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DOI
10.1093/femsec/fiz174

Publication date
2019

Document Version
Accepted author manuscript

Published in
FEMS Microbiology Ecology

The patterns of nitrogen fixation in haloalkaliphilic phototrophic communities of Kulunda Steppe soda lakes (Altai, Russia)

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Abstract

Nitrogen fixation (NF) of phototrophic microbial communities was studied in a number of soda lakes with a wide range of salinity (25-400 g/l) located in Kulunda Steppe (Altai, Russia) during several summer seasons (2011-2016). The phototrophic communities in these lakes were represented by the algal-bacterial *Ctenocladus*-communities or cyanobacterial biofilms dominated by different heterocystous and non-heterocystous cyanobacteria and purple sulfur bacteria *Ectothiorhodospira* sp. (up to 210 g/l) and endoevaporitic *Euhalothece*-communities dominated by extremely salt-tolerant unicellular cyanobacterium *Euhalothece* sp. as well as *Ectothiorhodospira* sp. (above 350 g/l). Salinity was the major factor influencing the composition and NF potential of the phototrophic communities. The "heterocystous" communities exhibited light-independent NF at total salinity up to 60 g/l. The "non-heterocystous" communities exhibited light-dependent NF in a range 50-100 g/l, but it was significantly suppressed at 100 g/l. At 160-210 g/l the dark heterotrophic NF was a prevailing process if communities didn't contain *Euhalothece* sp. At salt-saturating range above 350 g/l the light-dependent NF associated with the *Euhalothece*-communities was detected. A statistically significant positive correlation between the NF and diurnal light intensity was found in all samples of "non-heterocystous" communities in contrast to "heterocystous" communities with insignificant correlation coefficients.

Keywords: phototrophic nitrogen fixation; soda lakes; circadian rhythm; non-heterocystous cyanobacteria; *Ctenocladus*

Introduction

Soda lakes are one of the most productive ecosystems on Earth and nitrogen availability is one of the most important limiting factors in these ecosystems. Nitrogen fixation (NF) in moderately saline soda lakes (below 40 g/l) is usually attributed to heterocystous cyanobacteria. Blooms of *Anabaenopsis* spp.*, Cyanospira* spp.*, Nodularia spumigena* are frequent in the water column of Pyramid Lake in the USA (Galat *et al.* 1990), Alchichica in Mexico (Oliva *et al.* 2009), lakes of the East African Rift (Grant and Jones 2016; Krienitz and Schagerl 2016) and the Brazilian Pantanal region (Andreote *et al.* 2018; Costa *et al.* 2016). Shallow soda lakes of south Siberia are located in cryo-arid continental climate and subjected to high amplitude of the hydro-chemical fluctuations which cause significant successional changes of microbial communities. Although heterocystous cyanobacteria were described there at salinities up to 90 g/l (Burganskaya *et al.* 2018; Tsyrenova *et al.* 2011; Voronikhin 1929, 1932, 1934) they are considered to persist in phototrophic crusts with an ability to proliferate during wet periods as well as during short-term rains of the dry periods (Namsaraev *et al.* 2018a,b).

Available data show that the patterns of NF at highly saline and alkaline conditions differ from moderately saline lakes. At the salinities higher than 40 g/l the NF in soda habitats is still poorly studied. Most of the data were obtained for the stratified soda Mono Lake in California, USA (Herbst 1998; Oremland 1990; Steward *et al.* 2004) and shallow hypersaline Bitter-1 lake in Kulunda steppe, Russia (Tourova *et al.* 2014; Namsaraev *et al.* 2018c).

During studies on the Mono Lake (pH 9.8-10, salinity in the range 79-100 g/l depending on the year and depth of sampling) the highest rates of NF were found in the benthic phototrophic communities and no significant NF was detected in the water column (Herbst 1998; Oremland 1990; Steward *et al.* 2004). Benthic phototrophic communities (*Ctenocladus* aggregates) usually exhibited light-dependent NF stimulated by inhibitor of photosystem II (DCMU, or 3-(3,4-dichlorophenyl)-1,1-dimethylurea). The authors proposed that this process was mediated by non-heterocystous cyanobacteria. Surface sediment layers without cyanobacteria in Mono Lake exhibited anaerobic light-independent NF, indicating the involvement of chemotrophic microorganisms.

During our previous study on Bitter-1 soda lake located in Kulunda steppe (pH 9.6-10.53, salinity in the range 85-400 g/l depending on the year of sampling), we showed that the phototrophic communities of haloalkaliphilic bacteria also possess the light-dependent ability to fix molecular nitrogen in the whole range of salinity but the highest rate of the NF was found at salinity below 100 g/l (Namsaraev *et al.* 2018c).

The current study was focused on the analysis of the nitrogen-fixing activity and composition of the haloalkaliphilic phototrophic diazotrophic communities in a number of soda lakes with salinity from

25 to 400 g/l located in Kulunda Steppe. It provided us an opportunity to study the patterns of NF exhibited by the natural phototrophic communities in a broad range of salinities.

Materials and methods

Study sites and sampling

Diazotrophic activity of phototrophic communities was studied in the following soda lakes: Cock Soda Lake, Bitter-1 and Bitter-3 lakes, Tanatar V and VI, Picturesque Lake and Crooked Lake. The locality of these lakes, its names in Russian pronunciation and major properties are presented in **Table 1**.

Salinity and pH of the brines were measured using a WTW field potentiometer-conductometer (Germany). For the hypersaline conditions, the average pH values between the native brine and its 1:5 dilution was measured. The total salinity values were additionally verified gravimetrically in the laboratory after the brine filtration through 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).

Samples of phototrophic biomass were collected from the littoral area and shores of the lakes and from the surface of the moist soil surrounding lakes. Chlorophyll *a* concentration in samples was analyzed according to Namsaraev (2009) using extraction with 80% (v/v) acetone.

Light microscopy

Cyanobacterial morphology was examined in wet mounts under a Jenaval light microscope equipped with a Zeiss Bundle Canon PS G9 digital camera (Germany). Identification of cyanobacteria in environmental samples was performed according to previous studies (Namsaraev *et al.* 2018c; Samylina *et al.* 2014), determination manuals (Komárek and Anagnostidis 1998; Komárek 2013) and recent taxonomical papers (Abed *et al.* 2002; Dadheech *et al.* 2013).

Potential nitrogen fixation activity measurements by acetylene reduction assay and statistical analysis

The potential total NF of phototrophic communities was estimated by acetylene reduction rates (ARR). The acetylene reduction assay (ARA) was conducted according to Hardy *et al.* (1968), either

under natural light in the field (in 2011 and 2012) or in the laboratory immediately after returning from the field (in 2014, 2015 and 2016). All collected samples were stored at ambient temperature until their analysis in the laboratory (max. storage time – 5 days). The field measurements were conducted as described previously (Namsaraev *et al.* 2018c). Results were obtained from two biological replicates and presented as means±range of values.

The diurnal dynamics of diazotrophic activity was studied in the laboratory using 5 ml of phototrophic biomass slurry in native brines in a 50 ml flask with a grey rubber stopper. The headspace was flushed with argon gas and 1.5% (v/v) of C_2H_2 was added to the flask. Dark-incubated samples were wrapped in two layers of aluminum foil. After 15 minutes of equilibration, the gas samples were collected and analyzed to establish initial levels of ethylene. The incubation was held under ambient light and 0.5 ml gas samples were collected each hour during 24 h period. The samples were analyzed for C_2H_4 formation using a Chromatek Krystall 5000.1 GC (Russia) with a flame ionization detector. The experiments were conducted on 11-12 July 2014, 08-09 July 2015 and 06-07 July 2016. Each year the measurements started at 13:00. Light intensity was measured by the luxmeter «ТКА-PKM»/31 (Russia) every hour in 2014 and every half hour in 2015 and 2016 during 24 h period. The light intensity varied during the experiment from 0 to 4.2 kLux in 2014, from 0 to 5.91 kLux in 2015 and from 0 to 7.23 kLux in 2016. A potential stimulation of heterotrophic ARR was tested by adding 1 mM glucose to dark-incubated samples. Values of regular Pearson's correlation coefficients (CC) and cross-correlation functions (CCF) were calculated using series of light intensities (illumination) and ARR measured in this experiment. Series of light intensities consisted of averages calculated from two (in 2014) or three (in 2015 and 2016) values measured during the certain hour when ARR was determined. First, values of CC between series of illumination and ARR were calculated. Then, each couple of series (light intensity vs. ARR) was subjected to cross-correlation analysis (Box and Jenkins 1970). Since the analysis data in series needed to be equally spaced in time, several missing values (years 2015 and 2016) were evaluated using the cubic spline interpolation procedure according to McClarren (2018).

