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The isolation and characterization of actinobacteria from dominant benthic macroinvertebrates endemic to Lake Baikal

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Abstract The high demand for new antibacterials fosters the isolation of new biologically active compounds producing actinobacteria. Here, we report the isolation and initial characterization of cultured actinobacteria from dominant benthic organisms' communities of Lake Baikal. Twenty-five distinct strains were obtained from 5 species of Baikal endemic macroinvertebrates of amphipods, freshwater sponges, turbellaria worms, and insects (caddisfly larvae). The 16S ribosomal RNA (rRNA)-based phylogenic analysis of obtained strains showed their affiliation to *Streptomyces*, *Nocardia*, *Pseudonocardia*, *Micromonospora*, *Aeromicrobium*, and *Agromyces* genera, revealing the diversity of actinobacteria associated with the benthic organisms of Lake Baikal. The biological activity assays showed that 24 out of 25 strains are producing compounds active against at least one of the test cultures used, including Gram-negative bacteria and *Candida albicans*. Complete dereplication of secondary metabolite profiles of two isolated strains led to identification of only few known compounds, while the majority of detected metabolites are not listed in existing antibiotic databases.

Denis Axenov-Gribanov and Yuriy Rebets contributed equally to this work.

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Introduction

Actinobacteria are the largest source of biologically active compounds that are widely used in medicine, veterinary, agriculture, and others (Demain and Adrio 2008). Around 55 % of all antibacterial compounds are coming from the species from the genus *Streptomyces* and 11 % from other actinobacteria (Strohl 1997). The search for new antibiotics constantly continues in order to combat new challenges that are rising due to appearance of new pathogens and development of antibiotic resistance by existing pathogenic bacteria. This search involves isolation of new producing strains, including new actinobacteria. Nowadays, the necessary prerequisite for successful screening program for antibacterial producers is going for new loci (Monciardini et al. 2014). Classic sources of actinobacteria are exhausted, and discovery of new biologically active compounds is rather inefficient. Thus, the screening programs are often oriented to expand the isolation sources to new geographical/geological locations with extreme and/or unusual conditions or symbiotic or pathogenic interactions (Alvin et al. 2014; Nikapitiya 2012). It is a fact that bacterial strains obtained from unusual and extreme ecosystems and their biological communities tend to produce new biologically active compounds (Jenke-Kodama and Dittmann 2009). Successful examples of such programs are isolation of new actinobacteria from wasps and their nests (Kroiss et al. 2010; Madden et al. 2013), leaf-cutting ants (Currie et al. 2003), and other invertebrates, as well as from plants (Alvin et al. 2014; Mushegian et al. 2011), thermal springs (Valverde et al. 2012), radioactive wastes (Bagwell et al. 2008; Mao et al. 2007), and others. Microbial communities of marine sponges have been also studied as a promising source of pharmaceutical and biotechnical important chemicals for many years (Hentschel et al. 2012; Monciardini et al. 2014; Nikapitiya 2012).

The peculiarities and biodiversity of ancient Lake Baikal ecosystem are well known and described by Kozhova and Izmest'eva (1998), Timoshkin et al. (2001), and others. In view of the high degree of the endemicity fauna of Lake Baikal is the promising object for screening for new actinobacteria strains. Amphipods (*Amphipoda*, *Crustacea*), gastropods (*Gastropoda*, *Mollusca*), freshwater sponges (*Porifera*), planarians (*Planariidae*, *Plathelminthes*), and caddisfly (*Trichoptera*) are the largest groups of macroinvertebrates with the highest level of biodiversity and distribution (Kozhova and Izmest'eva 1998; Timoshkin et al. 2001). All tested organisms are inhabitants of the lake's littoral community, experiencing respective environmental and anthropogenic impacts. Baikal amphipod and gastropod groups are widely studied organisms in terms of ecology, taxonomy, and ecophysiology. Sponges are well studied with respect to their filtering activity and microbial consortia. Similarly, planaria have been well documented as parasitic symbionts of fishes and amphipods, and caddisfly larvae have been considered an important indicator for the biomonitoring of ancient ecosystems (Timoshkin et al. 2001).

Organisms inhabit Lake Baikal from the water's edge to maximum depths (1642 m). In terms of endemic peculiarities and stable environmental conditions, the majority occupy highly specialized ecological niches, including food spectrum, thermal regime, biotic interactions, and others. The greatest proportion of species richness (60–70 %) is concentrated in the littoral zone and represented mostly by benthic invertebrates (Timoshkin et al. 2001). They can accumulate high numbers of microorganisms and their spores due to their active water mass filtering capacities and/or active moving along the bottom of the lake. Dwellers and scavengers of Lake Baikal are of particular interest for actinobacteria isolation purposes.

Several attempts of characterization of Lake Baikal actinobacterial microbiome have been already published by Zakharova et al. (2013). The same researchers reported that actinobacteria represents around 16 % of the entire microbiome of the lake sediments. Studies of the microbiome of two endemic sponge species also demonstrated high occupancy by the actinobacteria species (Gladkikh et al. 2014). Terkina et al. (2002) reported isolation and initial classification of actinobacteria from water and sediments of the lake. Interestingly, these authors observed quite high enrichments of isolates from the lake sediments in *Micromonospora* species (up to 59 %), while *Streptomyces* were predominant in the water samples. The same group reported a high dosage of biologically active strains among Lake Baikal isolates, including those inhibiting the growth of pathogenic bacteria as well as tumor cells (Terkina et al. 2006). These studies clearly demonstrated the huge potential of the ecosystem of Lake Baikal for discovery of new species of bacteria with unique metabolic profiles. Thus, the main goal of our research was to investigate the possibility of isolation of new actinobacteria species from the atypical sources, mainly focusing on endemic invertebrates of Lake Baikal, with the focus on the biological activity of obtained species. Here, for the first time, we report the isolation of cultured actinobacteria from several dominant endemic benthic organisms of Lake Baikal including amphipods, planarians, caddisfly, and their analysis in terms of phylogeny and antibacterial activity. The presented data shows a great potential for the discovery of new producers of biologically active compounds from Lake Baikal opening a perspective for deeper investigations in this direction.

