira*. Although, at such salinity, sulfate-reducing bacteria were predicted to be inhibited due to the thermodynamic constraints of low-energy-generating catabolism (Oren 1999, 2011b), they were still detectable both by the specific molecular marker (*dsrB*) in situ (Foti et al. 2007, 2008) and also by cultivation approach, in particular the extremely salt-tolerant lithotrophic genera *Desulfonatronospira* and *Desulfonatronovibrio* and the acetate–propionate oxidizing genera *Desulfonatronobacter* and '*Candidatus*

Synthrophonatronum' (Sorokin et al. 2015a, b; Sorokin 2017).

Thus, the *nifH* gene screening suggests that, at 350–400 g/L, the nitrogen fixation in hypersaline soda lakes is probably performed by anaerobic heterotrophs and sulfate-reducing bacteria, and at salinity lower than 300 g/L oxygenic and anoxygenic phototrophic bacteria. This conclusion was verified in experiments with phototrophic enrichment cultures run at variable salt concentrations that demonstrated activation of heterocystous diazotrophic cyanobacteria of the genus *Nodularia* only at low salinity and the dominance of nonheterocystous filamentous potentially diazotrophic cyanobacteria of the genera *Geitlerinema* and *Nodosilinea* at intermediate salinities of 70–150 g/L. At conditions, close to salt saturation, the only oxygenic phototroph capable of diazotrophy seems to be *Euhalothece* (whose capacity for nitrogen fixation is still poorly studied), while, at anaerobic conditions, the anoxygenic phototrophic bacteria of the genus *Ectothiorhodospira* and sulfate-reducing bacteria might still be active at low level of activity.

Conclusion

Communities of haloalkaliphilic bacteria from the Bitter-1 hypersaline soda lake have the ability to fix molecular nitrogen in a wide range of salinity (from 20 to 400 g/L), which can be explained by the presence of several groups of diazotrophs with significantly different salinity tolerance. The rate of nitrogen fixation depends on the salinity and can have an interannual dynamics driven by the hydrology of the lake.

Acknowledgements This work was supported by the Program of fundamental research for state academies (No. 01201350928), Russian Academy of Sciences and Federal Agency of Scientific Organizations (Project Nos. 0104-2018-0033 and 0104-2018-0030). ZN was supported by the NRC "Kurchatov Institute". DS was supported by the the Gravitation-SIAM program of the Dutch Ministry of Education and Science (24002002).

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