To investigate the influence of brine dilution on the growth and NF of the community, the environmental sample from the Crooked Lake (2014) with a salinity of 210 g/l was diluted with distilled water down to 105 and 52.5 g/l. Before the addition of C_2H_2 the samples were left overnight at low light intensity for adaptation of the microbial community to the decreased salinity levels. The incubation time was 72 hours at 30^{o}C at the light intensity of 5 kLux.

NifH gene amplification, cloning and sequencing

Genomic DNA was extracted using a DNeasy PowerSoil kit (Qiagen) according to the manufacturer's instructions. Primers nifH-F (5′-AAAGGYGGWATCGGYAARTCCACCAC-3′) and

nifH-R (5′-TTGTTSGCSGCRTACATSGCCATCAT-3′) (Rösch *et al.* 2002) were used for amplification of the *nifH* gene. The PCR products were purified in 0.7% agarose gel using the Wizard SV Gel and PCR Clean-Up System kit (Promega, United States) according to the manufacturer's recommendations. Cloning was carried out with the pGEM-T Easy Vector System I (Promega, United States). The competent cells of *E.coli* DH10B were transformed on an Eppendorf multiporator (Germany). The target insert of gene *nifH* was sequenced using primer M13F (Sambrook *et al.* 1989). Sequencing was performed by the Sanger method on an ABI3730 DNA Analyzer sequencer (Applied Biosystems, USA) using the Big Dye Terminator v. Reagent kit. 3.1 Cycle Sequencing Kit (Applied Biosystems, USA), as recommended by the manufacturer. Clones sequences with >99% sequence similarity were clustered into operational taxonomic units (OTUs). The nucleotide sequences of the *nifH* genes determined in this work were deposited in GenBank with accession numbers MK604935 – MK604942.

Bacterial composition analysis by NGS.

Using the same DNA extracted for the *nifH* detection, amplification and sequencing of the V3-V4 of the 16S rRNA gene was performed in an Illumina HiSeq 2000 machine (paired-end 2x300 bp). The Illumina sequence reads were deposited in the Sequence Read Archive (SRA) at the NCBI under accession number SRR8662465. The raw data obtained from Illumina sequencing were analyzed using the QIIME pipeline (version 1.9.1) (Caporaso *et al.* 2010). Singletons, chloroplasts, and mitochondrial sequences were removed from the data set using mothur's remove.lineage function. (Schloss *et al.* 2009). All putative chimeras were checked by the Usearch tool using a chimera-free reference database according to the Uchime algorithm (Edgar *et al.* 2011). These high-quality reads were clustered into OTUs at 97% sequence similarity using UCLUST (Edgar 2010). The taxonomic classification of unique operational taxonomic units (OTUs) was processed using the RDP Classifier (Wang *et al.* 2007). The relative abundance of each group at different taxonomic levels (phylum, class, order, family, and genus) was used for subsequent analysis.

Phylogenetic analysis

Nucleotide sequences were aligned using the MAFFT (Katoh and Standley 2013). Phylogenetic analysis was performed using the IQ-TREE program (Nguyen *et al.* 2014) with selection of evolutionary model using ModelFinder (Kalyaanamoorthy *et al.* 2017) and estimating of branch supports using UFBoot2 (Hoang *et al.* 2017).

Results

Phototrophic communities of soda lakes

The majority of the Kulunda Steppe soda lakes was dominated by algal-bacterial communities with the filamentous chlorophyte *Ctenocladus circinnatus* (*Ctenocladus*-communities) (**Fig. 1 a-d, Table 2**). It formed flocculated aggregates that concentrated near the shore and gradually dried up. Contrary to other lakes the Bitter-1 lake was dominated by the ephemeral cyanobacterial biofilms (**Fig. 1 g**) and endoevaporitic communities (**Fig. 1 e**).

According to the composition of the dominant potentially diazotrophic phototrophs several types of microbial communities were distinguished in the studied lakes:

1. **"Heterocystous" communities.** *Ctenocladus*-communities where heterocystous cyanobacteria visually dominated among phototrophic bacteria. Non-heterocystous and unicellular cyanobacteria and purple sulfur bacteria *Ectothiorhodospira* sp. were present as minor components. Such communities were found in the brine within the salinity range 25-60 g/l (samples B3-15, T6-15/1 and CS-16/2 in **Table 2**).

2. **"Non-heterocystous" communities**. *Ctenocladus*-communities and cyanobacterial biofilms with the visual dominance of filamentous non-heterocystous and/or unicellular cyanobacteria as well as purple sulfur bacteria *Ectothiorhodospira* sp. Heterocystous cyanobacteria were either absent or detected sporadically as akinetes. Such communities were present in the range of salinity from 55 to 210 g/l (**Fig. 1 d, Table 2**).

3. ***Ctenocladus*-communities without visible phototrophic diazotrophs** (cyanobacteria and/or purple sulfur bacteria) were found in Cock Soda Lake in 2014 at the salinity 65 g/l (sample CS-14/1) and in Picturesque Lake in 2016 at the salinity 85 g/l (sample Pic-16/1) (**Fig. 1 b, Table 2**).

4. ***Euhalothece*-communities.** Endoevaporitic algal-bacterial communities (**Fig. 1 e-f**) developed between the throna crystals in the Bitter-1 lake at salinities 350-400 g/l (samples B1-11, B1-12 in **Table 2**). Green unicellular algae *Dunaliella* sp., unicellular cyanobacteria *Euhalothece* sp. and purple sulfur bacteria *Ectothiorhodospira* sp. dominated there. The diazotrophic potential of such communities has been studied earlier (Namsaraev *et al.* 2018c).

5. **Biological soil crusts (BSC)** developed on the shores of the lakes. They were represented by cyanobacterial biofilms on the moist soil between thickets of *Salicornia altaica* (**Fig. 1 h**). Filamentous non-heterocystous and heterocystous cyanobacteria usually dominated there (T6-14/1, T6-14/2, T6-16 in **Table 2**), therefore "heterocystous" and "non-heterocystous" BSC could be distinguished.

Diversity of phototrophic microorganisms

The filamentous and unicellular cyanobacteria dominated in the studied phototrophic communities. Heterocystous cyanobacteria were represented by two species of *Nodularia*, morphologically corresponding to *Nodularia harveyana* (**Fig. 2 g**) and *Nodularia spumigena* (**Fig. 2 h**) (Komárek 2013) which were described in Kulunda Steppe lakes earlier by Voronikhin (1929, 1934). The vegetative filaments of *N. harveyana* and *N. spumigena* were detected in the brine at salinities up to 60 g/l (**Fig. 3**). Higher salinity was unfavorable for the development of the heterocystous cyanobacteria but akinetes of *N. spumigena* were found at the salinity up to 210 g/l (Cr-14). The heterocystous cyanobacteria are rarely detected at the salinities higher than 70-100 g/l in NaCl-dominated lakes with neutral pH (Oren 2011, 2015) and higher than 30-40 (90) g/l in soda lakes (Krienitz and Schagerl 2016; Tsyrenova *et al.* 2011; Voronikhin 1929, 1934). The representatives of the genus *Nodularia* are able to grow up to 100 g/l NaCl in culture that is probably the upper range of salinity for heterocystous cyanobacteria from soda lakes (Tsyrenova *et al.* 2011).

Geitlerinema sp. (**Fig. 2 a**) and *Nodosilinea* sp. (**Fig. 2 b**) were the most abundant filamentous non-heterocystous cyanobacteria common up to 210 g/l (**Fig. 3**). *Halomicronema* sp. (**Fig. 2 e**) and *Phormidium* sp. (**Fig. 2 f**) were present in some of the samples. *Halomicronema* sp. was found in 2015 in the sample from the Cock Soda Lake at the salinity 85 g/l (CS-15) and in 2014 on a moist soil near Tanatar VI (T6-14/1, T6-14/2). Morphologically it resembled the type species *Halomicronema excentricum* (Abed *et al.* 2002), isolated from the solar saltern ponds in Eilat (Israel). *Phormidium* sp. was spread in the samples within a wide salinity range from 25 to 210 g/l and in the samples of moist soil (T6-14/1, T6-14/2). Morphologically it corresponded to *Phormidium etoshii* KR2008/49, isolated from saline-alkaline Etosha pans, Namibia (Dadheech et al. 2013). A similar morphotype was described as *Oscillatoria brevis* in the Kulunda Steppe lakes at the beginning of the XX century by Voronikhin (1934).