Materials and methods

Sample collection, preparation, and isolation of actinobacteria

The endemic species representing dominant taxa from Lake Baikal invertebrates' fauna were chosen for actinobacteria isolation: spongia (*Baicalospongia bacilifera*), amphipoda (*Pallasea concelloides*, *Brandtia* sp.), caddisfly *Trichoptera* sp. (larvae), and turbellaria (*Baikalobia variegata*). The animals were collected in February 2014 in Listvyanka village (South Baikal, N 51.867936, E 104.829715) using a benthic dragnet. The depth of sampling was 5–7 m. Immediately following sampling, the animals were placed into thermostatic conditions at 6 °C with constant aeration and transferred to the laboratory. The samples were washed with sterile water, fixed, and homogenized manually in 20 % sterile glycerol in ratio 1:10. As a negative control, water from tanks where animals were kept was used.

The isolation and cultivation of actinobacteria strains were performed on the solid nutrient media MS (soy flour, 20 g/L; D-mannitol, 20 g/L; agar, 20 g/L; pH 7.2) and Czapek agar ($NaNO_3$, 2 g/L; starch, 30 g/L; $MgSO_4$ $7H_2O$, 0.5 g/L; KCl, 0.5 g/L; FeSO4 7H2O, 0.01 g/L; K_2HPO_4, 1 g/L; agar, 20 g/L; pH 7.2) at 28 °C supplemented with antibiotics cycloheximide (50 μg/mL) and phosphomycin (100 μg/mL) (Kieser et al. 2000). Homogenates were diluted 1:10, 1:100, and 1:1000 in sterile 1 % saline solution, and dilutions were plated on MS media in 3 replicates. Plates were incubated for 14 days at 28 °C and checked every 24 h for actinomycetes colonies appearance. Actinomycetes were recognized based on colony morphology: solid density of colonies, growth inside of the agar media, and steady border of colonies (Kieser et al. 2000). Colonies with a leathery texture with or without aerial hyphae were transferred from the primary plates onto new MS media. An attempt was made to obtain in pure culture all colonies observed on the primary plates. In this way, 25 isolates were obtained. Active and rare strains were deposited in Russian Collection of Agricultural Microorganisms (RCAM), St.

Petersburg. Information about the strain deposition is available on www.arriam.spb.ru (accession numbers: 03463-03476).

16S rRNA gene sequencing and analysis

Strains were grown in 10 mL of TSB media at 28 °C for 3 days, and total DNA was isolated as described (Kieser et al. 2000). Amplification of the 16S ribosomal RNA (rRNA) gene was carried out with primers: 8F (AGA GTT TGA TYM TGG CTC AG) and 1510R (TAC GGY TAC CTT GTT ACG ACT T). Obtained fragments were gel purified using QIAquick Gel Extraction Kit (Qiagen, Venlo, Netherlands) and sequenced with the amplification primers 8F and 1510R generating almost the entire gene sequence (1365~1401 bp). The forward and reverse sequences were assembled with SeqMan DNAStar software (Lasergen, Houston, USA). The entire gene sequences were aligned with the bacterial 16S rRNA gene sequences from the RDP-II database (Wang et al. 2007). Ninety-one rDNA sequences were aligned using MAFFT v7.017 (gap open penalty 1.53, offset value 0.123, scoring matrix 200PAM/k=2, algorithm: auto) (Katoh 2013). A dendrogram was built using Geneious (Kearse et al. 2012) (Tamura-Nei genetic distance model, neighbor-joining method, *Escherichia coli* as an outgroup, bootstrap value 1000, consensus tree with 50 % support threshold). Sequences were deposited in the GenBank (accession numbers: KP749314-KP749323, KP749325-KP749338, and KT005998).

Metabolites analysis

Isolated strains were inoculated in 10 mL of TSB, grown for 2 days at 28 °C, and 2 mL of pre-culture were used to inoculate the 50 mL of production media. Nutrient media NL-19 (soy flour, 20 g/L; D-mannitol, 20 g/L; pH 7.2) and SG (glucose, 20 g/L; soy peptone, 10 g/L; $CaCO_3$, 2 g/L; $CoCl_2$, 0.001 g/L; pH 7.2) were used as production media. Strains were cultivated on both media at 28 °C for 4 days. Metabolites from cultural liquid were extracted with ethyl acetate (Sigma, St. Louis, USA). The compounds from biomass and solid MS media were extracted with acetone:methanol mixture (ratio 1:1). The obtained extracts were evaporated and dissolved in methanol. Samples were initially analyzed on low-resolution ion trap LC-MS amaZone system (Bruker, Billerica, USA). Samples were separated on an Ultimate 3000 HPLC system (Dionex, Sunnyvale, USA) using C18 column (Affymetrix, Santa Clara, USA) and linear gradient of acetonitrile against 0.1 % ammonium formate solution in water over time of 20 min with flow rate 0.5 mL/min. Detection was performed in both negative and positive modes. After an initial assessment, samples were analyzed by the ultra-high resolution mass spectrometry using LC-QTOF system maXis II (Bruker, Billerica, USA). HPLC conditions used were the same as for low-resolution LC-MS analysis. The mass detection was performed in both positive and negative modes with the detection range set to 160–2500.

Data were collected and analyzed by Brucker Compass Data Analysis software, version 4.1 (Bruker, Billerica, USA). The screening for known compounds was performed using Dictionary of Natural Products database version 6.1 (CRC Press, Boca Raton, USA), using the following parameters for search: accurate molecular mass, absorption spectra, and source of compounds isolation (Whittle et al. 2003). Compounds were considered to be similar when the difference in accurate mass was less than 0.02 and absorption spectrum was identical.