Unicellular cyanobacteria were represented mainly by *Euhalothece* sp. (**Fig. 2 c**) which prevailed at the highest salinity values (**Fig. 3**). Besides, occasional colonies of *Chroococcus turgidus* were present in one sample (T6-15/1).

The purple sulfur bacteria *Ectothiorhodospira* sp. were present in the whole range of salinity up to 400 g/L (**Fig. 3**). In the environmental samples it usually occurred as dense colonies enclosed in a slime matrix which, probably, protected the cells from oxygen and elevated salinity (**Fig. 1 c**).

Earlier the *nifH* gene was detected in the genomes of *Geitlerinema* sp., *Nodosilinea* sp., *Euhalothece* sp. and *Nodularia* sp. (Namsaraev *et al.* 2018c), while the evidence for *Halomicronema* sp., *Phormidium* sp. and *Ch. turgidus* is still missing. The *Ectothiorhodospira* sp. possesses the *nifH*

295 genes and therefore can be an important diazotroph in soda lakes (Namsaraev *et al.* 2018c; Tourova *et*
296 *al.* 2014).
297
298 **The effect of salinity on diazotrophic activity**
299
300 Nitrogen fixation activity was detected by the ARA in the whole studied range of salinity from 25
301 to 400 g/L (**Fig. 4, Table 2**).
302 In the "heterocystous" communities (25 g/l in Bitter-3 in 2015 and 60 g/l in Tanatar VI in 2015)
303 the light and dark ARR were comparable with each other. There was a significant decrease in ARR with
304 an increase in salinity (19.93±2.93 and 2.29±0.28 nmol C_2H_4/ml·h at 25 and 60 g/l, respectively).
305 In the "non-heterocystous" communities in the range of salinity 55-90 g/l we detected the highest
306 rates of light ARR (up to 24.64±4.76 nmol C_2H_4/ml·h) comparable to those measured in Mono Lake at
307 50 and 75 g/l (Herbst 1998). At a higher salinity (100 g/l) the ARR severely declined to 1.35±0.12 nmol
308 C_2H_4/ml·h. The light-dependent rates significantly exceeded dark rates and the addition of glucose never
309 stimulated dark NF.
310 At salinity range160-210 g/l the "non-heterocystous" communities still existed (samples CS-12,
311 T6-11, B1-14, B3-12 and Cr 14). They exhibited low NF potential, but the light or dark dependence of
312 ARR was different (**Table 2**). Dark ARR exceeded light ARR in samples CS-12 and T6-11 indicating
313 that phototrophic diazotrophs possibly were less active than chemotrophs in Kulunda Steppe soda lakes.
314 In contrast, light ARR exceeded dark ARR in samples B1-14 and B3-12 (**Table 2**). This effect can be
315 explained by the appearance of extremely salt-tolerant *Euhalothece* sp. among dominating phototrophs.
316 Sample Cr-14 was not active: algae (*C. circinnatus*) and cyanobacteria were mostly present in dormant
317 forms at natural salinity 210 g/l. To investigate the influence of brine dilution on the growth and NF, we
318 placed this sample to diluted conditions (105 and 52.5 g/l). Dilution of brine quickly stimulated
319 proliferation of cyanobacteria (**Fig. S1**) and light ARR: 0.01±0.00, 0.20±0.02 and 0.29±0.11 nmol
320 C_2H_4/ml·h at 210, 105 and 52.5 g/l, respectively. The dark ARR didn't increase during this experiment.
321 These data coincide with previously obtained results for hypersaline lake Bitter-1(Namsaraev *et al.*
322 2018c).
323 At salinity range 350-400 g/l a low intensity of light-dependent NF (1.24±0.16 and 0.56±0.01
324 nmol C_2H_4/ml·h at 350 and 400 g/l, respectively) exhibited by *Euhalothece*-communities was detected
325 (**Fig. 4**). Biological soil crusts (BSC) were common on the shore of the lake Tanatar VI .
326 "Heterocystous" BSC exhibited NF potential (T6-14/1). "Non-heterocystous" BSC (T6-14/2, T6-16)
327 were exhibited very low light-dependent NF (**Table 2**).
328
329 **The case of sample Pic-16/1 from Picturesque Lake**

The sample Pic-16/1 was collected at the salinity 85 g/l and it is remarkable due to the high activity of the light-dependent ARR (17.12±3.32 nmol C_2H_4/ml·h), but the virtual absence of the recognizable morphotypes of potentially diazotrophic cyanobacteria or purple bacteria (**Fig. S2, Table 2**). Unlike other samples in our study dark ARR exhibited by sample Pic-16/1 was also significant (7.59 nmol C_2H_4/ml·h), indicating a possible contribution of chemotrophic bacteria into cumulative activity. To clarify which organisms were responsible for ARR in light and dark we have analyzed the diversity of *nifH* (**Fig. 5**) and 16S rRNA (**Fig. S3**) genes in the sample Pic-16/1. A clone library of *nifH* sequences (88 clones) included several phylotypes. The dominant phylotypes were identical to the *nifH* gene of the filamentous cyanobacterium *Nodosilinea* sp. (34 clones) and diverse representatives of *Deltaproteobacteria* (49 clones). Accordingly, *Nodosilinea* sp. was present in the sample as a minor component, which was also confirmed by a low representation of its sequences in the 16S rRNA gene library (**Fig. S3**). *NifH* and 16S rRNA genes belonging to other cyanobacteria (*Geitlerinema* sp., *Nodularia* sp.) were not detected in the sample Pic-16/1. Minor phylotypes were represented by *nifH* sequences related to *Ectothiorhodospira haloalkaliphila* (5 clones) and unclassified Proteobacteria (3 clones) related to unclassified phylotype (6KL-otu2-3 in **Fig. 5**) earlier detected in Tanatar V (Tourova *et al.* 2014). It was assumed that this branch either belongs to a known taxon whose *nifH* gene sequence is still lacking in public databases or represents yet unknown taxon within *Gammaproteobacteria*. Thus, sample Pic-16/1 contained a small but highly active population of non-heterocystous cyanobacterium *Nodosilinea* sp., detectable only by using functional molecular marker *nifH*. Therefore, this sample can be classified as "non-heterocystous" community. The dark NF in this sample can be attributed to the representatives of sulfate-reducing bacteria (**Fig. 5**).

Diurnal dynamics of nitrogen fixation

Two types of the diurnal dynamics of NF were observed in the "heterocystous" and "non-heterocystous" *Ctenocladus*-communities and cyanobacterial biofilms from the studied lakes:

1) Hourly ARR were high during both daylight time and at night. This pattern was observed in the "heterocystous" communities. Values of regular Pearson's correlation coefficients (CC) between illumination and ARR were insignificant at the 0.05 significance level for samples with this type of community (**Table 3)** indicating light-independent NF.

2) Hourly ARR were high during the whole daylight period and decreased or stopped after the sunset (**Fig. 6**). This type of daily pattern was typical to the "non-heterocystous" communities including "exceptional" sample Pic-16/1. Values of CC were not very high, although significant at the 0.05 significance level (**Table 3**). Values of cross-correlation function (CCF) at lags 1, 2 or 3 were much

higher than values of CC for the part of the samples (CS-14/1, CS-14/2, B1-14, B3-14, B1-16/2 and Pic 16/1 in **Table 3**). It means that the peaks of ARR took place 1, 2 or 3 hours later than the peaks of illumination. Interestingly, the lag values correlated to the composition of microorganisms in the community. For example, samples CS-14/1 and Pic-16/1 without visible phototrophic diazotrophs showed higher CCF values at 1-hour delay while samples B1-14 and B1-16/2 with non-heterocystous and unicellular cyanobacteria revealed higher CCF values at 2-hours delay. The samples with non-heterocystous cyanobacteria and purple bacteria (T6-14/2, T6-15/2, CS-16/1, CS-16/2) showed a faster response to light without delay (**Table 3**). Additionally, composition of microorganisms in the community may influence the values of CC and CCF. For example, the presence of rare vegetative filaments of heterocystous cyanobacteria in the "non-heterocystous" communities significantly reduced the values of CC/CCF: 0.89/0.88 for the sample CS-16/1 with non-heterocystous cyanobacteria and purple sulfur bacteria vs. 0.52/0.52 for the sample CS-16/2 with non-heterocystous cyanobacteria, purple sulfur bacteria and rare heterocystous cyanobacteria (**Table 3**).

The relationships between community composition and values of CC and CCF mentioned above were not studied in details in this work. Quantitative estimates of abundance of separate groups of diazotrophs in the community are necessary for unambiguous discussion on this problem.

Discussion

Wide fluctuations of the environmental conditions in the studied lakes allowed us to study the patterns of nitrogen fixation in the range of salinity between 25 and 400 g/L. To our knowledge this is the widest range of salinity analyzed in the papers devoted to nitrogen fixation in soda lakes.

The obtained data show that the salinity levels of around 60, 90-100 and 200 g/L can be considered as boundaries with a drastic shift in activity and composition of phototrophic communities.

At the salinity below 60 g/L the phototrophic diazotrophic community was dominated by heterocystous cyanobacteria of the genus *Nodularia*. With the increase of salinity the activity of NF in this type of community decreased and the community was replaced by "non-heterocystous" type. For example, the lake Tanatar VI was sampled at 100 g/L in 2014 and 60 g/L in 2015. In the first case no vegetative cells of heterocystous cyanobacteria were observed in the lake, but during second sampling the lake was dominated by heterocystous cyanobacteria. Also, the samples of soil crusts collected from the shore of Tanatar VI in 2014 contained heterocystous cyanobacteria and exhibited high ARR (7.14 nmol C_2H_4/g·h, sample T6-14/1) (**Table 2**). As heterocystous cyanobacteria are characteristic for alkaline soils (Shtina *et al.* 1998), the example of sample T6-14/1 can point out the general survival strategy of heterocystous cyanobacteria during dry salt-concentration periods and their contribution to the supply of bound nitrogen to soda lake ecosystems during both wet and dry periods.

At the salinity between 60 and 200 g/L the diazotrophic community was dominated by non-heterocystous cyanobacteria. Within this range at 90-100 g/L we detected a sharp decline of light-dependent ARR. The similar decline of ARR around 100 g/l was observed earlier in Mono Lake (Herbst 1998), as well as in hypersaline environments with neutral pH. For example, maximal ARR was detected in the range of 10-70 g/l and was almost absent at 100 g/l in planktonic communities of the Great Salt Lake (Marcarelli *et al.* 2006). The similar salinity response was observed in Bahamian hypersaline lagoons (Pinckney *et al.* 1995).

At the range 160-210 g/l the "non-heterocystous" communities still existed in Kulunda Steppe soda lakes, but phototrophic diazotrophs became less active than chemotrophic: dark NF prevailed over light NF or was comparable with it. This is in agreement with the data from Mono Lake showing that most or all of the ARR remaining at 150 g/l was attributable to the activity of anaerobic chemotrophs, most probably sulfate reducing bacteria (Herbst 1998). Interestingly, communities without *Euhalothece* sp. were not active or exhibited prevailing dark NF indicating that the salinities were too high for phototrophic components. For example, the sample from the Crooked Lake (Cr-14) at 210 g/l was inactive, but this situation quickly reversed to proliferation of active forms upon brine dilution from 210 to 105 and 52.5 g/l. At the same time the light-dependent ARR values increased 20-30 times, whereas the dark ARR didn't increase. In contrast, samples containing *Euhalothece* sp. among dominating phototrops (B1-14, B3-12) exhibited light-dependent ARR (**Table 2**). These cyanobacteria are extremely salt-tolerant (Garcia-Pichel et al. 1998; Mikhodyuk et al. 2008). In Kulunda Steppe soda lakes they dominate in endoevaporitic communities which also exhibited light-dependent NF at 350-400 g/l. Thus, 200 g/l is a boundary when salinity becomes favorable for *Euhalothece* sp. and "non-heterocystous" community starts to converse to the *Euhalothece*-community (**Fig. 3**).

The contribution of anoxygenic purple sulfur bacteria *Ectothiorhodospira* sp. to NF by natural communities is still not clear. Although it occurs in a whole range of salinity in environmental samples (**Fig. 3**), possess *nifH* genes and the ability for anaerobic growth in the nitrogen-free medium at extreme salinity (Namsaraev *et al.* 2018c), the environmental "non-heterocystous" communities with a dominance of *Ectothiorhodospira* sp. and cyanobacteria as a minor component (T5-15/1, T5-15/2, Pic-16/2) exhibited only low diazotrophic activity (**Table 2**). Also, the majority of such communities was represented by dense biomass of algae *C. circinnatus* driven by a wind to the shallow littoral zone. Most likely these communities were not limited in available nitrogen because of the decomposition of *Ctenocladus* biomass.

Cyanobacteria are considered to be the organisms that determine patterns of NF in cyanobacterial mats. Different species may exhibit various types of temporal and spatial separation of photosynthesis and NF which provides specific diurnal dynamics of the whole community (Stal 2012, 2015). For example, the NF in heterocystous cyanobacteria in most cases is light-dependent and cease in the night

as a result of insufficient energy supply to heterocysts (Stal 2012). But contrary to this, “heterocystous” communities in Kulunda Steppe soda lakes exhibited light-independent NF, showing unusual behavior. Such behavior is exceptional but known for some heterocystous cyanobacteria from different habitats (Griffiths *et al.* 1987; Huber 1986; Stal 2015).

Patterns of 24-hours ARR measured in “non-heterocystous” communities during several years in a variety of Kulunda Steppe soda lakes at different salinities were always significantly dependent on the light intensity. These patterns were also unusual in comparison with marine cyanobacterial mats with non-heterocystous cyanobacteria with typical peaks of NF around sunset or sunrise (Stal 2012). But the examples of exceptional behavior similar to our case are also known: *Coleofasciculus*-dominated mat from the North Sea beach of Schiermonnikoog in the Netherlands exhibited constantly fluctuating diazotrophic activity with the maximal ARR occurring during the light period (Bolhuis *et al.* 2010). Thereby, diurnal dynamics of NF may be a result of a combined contribution of phylogenetically and ecophysiologically different bacteria. Similar to the light-controlled phototrophic diazotrophs, the chemotrophs (*Proteobacteria*, *Bacteroidetes*) may have their own circadian control of NF related genes which can be influenced by light, temperature or metabolites from neighboring species (Hörnlein *et al.* 2018). But the cumulative contribution of different components of the diazotrophic communities leading to the observed patterns of NF is something still unexplored neither in soda lakes nor in other habitats.

Conclusion

Highly productive shallow soda lakes of the Kulunda Steppe (Altai Region, Russia) represent a spectacular example of double-extreme habitats whereby microbial nitrogen fixation is still not well understood. We found that nitrogen fixation occurs in a wide range of salinity (25-400 g/l) there. This study suggests that several groups of photo- and heterotrophic bacteria with significantly different salinity tolerance play a role in the observed cumulative NF activity in these lakes. The specific patterns of diurnal NF exhibited by studied communities differ from those typical for well-studied marine microbial mats with non-heterocystous cyanobacteria.

Funding

This work was supported by the Russian Foundation for Basic Research [Grant numbers 19-04-00377, 19-04-00401] and Ministry of Science and Higher Education of the Russian Federation. Z.B. Namsaraev was supported by NRC "Kurchatov Institute".

Acknowledgements

This study was performed using the scientific equipment of the Core Research Facility 'Bioengineering' (Research Center of Biotechnology RAS).

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Table 1. The studied soda lakes and its hydrochemical properties during years when nitrogen fixation was investigated.

Lake (name in Russian pronunciation)	Locality in Altai Region	Coordinates (Google maps)	Year	pH	Salinity (g/l)	Alkalinity (M)	
						CO_3^{2-}	Total
Cock Soda Lake (Petukhovskoe Sodovoe)	Klyuchevskoi district	52°6′20.52″N 79°9′22.19″E	2011	10.2	100	1.0	1.1
			2012	9.8	200	2.4	2.7
			2014	10.0	65	0.4	0.5
			2015	10.0	85	0.3	0.6
			2016	9.9	55	0.5	0.6
Bitter-1 (Gorchina 1)	Mikhailovsky district	51°40'19.1"N 79°54'20.4"E	2011	9.9	350	3.8	4.4
			2012	10.2	400	4.4	4.9
			2014	10.3	200	1.7	2.1
			2016	10.2	85	1.0	1.3
Bitter-3 (Gorchina 3)	Mikhailovsky district	51°40'00.4"N 79°54'43.9"E	2011	10.3	90	0.8	1.0
			2012	9.9	200	2.6	3.0
			2014	10.5	60	0.4	0.5
			2015	10.5	25	0.5	0.6
Tanatar V	Mikhailovsky district	51°37'27.4"N 79°50'26.9"E	2015	10.1	100	0.8	1.0
Tanatar VI	Mikhailovsky district	51°37'08.4"N 79°48'53.0"E	2011	10.0	160	1.3	1.7
			2012	9.8	250	3.2	3.4
			2014	10.1	100	0.5	0.6
			2015	10.2	60	0.5	0.6
			2016	9.9	60	0.5	0.6
Picturesque Lake (Zhivopisnoe)	Mikhailovsky district	51°43'35.3"N 79°52'24.5"E	2016	9.7	85	0.6	0.7
Crooked Lake (Krivoe)	Uglovsky district	51°39'38.4"N 80°08'46.2"E	2014	9.1	210	0.7	1.0

600 **Table 2.** The phototrophic communities in soda lakes of Kulunda Steppe and their acetylene reduction
601 rates (ARR).