Biological activity assay of extracts from isolated strains

Antimicrobial activities of extracted metabolites were assayed by disk diffusion method (Ruangpan and Tendencia 2004). For this, 40 μL of each extract was loaded on 6-mm-diameter paper disk. Antibacterial activities of metabolites produced by strains grown on MS solid media were assayed by agar diffusion method. For this, 8-mm-diameter agar block of each culture was placed on the test cultures. Test cultures of *Bacillus subtilis* ATCC 6633, *Staphylococcus carnosus* ATCC 51365, *Pseudomonas putida* KT 2440, *E. coli* ATCC 25922 (TolC), *E. coli* K12, and *Saccharomyces cerevisiae* BY4742 were plated from the liquid cultures on LB broth or YPD (yeast) plates, dried for 20 min prior disk/agar blocks were applied. Several extracts that showed activity against *S. cerevisiae* were tested against *Candida albicans* DSM1665. The test cultures were obtained from Leibniz-Institut DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The zones of inhibition were measured manually with accuracy ±1 mm.

Results and discussion

Isolation and phylogenetic characterization of actinobacteria from Lake Baikal endemic invertebrates

Several reports on characterization of microbial communities of water and sediments of Lake Baikal have been published earlier (Terkina et al. 2002, 2006). These publications revealed high enrichments of the lake microbiome with the actinobacteria species. Attempts on characterization of the microbiome of Baikal freshwater sponges have been reported as well (Jenke-Kodama and Dittmann 2009; Kaluzhnaya et al. 2011). We assumed that microbial specimens associated with the endemic fauna of Lake Baikal should preserve the high degree of endemicity as well. Isolated evolution of the lake biota further increases the chances that the secondary

metabolism genes of the bacterial community also evolved separately from the general pool significantly raising the possibility to find novel chemical scaffolds with new biological activities. The macroinvertebrates of Lake Baikal such as amphipods, sponges, turbellaria, and caddisfly are characterized by high endemism (100, 80, 90, and 30 % of representatives, respectively) (Russinek et al. 2012). Due to this, several endemic species from these dominant taxa of littoral and sublittoral zones were used as a source of new actinobacteria strains. Five species were collected in two replicates and used as the sources for actinobacteria isolation (Table 1).

Initial screening provided 25 independent isolates based on morphological features (Fig. S1). As can be seen from Table 1, the majority of actinomycetes were obtained from the samples originating from net-spinning caddisfly *Trichoptera* sp. larvae and amphipods *Brandtia* sp. Surprisingly, only two strains were isolated from the two sponge's samples. The previous studies have shown that freshwater sponges are a great reservoir of actinobacteria representing 12–26 % of their microbial community (Costa et al. 2013; Gladkikh et al. 2014; Kaluzhnaya et al. 2012; Kaluzhnaya and Itskovich 2014). However, published reports are based on cultivation independent approaches, rather than on direct isolation of bacteria, thus encountering all microbiome including uncultured species. In order to fully uncover the potential for actinobacteria isolation from sponges, the media composition would have to be adjusted accordingly as well as analysis of samples collected in different seasons, especially during peaks of phytoplankton vegetation.

Phylogenic analysis based on 16S RNA showed that the majority of them belongs to the genus *Streptomyces* (15 out of 25) (Fig. S2; Table 1). This is typical since this genus is usually dominant in all types of samples. Furthermore, *Streptomyces* species were previously found to be dominant actinobacteria in water and sediments of Lake Baikal (Terkina et al. 2002). The second most "abundant" group of isolates belonged to genus *Nocardia* (6 out of 25)—5 isolates out of 6 from amphipods and 1 from caddisfly larvae. *Nocardia* are typically isolated from soil samples. However, these bacteria could be also found in diverse environments such as air, water, plants, and rotten materials (Faghri et al. 2014). The large portion of *Nocardia* isolates was reported from the termite

Table 1 Actinobacteria strains isolated from macroinvertebrates of Lake Baikal

Macroinvertebrates sample		Strain name	Query score	Sequence ID in Genbank	Strain ID in RCAM
Taxon	Species				
Spongia	*Baikalospongia bacilifera*	*Streptomyces* sp. IB2014/01-2	KP749314	100	
		Pseudonocardia sp. IB2014/02-2	KP749315	99	03471
Turbellaria	*Baikalobia variegata*	*Micromonospora* sp. IB2014/08-1	KP749316	100	03473
Trichoptera	*Trichoptera* sp. larvae	*Streptomyces* sp. IB2014/010-1	KP749317	99	03463
		Streptomyces sp. IB2014/010-2	KP749318	99	
		Streptomyces sp. IB2014/010-3	KP749319	99	
		Streptomyces sp. IB2014/011-1	KP749320	100	03464
		Streptomyces sp. IB2014/011-2	KP749321	100	
		Streptomyces sp. IB2014/011-3	KP749322	99	
		Streptomyces sp. IB2014/011-6	KP749323	99	
		Nocardia sp. IB2014/011-8	KT005998	98	03476
		Streptomyces sp. IB2014/011-10	KP749325	99	
		Agromyces sp. IB2014/011-11	KP749326	98	03465
		Streptomyces sp. IB2014/011-12	KP749327	99	
		Streptomyces sp. IB2014/011-13	KP749328	99	
Amphipoda	*Pallasea cancellus*	*Nocardia* sp. IB2014/014-5	KP749329	99	03466
	Brandtia sp.	*Streptomyces* sp. IB2014/016-1	KP749330	100	
		Streptomyces sp. IB2014/016-2	KP749331	100	
		Streptomyces sp. IB2014/016-5	KP749332	100	
		Streptomyces sp. IB2014/016-6	KP749333	99	03467
		Aeromicrobium sp. IB2014/016-9	KP749334	99	03468
		Nocardia sp. IB2014/017-1	KP749335	99	
		Nocardia sp. IB2014/017-3	KP749336	98	03469
		Nocardia sp. IB2014/017-6	KP749337	97	
		Nocardia sp. IB2014/017-7	KP749338	99	03470