Year	Type of sample (salinity of brine, g/l)	Sample code	Type of phototrophic community and dominant morphotypes of phototrophic microorganisms	Chl *a* (μg/ml)	ARR (nmol C_2H_4/ml·h)	
					Light	Dark
Cock Soda lake						
2011	brine (100)	CS-11	*Ctenocladus*-community with cyanobacteria *Geitlerinema* sp. and purple bacteria *Ectothiorhodospira* sp.	13.27	1.35±0.12	0.02±0.01
2012	drying mud (200)	CS-12	*Ctenocladus*-community with cyanobacteria *Geitlerinema* sp., *Nodosilinea* sp. and purple bacteria *Ectothiorhodospira* sp.	44.30	0.46±0.21	0.72±0.20
2014	brine (65)	CS-14/1	*Ctenocladus*-community (cyanobacteria are not visible)	4.79	0.12±0.04	0.01±0.01
		CS-14/2	*Ctenocladus*-community with cyanobacteria *Geitlerinema* sp., *Nodosilinea* sp.	3.67	1.52±0.16	0.01±0.01
2015	brine (85)	CS-15	*Ctenocladus*-community with cyanobacteria *Geitlerinema* sp., *Halomicronema* sp.	11.95	0.16±0.09	0.04±0.03
2016	brine (55)	CS-16/1	*Ctenocladus*-community with cyanobacteria *Geitlerinema* sp., *Nodosilinea* sp. and purple bacteria *Ectothiorhodospira* sp. Rare akinetes are present.	29.62	16.56±7.24	0.03±0.03
		CS-16/2	*Ctenocladus*-community with cyanobacteria *Geitlerinema* sp., *Nodosilinea* sp., rare heterocystous *N. harveyana, N. spumigena* and purple bacteria *Ectothiorhodospira* sp.	11.24	13.22±2.84	0.05±0.05
Bitter-1						
2011	brine under silt crust (350)	B1-11	Endoevaporitic community with green alga *Dunaliella* sp. and cyanobacterium *Euhalothece* sp., as well as purple bacteria *Ectothiorhodospira* sp.	32.0	1.24±0.16	0
2012	brine under silt crust (400)	B1-12	Endoevaporitic community with green alga *Dunaliella* sp. and cyanobacterium *Euhalothece* sp., as well as purple bacteria *Ectothiorhodospira* sp.	19.3	0.56±0.01	0.16±0.07
2014	brine (200)	B1-14	Cyanobacterial films with the dominance of *Geitlerinema* sp., *Euhalothece* sp., *Nodosilinea* sp., and purple bacteria *Ectothiorhodospira* sp.	2.59	0.32±0.10	0.01±0.00
2016	brine (85)	B1-16/1	*Ctenocladus*-community with cyanobacteria *Geitlerinema* sp., *Nodosilinea* sp., *Euhalothece* sp.	17.19	9.00±2.56	0.62±0.37
		B1-16/2	Cyanobacterial films with the dominance of *Nodosilinea* sp., *Euhalothece* sp., *Geitlerinema* sp.	13.12	8.00±2.74	1.12±1.10
Bitter-3						
2011	brine (90)	B3-11	*Ctenocladus*-community with cyanobacteria *Nodosilinea* sp., *Geitlerinema* sp. and purple bacteria *Ectothiorhodospira* sp.	27.83	24.64±4.76	0.25±0.11
2012	brine (200)	B3-12	*Ctenocladus*-community with cyanobacteria *Geitlerinema* sp., *Nodosilinea* sp. and *Euhalothece* sp.	46.2	0.32±0.03	0
2014	brine (60)	B3-14	*Ctenocladus*-community with cyanobacteria *Nodosilinea* sp. (also akinetes of *N. harveyana*) and purple bacteria *Ectothiorhodospira* sp.	1.40	0.13±0.07	0
2015	brine (25)	B3-15	*Ctenocladus*-community with cyanobacteria *Nodosilinea* sp., *N. harveyana* (and also *Geitlerinema* sp., *Phormidium* sp, *Euhalothece* sp.) and purple bacteria *Ectothiorhodospira* sp.	5.15	19.93±2.93	16.56
Tanatar V						
2015	compressed biomass in brine (100)	T5-15/1	*Ctenocladus*-community with cyanobacteria *Geitlerinema* sp. and purple bacteria *Ectothiorhodospira* sp.	*15.44*	*0.10±0.06*	*0.02±0.00*
	compressed biomass in mud	T5-15/2	*Ctenocladus*-community with purple bacteria *Ectothiorhodospira* sp. (cyanobacteria *Geitlerinema* sp., as a minor component)	*40.95*	*0.12±0.09*	*0.02±0.00*
Tanatar VI						

2011	brine (160)	T6-11	*Ctenocladus*-community with cyanobacteria *Geitlerinema* sp., *Nodosilinea* sp. and *Euhalothece* sp.	15.07	1.02±0.07	1.65±0.01
2014	moist soil (100)	T6-14/1	Cyanobacterial films with *Nodosilinea* sp., *Phormidium* sp., *N. harveyana* (vegetative cells and akinetes), *Halomicronema* sp.	*16.56*	*7.14*	*n/d*
		T6-14/2	Cyanobacterial films with the dominance of *Geitlerinema* sp., *Halomicronema* sp. and *Phormidium* sp., *Nodosilinea* sp. is also present	*7.74*	*0.11±0.01*	*0.05±0.01*
2015	brine (60)	T6-15/1	*Ctenocladus*-community with cyanobacteria *N. harveyana*, *Chroococcus turgidus*	10.06	2.29±0.28	2.52±0.27
	drying mud	T6-15/2	*Ctenocladus*-community with cyanobacteria *Geitlerinema* sp.	11.79	1.23±0.18	0.04±0.02
2016	moist soil (60)	T6-16	Cyanobacterial films with *Nodosilinea* sp., *Geitlerinema* sp., *Euhalothece* sp.	*64.46*	*0.07±0.02*	*0.01±0.00*
Picturesque lake						
2016	brine (85)	Pic-16/1	*Ctenocladus*-community (cyanobacteria are not visible)	13.71	17.12±3.32	7.59
		Pic-16/2	*Ctenocladus*-community with purple bacteria *Ectothiorhodospira* sp. (cyanobacteria *Geitlerinema* sp. as a minor component)	60.43	0.11±0.05	0.04±0.01
Crooked lake						
2014	brine (210)	Cr-14	Remains of *Ctenocladus*-community with dominance of *Geitlerinema* sp. and *Phormidium* sp., *N. spumigena* (akinetes)	3.04	0.01±0.00	0

602 *n/d* not determined; *values in italics* mean that amount of Chl *a* is given in μg/g and ARR is given in
603 nmol C_2H_4/g·h

Table 3. Values of regular Pearson's correlation coefficients (CC) and values of cross-correlation function (CCF) between illumination (first variable) and ARR (lagged variable) of phototrophic biomass from various sampling sites.

Year	Lake	Sample code	Type of community	CC	p-value	CCF †	Delay (h)	p-value
2014	Cock Soda lake	CS-14/1	*Ctenocladus*-community without visible cyanobacteria	**0.58**	**0.0028**	**0.73**	**1**	**0.0020**
		CS-14/2	*Ctenocladus*-community with non-heterocystous cyanobacteria	**0.64**	**0.0008**	**0.76**	**1**	**0.0015**
	Bitter 1	B1-14	Films with non-heterocystous and unicellular cyanobacteria and purple bacteria	**0.53**	**0.0076**	**0.83**	**2**	**0.0008**
	Bitter 3	B3-14	*Ctenocladus*-community with non-heterocystous cyanobacteria and purple bacteria	**0.55**	**0.0058**	**0.85**	**3**	**0.0008**
	Tanatar VI	T6-14/1	Films with vegetative heterocystous cyanobacteria	0.19	0.3849	-	-	-
		T6-14/2	Films with non-heterocystous cyanobacteria	**0.67**	**0.0004**	**0.67**	**0**	**0.0035**
2015	Bitter 3	B3-15	*Ctenocladus*-community with vegetative heterocystous cyanobacteria and purple bacteria	0.28	0.1818	-	-	-
	Tanatar VI	T6-15/1	*Ctenocladus*-community with vegetative heterocystous cyanobacteria	0.10	0.6520	-	-	-
		T6-15/2	*Ctenocladus*-community with non-heterocystous cyanobacteria	**0.82**	**0.0000**	**0.83**	**0**	**0.0005**
2016	Cock Soda lake	CS-16/1	*Ctenocladus*-community with non-heterocystous cyanobacteria and purple bacteria	**0.89**	**0.0000**	**0.88**	**0**	**0.0003**
		CS-16/2	*Ctenocladus*-community with non-heterocystous and rare vegetative heterocystous cyanobacteria and purple bacteria	**0.52**	**0.0085**	**0.52**	**0**	**0.0191**
	Bitter 1	B1-16/1	*Ctenocladus*-community with non-heterocystous and unicellular cyanobacteria	**0.57**	**0.0037**	**0.61**	**0**	**0.0065**
		B1-16/2	Films with non-heterocystous and unicellular cyanobacteria	**0.56**	**0.0040**	**0.65**	**2**	**0.0059**
	Picturesque lake	Pic-16/1	*Ctenocladus*-community without visible cyanobacteria	**0.52**	**0.0085**	**0.58**	**1**	**0.0112**

† – Maximal significant value of CCF for a certain couple of series (illuminance vs. ARR). Significant (at least at 0.05 s.l.) values are in **bold**.

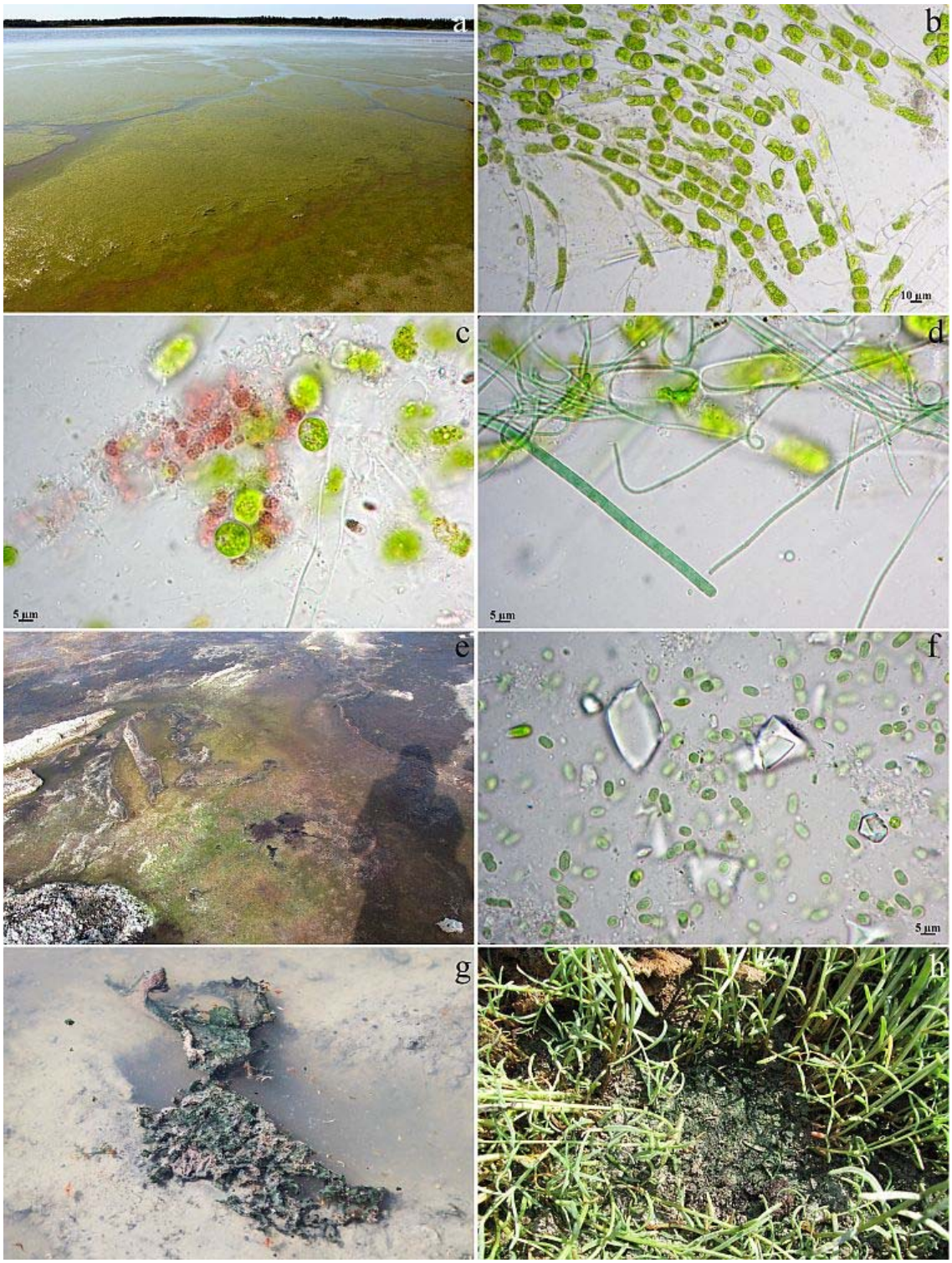


Fig. 1. General view (a, e, g, h) and light microscopy (b-d, f) of phototrophic communities from Kulunda Steppe soda lakes. a) *Ctenocladus*-community in Cock Soda Lake, 2011; b) filaments of *C. circinnatus*; c) *C. circinnatus* with colonies of purple bacteria *Ectothiorhodospira* sp.; d) *C. circinnatus* with non-heterocystous cyanobacteria *Nodosilinea* sp. and *Geitlerinema* sp.; e) endoevaporitic *Euhalothece*-community in Bitter-1 lake, 2012; f) unicellular cyanobacteria *Euhalothece* sp. and green algae *Dunaliella* sp. between the crystals of throne; g) cyanobacterial biofilms in brine in Bitter-1 lake, 2014; h) biological soil crust on the moist soil between thickets of *Salicornia altaica* on the shoe of Tanatar VI, 2014.