nests where they represent 8 % of all actinobacteria (Sujada et al. 2014). Recently, *Nocardia soli* was isolated from Baikal freshwater sponges by applying a new procedure for bacteria cultivation (Jung et al. 2014). Terkina and co-authors did not observe any *Nocardia* species in both water and sediment samples from Lake Baikal (Terkina et al. 2002). This makes us believe that the observed enrichment of amphipod's samples with the *Nocardia* reflects the specialized rather than random association/interaction between actinobacteria and hosts. Only two isolates were obtained from the sponge *B. bacilifera* (Table 1). Typical actinobacteria strains associated with the marine sponges belong to the *Micromonospora* and *Salinispora* genera (Abdelmohsen et al. 2014; Vicente et al. 2013). However, the only *Micromonospora* strain obtained in this work originated from the *Turbellaria* worms while the sponge samples gave *Streptomyces* and *Pseudonocardia* species. Three representatives of the less abundant and studied genera of actinomycetes, including *Agromyces* and *Aeromicrobium*, were also found to be affiliated with the Baikal invertebrates (Fig. 1, S1; Table 1). Whereas *Agromyces* species are usually isolated from soil samples and marine sediments (Gledhill and Casida 1969), *Aeromicrobium* are more abundant and were typically recovered from soil, marine sediments, water, and even from air (Tang et al. 2008). Both strains *Aeromicrobium* sp. IB2014/016-9 and *Agromyces* sp. IB2014/011-11 *16S* rRNA are forming a tight clade with several representatives of the respective genera (Fig. 1). This finding indicates the diversity of species of actinomycetes that could be isolated with the used approach.

Analysis biological activity of isolated strains

In the agar block diffusion test, six strains were active against both tested Gram-positive *B. subtilis* and *S. carnosus* and seven were inhibiting growth of the TolC antibiotic susceptible mutant of *E. coli* but not *E. coli* K12 or *P. putida* (Table 2, S1). Only one strain *Streptomyces* sp. IB2014/011-6 was able to prevent growth of *E. coli* K12. Most of the strains showed cross activity against both Gram-positive and *E. coli* TolC cultures. Two strains (*Micromonospora* sp. IB2014/08-1 and *Streptomyces* sp. IB2014/016-6) were active strictly against *B. subtilis* and *S. carnosus*, while *Streptomyces* sp. IB2014/010-2 and *Nocardia* sp. IB2014/014-5 only inhibited growth of *E. coli* TolC. Methanol extracts from the MS agar grown strains were also tested for antibacterial activity. However, not in all cases where the agar block test gave positive results the activity was observed in extract (Table 2, S1). This could be explained by the nature of the active substances and their solubility in solvents used in extract preparation. Interestingly, almost all strains in both tests were found to be active against *S. cerevisiae* (19 out of 25) but not against *C. albicans*.

Media composition is often a determining factor for production of different secondary metabolites. Two different liquid media (NL19 and SG) were chosen for metabolite production (Kieser et al. 2000). As mentioned above, the metabolites were extracted separately from the cultural fluid and the biomass. Eleven extracts from the biomass and nine from the supernatant of the strains grown in NL19 media were active against Gram-positive bacteria (Table 2, S2). Also, two extracts from biomass of *Streptomyces* sp. IB2014/016-1 and *Streptomyces* sp. IB2014/016-6 were active against *E. coli* TolC, one of which, *Streptomyces* sp. IB2014/016-6, was able to hinder growth of *P. putida* and *E. coli* K12. At the same time, biomass extract of *Nocardia* sp. IB2014/017-7 was specifically active against *P. putida* but not any other test cultures tested. The biomass extract of *Streptomyces* sp. IB2014/016-5 was active against both yeast *S. cerevisiae* and *C. albicans* (Table 2, S2). The extracts from cultural fluids of eight strains were inhibiting growth of *E. coli* TolC, but only three of them (*Streptomyces* sp. IB2014/010-1, *Streptomyces* sp. IB2014/011-1, and *Streptomyces* sp. IB2014/016-6) were also active against *E. coli* K12, and only one *Streptomyces* sp. IB2014/010-1 was hitting *Pseudomonas* growth. One extract cultural liquid of *Streptomyces* sp. IB2014/016-2 was active against yeasts.

A similar situation was observed in the case of extracts from the strains grown in SG (Table 2, S3). Nine of the biomass extracts and nine of the cultural fluids extracts were active against the Gram-positive test cultures. At the same time, three strains were inhibiting growth of *E. coli* TolC, two of which also were active against *E. coli* K12 (*Streptomyces* sp. IB2014/016-2 and *Streptomyces* sp. IB2014/016-6). The *Streptomyces* sp. IB2014/016-6 during growth in both NL19 (biomass extract) and SG media (cultural liquid extract) was found to produce compounds inhibiting *P. putida*. Three out of four strains capable to inhibit growth of *S. cerevisiae* were also active against *C. albicans* (*Streptomyces* sp. IB2014/01-2, *Streptomyces* sp. IB2014/016-2, and *Streptomyces* sp. IB2014/016-5) (Table 2 S3). *Streptomyces* sp. IB2014/016-6 was active against *C. albicans* but not *S. cerevisiae*.