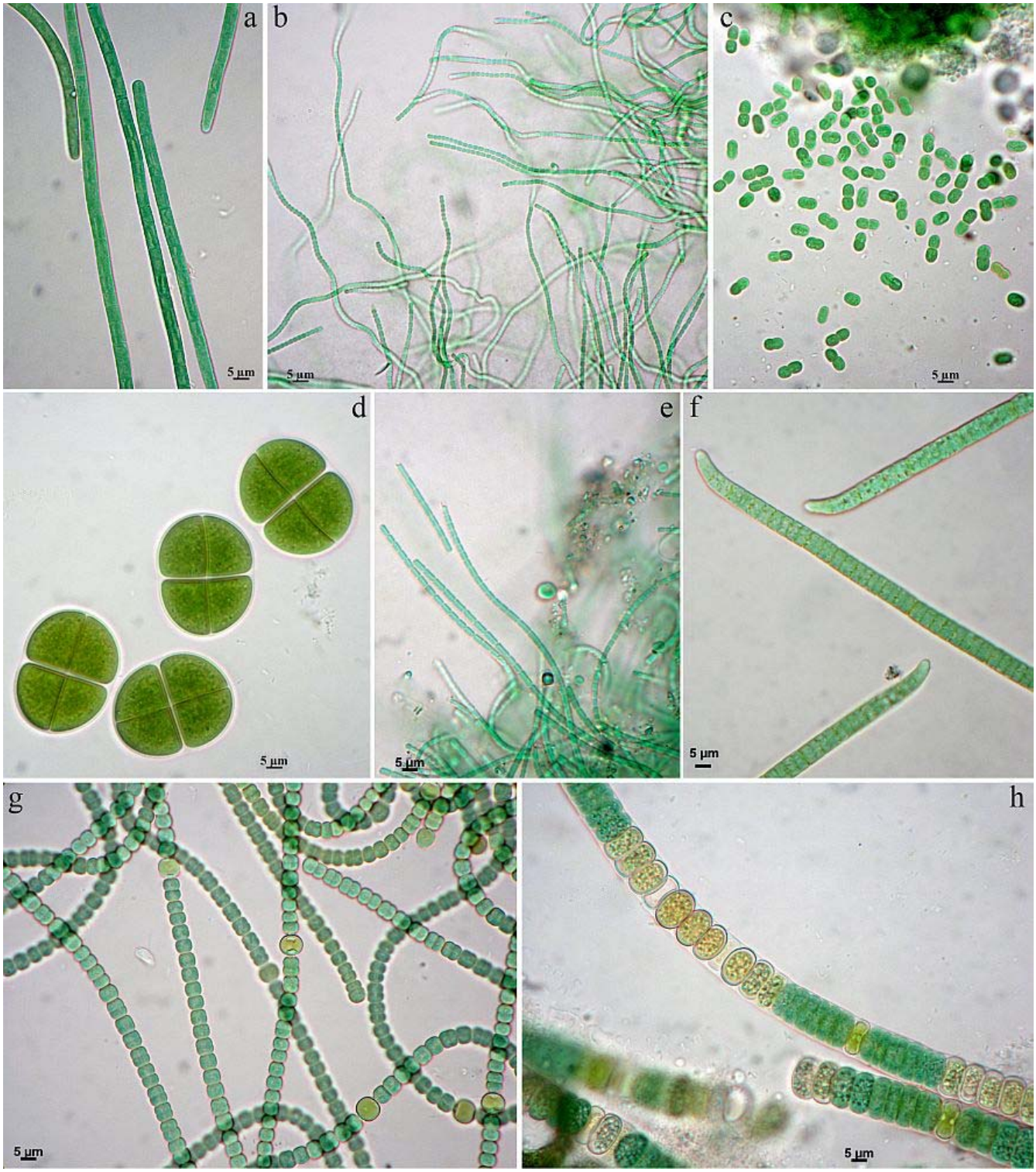
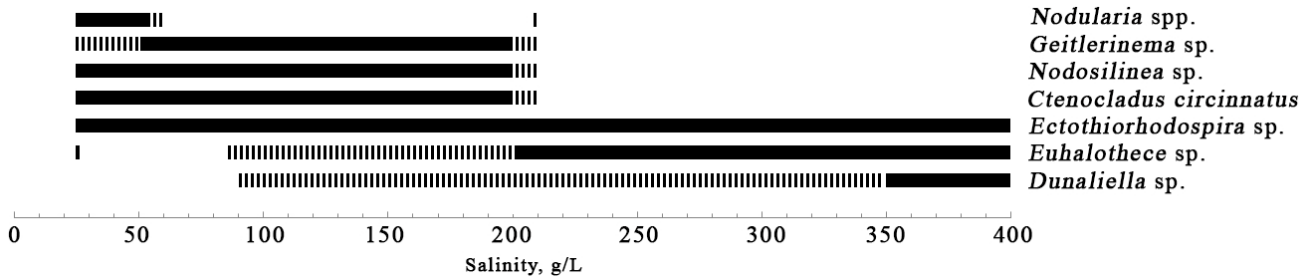


Fig. 2. Morphotypes of cyanobacteria detected in the samples from Kulunda Steppe soda lakes: a) *Geitlerinema* sp., b) *Nodosilinea* sp., c) *Euhalothece* sp., d) *Chroococcus turgidus*, e) *Halomicronema* sp., f) *Phormidium* sp., g) *Nodularia harveyana*, h) *Nodularia spumigena*.



627

628 **Fig. 3.** The range of salinity where different phototrophs were detected in the samples under study.
629 Filled line – a range with mass development, dashed line – a range with occurrence from frequent to
630 sporadic.

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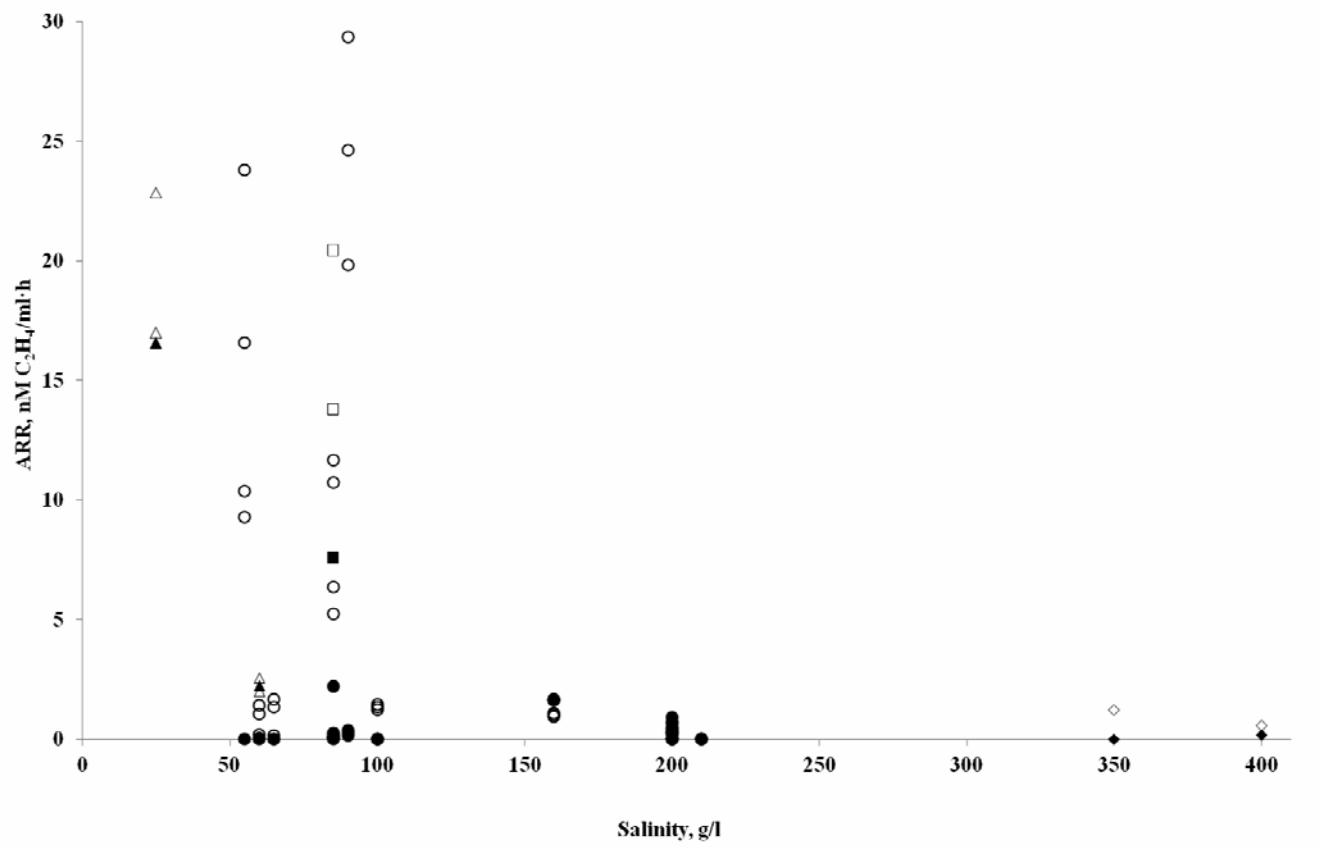

634

635 **Fig. 4.** Acetylene reduction rates (ARR) in brine under different salinities. Δ – "heterocystous"
636 communities, ○ – "non-heterocystous" communities, □ – sample Pic-16/1, ◊ - *Euhalothece*-
637 communities. Empty marks – light ARR, black marks – dark ARR.