Dereplication of secondary metabolite profiles of biologically active strains

Modern mass spectrometry methods allow performing not only general analysis of secondary metabolites but also combined with the existing databases provide the possibility for identification of individual compounds. Two different LC-MS protocols were used in this study. When low-resolution technique allows estimating the major components of extracts, the high-resolution experiments open a possibility for dereplication of metabolites. The extracts from all strains grown in all tested conditions were subjected to low-

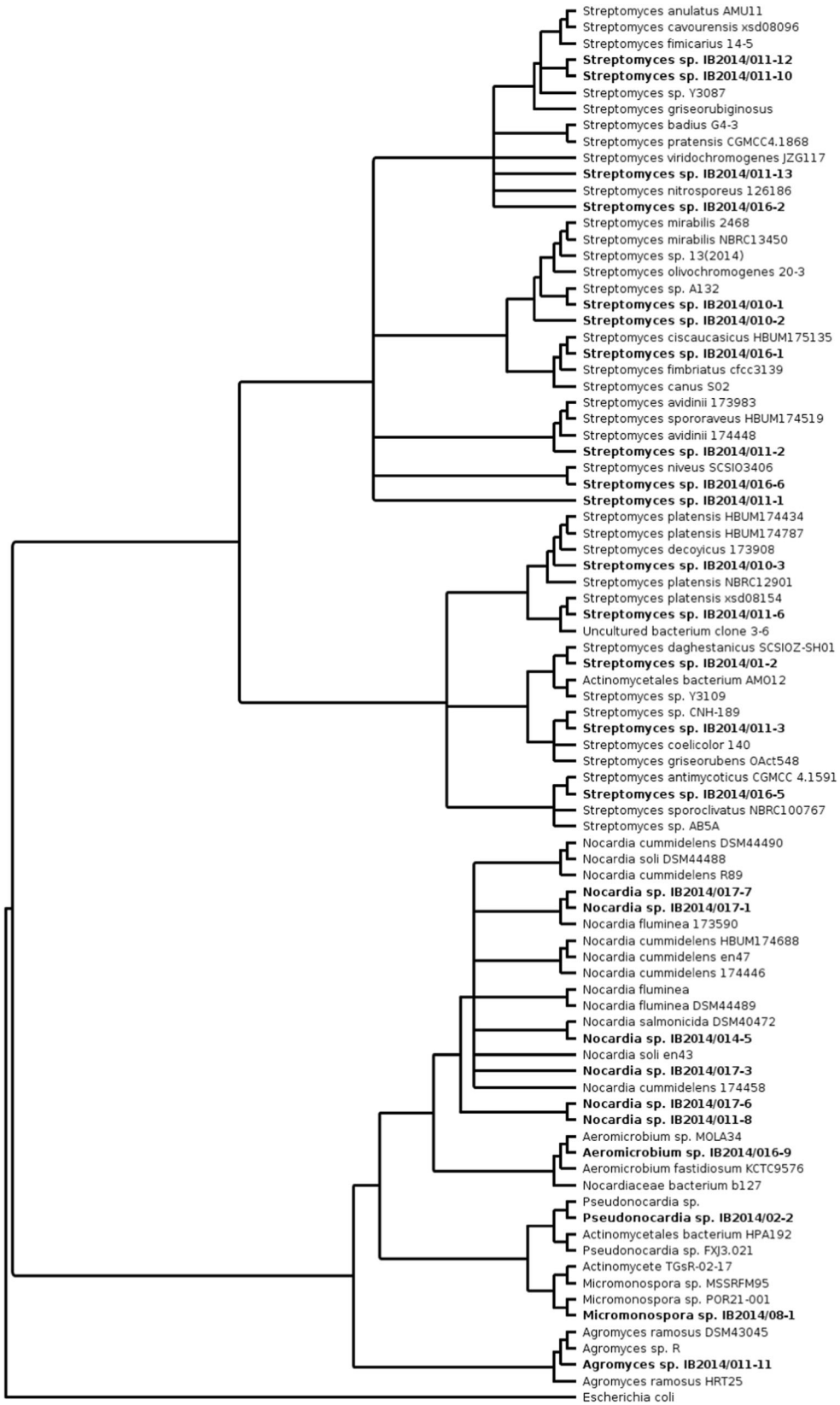

Fig. 1 Phylogenetic tree of actinobacteria isolates based on the 16S rRNA gene sequences

resolution LC-MS analysis in order to estimate the number of major compounds that are produced. In all, samples from 3 to 93 peaks on MS and UV chromatograms were observed (Table S4). It is not clear if several peaks in one extract

Table 2 Antibacterial and antifungal activity of isolated strain when grown on different media

Strain	MS agar		NL19		SG biomass	
	A. column	Extract	Biomass	Cul. liq.	Biomass	Cul. liq.
Streptomyces sp. IB2014/01-2	Sa	Sa	Sa; Ca	Ec*	Sa; Ca	
Pseudonocardia sp. IB2014/02-2	Sa	Sa				Ec*; Sc
Micromonospora sp. IB2014/08-1	Bs; Sc; Sa	Sa		Bs; Ec*		
Streptomyces sp. IB2014/010-1	Sa	Sa	Bs	Bs; Sc; Ec; Pp	Bs	
Streptomyces sp. IB2014/010-2	Ec*; Sa	Sa	Bs		Bs	
Streptomyces sp. IB2014/010-3	Ec*; Sc; Bs	Sc; Pp;	Sc	Sc;		
Streptomyces sp. IB2014/011-1	Bs; Sc; Ec*; Sa	Bs; Sc; Sa	Bs; Sc	Bs; Sc; Ec	Bs	Bs; Sc
Streptomyces sp. IB2014/011-2						
Streptomyces sp. IB2014/011-3	Sa	Sa		Ec*		
Streptomyces sp. IB2014/011-6	Bs; Sc; Ec	Bs; Sc; Sa	Bs; Sc; Sa		Bs; Sc; Sa	Bs; Sc; Sa
Nocardia sp. IB2014/011-8	Sa	Sa				
Streptomyces sp. IB2014/011-10	Sa	Sa				
Agromyces sp. IB2014/011-11	Sa	Sa				
Streptomyces sp. IB2014/011-12	Bs; Sc; Ec*; Sa	Bs; Sc; Sa	Sc	Bs; Sc	Bs	Bs; Sc
Streptomyces sp. IB2014/011-13		Sa				Bs; Sc
Nocardia sp. IB2014/014-5	Ec*;	Sa				
Streptomyces sp. IB2014/016-1	Sa	Bs; Sa	Bs; Sc; Ec*	Bs	Bs; Sc	Bs; Sc
Streptomyces sp. IB2014/016-2	Sa	Sc; Sa	Bs; Sc; Sa	Bs; Sc; Sa	Bs; Sc; Sa; Ca	Bs; Sc; Ec; Sa; Ca
Streptomyces sp. IB2014/016-5	Bs; Sc; Ec*; Sa	Sc; Sa	Bs; Sc; Sa; Ca	Bs; Sc	Bs; Sc; Sa	Bs; Sc; Sa; Ca
Streptomyces sp. IB2014/016-6	Sc; Sa	Sa	Bs; Sc; Ec; Pp	Bs; Sc; Ec;	Bs; Sc; Ec;	Bs; Sc; Ec; Pp; Ca
Aeromicrobium sp. IB2014/016-9	Sa	Sa		Ec*		
Nocardia sp. IB2014/017-1	Sa	Sa	Bs			
Nocardia sp. IB2014/017-3		Sa		Ec*		
Nocardia sp. IB2014/017-6	Sa	Sa				
Nocardia sp. IB2014/017-7	Sa	Sa	Pp			

Bs Bacillus subtilis, *Sc Staphylococcus carnosus*, *Ec Escherichia coli* K12 and TolC, *Ec* Escherichia coli* TolC, *Sa Saccharomyces cerevisiae*, *Ca Candida albicans*, *Pp Pseudomonas putida*

correspond to different individual metabolites or one family of compounds.

The majority of pathogenic Gram-negative bacteria are not controlled by drugs. Thus, strains producing metabolites capable to inhibit growth of *E. coli* and *P. putida* in our tests are of particular interest. We conducted complete dereplication of metabolic profiles of *Streptomyces* sp. IB2014/010-1 and *Streptomyces* sp. IB2014/016-6 (Table S5, S6). In addition to the characterization on low-resolution mass spectrometry platform, active extracts from these strains were also subjected to high-resolution mass spectrometry analysis. Five compounds out of 103 for strain *Streptomyces* sp. IB2014/010-1 and 10 out of 90 for strain *Streptomyces* sp. IB2014/016-6 were preliminary predicted based on characteristics from the Dictionary of Natural Products database using search parameters described in the method section. Only high-molecular detected compounds are given below.

We found that *Streptomyces* sp. IB2014/010-1 is producing variapeptin, a hexadepsipeptide antibiotic with antibacterial activity of azinothricin family (Table 3; S5; Fig. S2) (Nakagawa et al. 1990a, 1990b). It is produced in small quantities when compared to other major peaks on chromatograms (Fig. S2). Variapeptin was isolated in 1990 from extract of *Streptomyces variabilis* K2919 and shown to inhibit growth of numerous Gram-positive bacteria, but not Gram-negative or fungus. Later, with the expansion of this family of compounds, they were re-discovered as an anticancer drug of general action (Hale and Cai 1996). Other metabolites produced by *Streptomyces* sp. IB2014/010-1, including several major peaks, do not show any hits in DNP thus making us believe that the majority of them might be new (Table S5).

Streptomyces sp. IB2014/016-6 was found to produce three compounds of minalemine family: minalemine A (CRC number: DYN62-X), minalemine B (CRC number: DYN64-Z), and minalemine C (CRC number: DYN66-B) (Table 3; S6; Fig. S3) (Exposito et al. 1998). The detected molecular mass and absorption spectra of the identified metabolites correspond to the described features of minalemines (Exposito

Table 3 Compounds identified with DNP from extracts of isolated strain active against Gram-negative bacteria and their features

Strain	*Streptomyces* sp. IB2014/010-1	*Streptomyces* sp. IB2014/016-6		
Compound name	Variapeptin	Minalemine A	Minalemine B	Minalemine C
Dictionary of natural products				
CRC number	JVG29-C	DYN62-X	DYN64-Z	DYN66-B
Calculated mass	944.5218	596.4849	610.5006	624.5162
Molecular formula	$C_{46}H_{72}N_8O_{13}$	$C_{29}H_{60}N_{10}O_3$	$C_{30}H_{62}N_{10}O_3$	$C_{31}H_{64}N_{10}O_3$
UV maxima	222 (ε10000) (MeOH)	222 (MeOH)	222 (MeOH)	222 (MeOH)
Biological source	*Streptomyces variabilis* K2919	ascidian *Didemnun rodriguesi*		
Experimental data				
Detected mass, m/z	945.5099	597.5132	611.5235	625.5372
Accurate mass	944.5019	596.5052	610.5155	624.5298
UV maxima, nm	222 (MeOH)	226 (MeOH)	226 (MeOH)	226 (MeOH)
Δ Accurate mass	0.02	0.02	0.02	0.02

et al. 1998, 2001; Whittle et al. 2003). Minalemines are linear peptide secondary metabolites, which were previously identified in extracts from marine tunicates (Fig. S3) (Exposito et al. 1998). No biological activity was described for these metabolites. The core part of minalemines consist of L-leucine and an unusual amino diacid, 3-(*N*-carboxymethyl)-aminodecanoic acid (Ncma), that is rarely found in secondary metabolites (Duncan et al. 2002; Jin et al. 2010). The core of minalemines is connected with an aminogroup of two different ω-aminoguanidines: agmatine and homoagmatine. The individual minalemines differ in the chain length of the Ncma (10, 11, or 12 carbons A, B, and C, respectively) and in the presence or absence of a sulfamic acid group (D, E, F). Although minalemines were originally isolated from the marine tunicates, we observed presence of three out of six of these compounds (minalemines A-C) in the extract of *Streptomyces* sp. IB2014/016-6. This makes us believe that minalemines are not direct products of ascidian *Didemnun rodriguesi* but rather are synthesized by some actinomycetes associated with this organism. We failed to find minalemines D-F in extracts of *Streptomyces* sp. IB2014/016-6. Most probably, modification leading to conversion of minalemines A-C to D-F (addition of sulfamic acid) is indeed conducted by the *D. rodriguesi* representing an interesting example of interplay between secondary metabolism of actinomycetes and metabolic pathways/activity of its host.