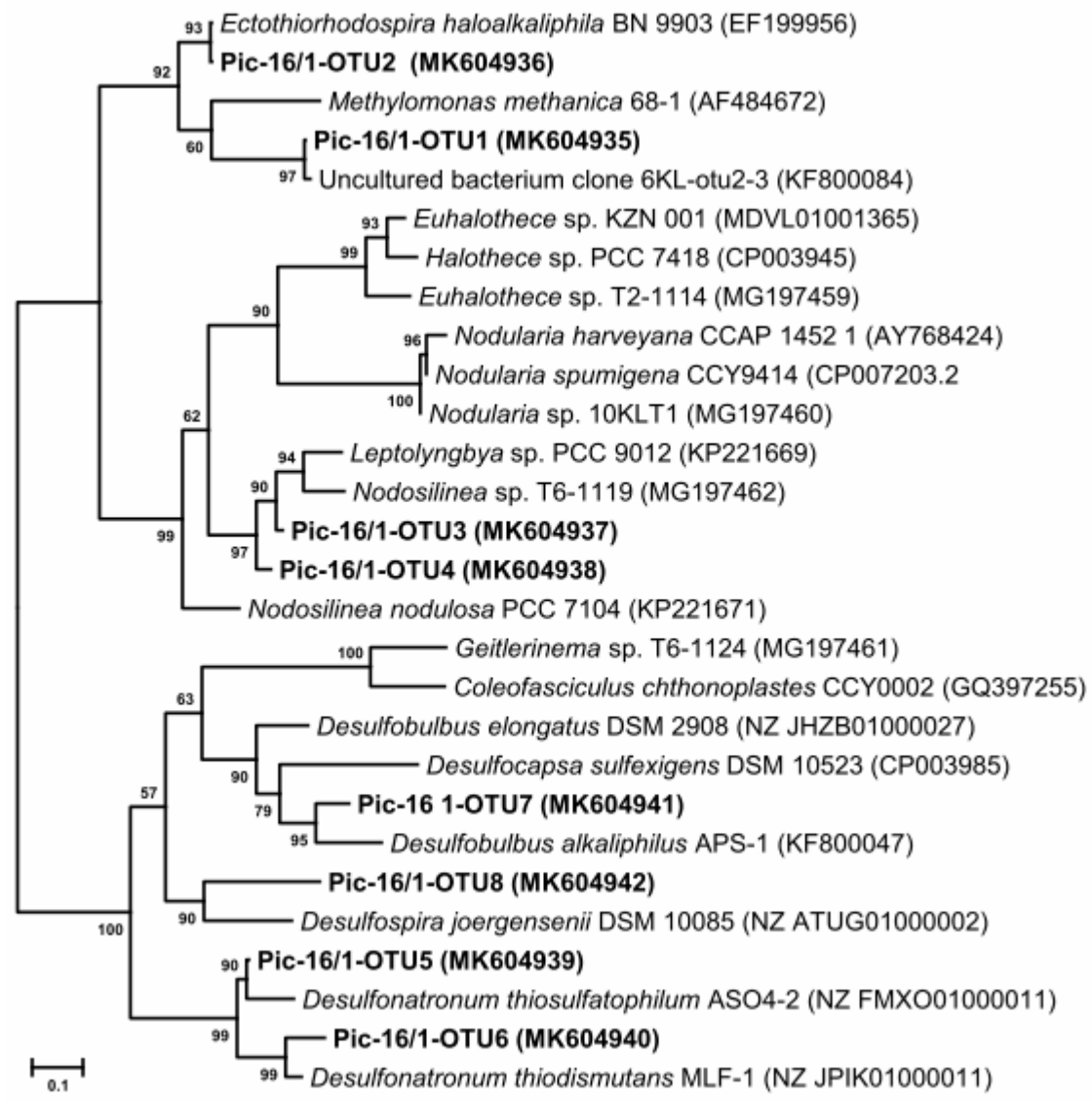

Fig. 5. Maximum-likelihood phylogenetic tree based on *nifH* gene sequences (426 nucleotide sites) obtained from the sample Pic-16/1 (Picturesque Lake). The tree was reconstructed with evolutionary model TN+F+I+G4. The scale bar represents nucleotide substitutions per site.

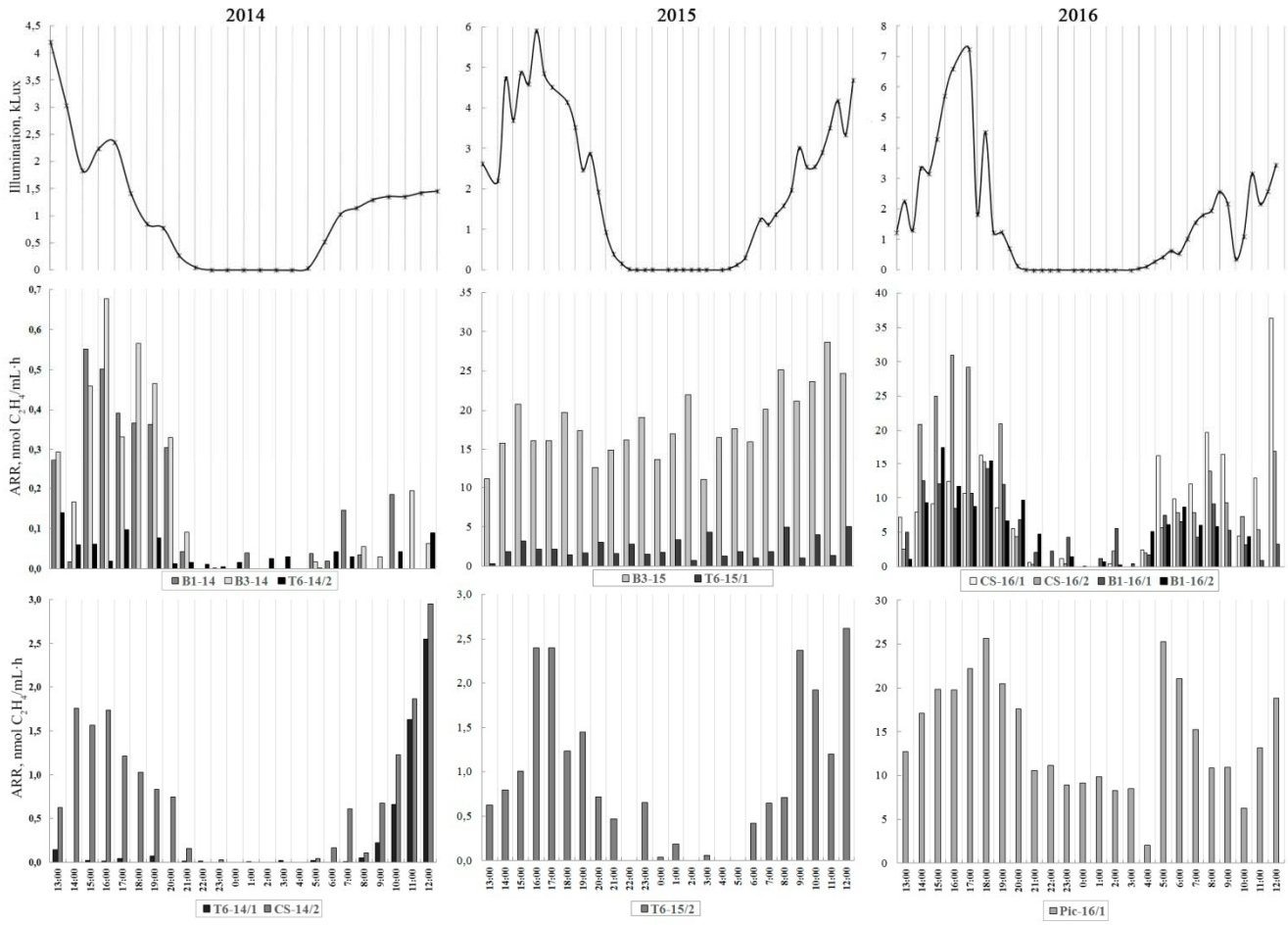

Fig. 6. Diurnal dynamics of ARR shown by phototrophic microbial communities from different lakes at the beginning of July 2014 (a-c), 2015 (d-f) and 2016 (g-i). Sample codes correspond to those given in tables 2 and 3. a, d, g) illumination (kLux); e) ARR exhibited by "heterocystous" communities dominated by *Nodularia* spp. (samples B3-15 and T6-15/1); i) ARR exhibited by the sample Pic-16/1 (*Ctenocladus*-community without visible cyanobacteria).

Fig. S1. Microphotographs of natural and 4-times diluted (activated for growth) sample Cr-14 collected from Crooked Lake in 2014. The sample represents remains of "non-heterocystous" *Ctenocladus*-community with *Geitlerinema* sp., *Phormidium* sp. and akinetes of *Nodularia spumigena*.

Fig. S2. Field photos of *Ctenocladus*-community from Picturesque Lake in 2016 and microphotographs of sample Pic-16/1 which represents *Ctenocladus*-community without visible cyanobacteria and purple sulfur bacteria.

Fig. S3. Maximum-likelihood phylogenetic tree based on 16S gene sequenses (484 nucleotide sites) obtained from the sample Pic-16/1. The tree was reconstructed with evolutionary model TIM3e+I+G4. The scale bar represents mucleotide substitutions per site.