In conclusion, we have isolated 25 actinobacteria strains from the endemic invertebrate species of Lake Baikal proving this ecosystem to be a promising source of new actinomycetes. Among them, representatives of genus *Streptomyces* and *Nocardia* were dominant taxa, depending on a source of isolation. As mentioned above, the high number of *Nocardia* sp. strains were found in amphipoda *Brandtia* sp. Several rare and less studied actinobacteria strains were isolated as well, including new representatives of *Aeromicrobium* and *Agromyces* genera. A large fraction of the isolated strains was able to inhibit growth of Gram-positive bacteria and budding yeast. At the same time, several strains were found to be capable to prevent the growth of Gram-negative test cultures and pathogenic *C. albicans*. These strains for sure represent a particular interest for deeper investigation with complete phylogenetic classification and identification and characterization of active compounds. In general, even after the initial estimation, we can state that the majority of secondary metabolites accumulated by the isolated strains are not described in available antibiotic databases. This fact proves that the chosen strategy for isolation of new actinobacteria from the unique ecological niches could be successful in order to obtain new biologically active compounds.

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Conflict of interest The authors declare that they have no competing interests.

Statement of human rights All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Statement on the welfare of animals This article does not contain any studies with human participants or vertebrate animals performed by any of the authors.

Mentioned in the article were Baikalian macroinvertebrate species not involved in endangered or protected species. No specific permissions were required for the sampling of invertebrate species.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

Abdelmohsen UR, Bayer K, Hentschel U (2014) Diversity, abundance and natural products of marine sponge-associated actinomycetes. Nat Prod Rep 31:381–399. doi:10.1039/C3np70111e

Alvin A, Miller KI, Neilan BA (2014) Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. Microbiol Res 169:483–495. doi:10.1016/j.micres.2013.12.009

Bagwell CE, Bhat S, Hawkins GM, Smith BW, Biswas T, Hoover TR, Shimkets LJ (2008) Survival in nuclear waste, extreme resistance, and potential applications gleaned from the genome sequence of *Kineococcus radiotolerans* SRS30216. PLoS One 3:e3878. doi:10.1371/journal.pone.0003878

Costa R, Keller-Costa T, Gomes NCM, da Rocha UN, van Overbeek L, van Elsas JD (2013) Evidence for selective bacterial community structuring in the freshwater sponge *Ephydatia fluviatilis*. Microb Ecol 65:232–244. doi:10.1007/s00248-012-0102-2

Currie CR, Scott JA, Summerbell RC, Malloch D (2003) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 423:461–461. doi:10.1038/Nature01563

Demain AL, Adrio JL (2008) Contributions of microorganisms to industrial biology. Mol Biotechnol 38:41–55. doi:10.1007/s12033-007-0035-z

Duncan SJ, Grüschow S, Williams DH, McNicholas C, Purewal R, Hajek M, Moore M (2002) Isolation and structure elucidation of chlorofusin, a novel p53-MDM2 antagonist from a *Fusarium* sp. J Am Chem Soc 124:14503–14503. doi:10.1021/Ja025114k

Exposito MA, López B, Fernández R, Vázquez M, Debitus C, Iglesias T, Riguera R (1998) Minalemines A-F: sulfamic acid peptide guanidine derivatives isolated from the marine tunicate *Didemnun rodriguesi*. Tetrahedron Lett 54:7539–7550. doi:10.1016/S0040-4020(98)00388-3

Exposito A, Fernandez-Suarez M, Iglesias T, Munoz L, Riguera R (2001) Total synthesis and absolute configuration of minalemine A, a guanidine peptide from the marine tunicate *Didemnum rodriguesi*. J Org Chem 66:4206–4213. doi:10.1021/Jo010076t

Faghri J, Bourbour S, Moghim S, Meidani M, Safaei HG, Hosseini N, Sedighi M (2014) Comparison of three phenotypic and deoxyribonucleic acid extraction methods for isolation and Identification of *Nocardia* spp. Adv Biomed Res 3:151. doi:10.4103/2277-9175.137839

Gladkikh AS, Kalyuzhnaya OV, Belykh OI, Ahn TS, Parfenova VV (2014) Analysis of bacterial communities of two Lake Baikal endemic sponge species. Microbiology 83:787–797. doi:10.1134/S002626171406006x

Gledhill WE, Casida LE (1969) Predominant catalase-negative soil bacteria. III. *Agromyces*, gen. n., microorganisms intermediary to *Actinomyces* and *Nocardia*. Appl Microbiol 18:340–349

Hale KJ, Cai JQ (1996) Synthetic studies on the azinothricin family of antitumour antibiotics. 5. Asymmetric synthesis of two activated esters for the northern sector of A83586C. Tetrahedron Lett 37:4233–4236. doi:10.1016/0040-4039(96)00804-0

Hentschel U, Piel J, Degnan SM, Taylor MW (2012) Genomic insights into the marine sponge microbiome. Nat Rev Microbiol 10:641–675. doi:10.1038/Nrmicro2839

Jenke-Kodama H, Dittmann E (2009) Evolution of metabolic diversity: insights from microbial polyketide synthases. Phytochemistry 70:1858–1866. doi:10.1016/j.phytochem.2009.05.021

Jin JM, Lee S, Lee J, Baek SR, Kim JC, Yun SH, Lee YW (2010) Functional characterization and manipulation of the apicidin biosynthetic pathway in *Fusarium semitectum*. Mol Microbiol 76:456–466. doi:10.1111/j.1365-2958.2010.07109.x

Jung D, Seo EY, Epstein SS, Joung Y, Han J, Parfenova VV, Ahn TS (2014) Application of a new cultivation technology, I-tip, for studying microbial diversity in freshwater sponges of Lake Baikal, Russia. FEMS Microbiol Ecol 90:417–423. doi:10.1111/1574-6941.12399

Kaluzhnaya OV, Itskovich VB (2014) Phylogenetic diversity of microorganisms associated with the deep-water sponge *Baikalospongia intermedia*. Rus J Genet 50:667–676. doi:10.1134/S1022795414060052

Kaluzhnaya OV, Itskovich VB, McCormack GP (2011) Phylogenetic diversity of bacteria associated with the endemic freshwater sponge *Lubomirskia baicalensis*. World J Microbiol Biotechnol 27:1955–1959. doi:10.1007/s11274-011-0654-1

Kaluzhnaya OV, Krivich AA, Itskovich VB (2012) Diversity of 16S rRNA genes in metagenomic community of the freshwater sponge *Lubomirskia baicalensis*. Rus J Genet 48:855–858. doi:10.1134/S1022795412070058

Katoh S (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 30:772–780

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Drummond A (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649. doi:10.1093/bioinformatics/bts199

Kieser BM, Buttner MJ, Charter KF, Hopwood D (2000) Practical streptomyces genetics. John Innes Foundation, Norwich

Kozhova O, Izmest'eva L (1998) Lake Baikal. Evolution and biodiversity. Backhuys Publ, Leiden

Kroiss J, Kaltenpoth M, Schneider B, Schwinger MG, Hertweck C, Maddula RK, Svatoš A (2010) Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. Nat Chem Biol 6:261–263. doi:10.1038/Nchembio.331

Madden AA, Grassetti A, Soriano JAN, Starks PT (2013) Actinomycetes with antimicrobial activity isolated from paper wasp (*Hymenoptera*: *Vespidae*: *Polistinae*) nests. Environ Entomol 42:703–710. doi:10.1603/En12159

Mao J, Tang Q, Zhang Z, Wang W, Wei D, Huang Y, Goodfellow M (2007) *Streptomyces radiopugnans* sp. *nov.* a radiation-resistant actinomycete isolated from radiation-polluted soil in China. Int J Syst Evol Microbiol 57:2578–2582. doi:10.1099/ijs.0.65027-0

Monciardini P, Iorio M, Maffioli S, Sosio M, Donadio S (2014) Discovering new bioactive molecules from microbial sources. Microb Biotechnol 7:209–220. doi:10.1111/1751-7915.12123

Mushegian AA, Peterson CN, Baker CCM, Pringle A (2011) Bacterial diversity across individual lichens. Appl Environ Microbiol 77:4249–4252. doi:10.1128/Aem.02850-10

Nakagawa M, Hayakawa Y, Adachi K, Seto H (1990a) A new depsipeptide antibiotic, variapeptin. Agric Biol Chem 54:791–794

Nakagawa M, Hayakawa Y, Furihata K, Seto H (1990b) Structural studies on new depsipeptide antibiotics, variapeptin and citropeptin. J Antibiot 43:477–484

Nikapitiya C (2012) Bioactive secondary metabolites from marine microbes for drug discovery. Adv Food Nutr Res 65:363–387. doi:10.1016/B978-0-12-416003-3.00024-X

Ruangpan L, Tendencia EA (2004) Disk diffusion method. Laboratory manual of standardized methods for antimicrobial sensitivity tests for bacteria isolated from aquatic animals and environment. SEAFDEC, Aquaculture Department, Tigbauan, pp 13–29

Russinek OT, Takhteev VV, Gladkochub DP, Khodzher TV, Budnev NM (2012) Baikalogy. Nauka, Nowosibirsk

Strohl WR (1997) Industrial antibiotics: today and the future. In: Strohl WR (ed) Biotechnology of antibiotics, 2nd edn. Marcel Dekker, New York, pp 1–47

Sujada N, Sungthong R, Lumyong S (2014) Termite nests as an abundant source of cultivable actinobacteria for biotechnological purposes. Microbes Environ 29:211–219. doi:10.1264/jsme2.ME13183

Tang Y, Zhou G, Zhang L, Mao J, Luo X, Wang M, Fang C (2008) *Aeromicrobium flavum* sp. *nov.* isolated from air. Int J Syst Evol Microbiol 58:1860–1863. doi:10.1099/ijs.0.65443-0

Terkina IA, Drukker VV, Parfenova VV, Kostornova TY (2002) The biodiversity of actinomycetes in Lake Baikal. Microbiology 71: 346–349. doi:10.1023/A:1015871115187

Terkina IA, Parfenova VV, Ahn TS (2006) Antagonistic activity of actinomycetes of Lake Baikal. Appl Biochem Microbiol 42:173–176. doi:10.1134/S0003683806020104

Timoshkin OA, Sitnikova TY, Rusinek OT, Pronin NM, Proviz VI, Melnik NG, Kamaltynov RM, Mazepova DF, Shoshnin AV (2001) Index of animal species inhabiting Lake Baikal and its catchment area. Nauka, Novosibirsk

Valverde A, Tuffin M, Cowan DA (2012) Biogeography of bacterial communities in hot springs: a focus on the actinobacteria. Extremophiles 16:669–679. doi:10.1007/s00792-012-0465-9

Vicente J, Stewart A, Song B, Hill RT, Wright JL (2013) Biodiversity of actinomycetes associated with Caribbean sponges and their potential for natural product discovery. Mar Biotechnol 15:413–424. doi:10.1007/s10126-013-9493-4

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267. doi:10.1128/Aem.00062-07

Whittle M, Willett P, Klaffke W, van Noort P (2003) Evaluation of similarity measures for searching the dictionary of natural products database. J Chem Inf Comput Sci 43:449–457. doi:10.1021/Ci025591m

Zakharova YR, Galachyants YP, Kurilkina MI, Likhoshvay AV, Petrova DP, Shishlyannikov SM, Likhoshway YV (2013) The structure of microbial community and degradation of diatoms in the deep near-bottom layer of Lake Baikal. Plos One 8:1–12 8. doi:10.1371/journal.pone.0059977 ARTN e59977