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Formation of Spicules During the Long-term Cultivation of Primmorphs from the Freshwater Baikal Sponge *Lubomirskia baikalensis*

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Abstract

Sponges (phylum Porifera) are phylogenetically ancient Metazoa that use silicon to form their skeletons. The process of biomineralization in sponges is one of the important problems being examined in the field of research focused on sponge biology. Primmorph cell culture is a convenient model for studying spiculogenesis. The aim of the present work was to produce a long-term primmorph culture from the freshwater Baikal sponge *Lubomirskia baikalensis* (class Demospongiae, order Haplosclerida and family Lubomirskiidae) in both natural Baikal water and artificial Baikal water to study the influence of silicate concentration on formation and growth of spicules in primmorphs. Silicate concentration plays an important role in formation and growth of spicules, as well as overabundance of silica leads to destruction of cell culture primmorphs. We also found that the composition of chemical elements (Si, O, C, and Na) varied along the length of growing spicules at cultivation in different media. The long-term culture of Baikal sponge primmorphs will be necessary for further investigations, and this system may serve as a powerful *in vitro* model to study spiculogenesis in Baikal siliceous sponges during the early stages of intracellular spicule formation to identify genes that affect biomineralization.

Keywords: Primmorphs, Spiculogenesis, Silica spicules, Microanalysis, Baikal sponges

Introduction

Sponges (phylum Porifera) are the phylogenetically oldest Metazoa. Demosponges recorded before the end the Marinoan glaciation (635Myr ago) exist at present [1]. Processes of biomineralization and biosilification in sponges are the subject of active research and debate. Three sponge clades (class Demospongiae, order Hexactinellida) produce silica skeletons [2]. Their skeletons consist of spicules that form species-specific shapes. Their mineral components consist of silicon dioxide, silicon in amorphous opal-A and the bonded protein spongin (an analogue of collagen) [3,4]. The secretion of silica spicules in sponges is an intracellular process [3,5]. Many silica spicule-forming sponges have been characterized as containing and expressing silicatein genes [6-9]. The exact mechanism(s) of the action of silicatein remain undetermined, but the investigation of this compound is considered to be particularly important for the use of silica-based materials *in vitro* [10]. Formation of spicules is a genetically controlled process and highly relevant to nanobiotechnology [6,11-13].

It is very difficult to investigate the mechanism of spicule formation (spiculogenesis) at the cellular and molecular levels in adult sponges because spicules contain a wide variety of proteins that are involved in metabolism of silica and formation of spicules (biogenic silica). To avoid this complexity, the use of sponge cell cultures (primmorphs) has been suggested [14-16]. Primmorphs are 3D cell aggregates from sponge cells being a suitable model for studying spiculogenesis [8,17-21]. They are formed by dissociation of sponge cells and subsequent growth of aggregates. Previously, a primmorph system from *Suberites domuncula* was used to demonstrate that silicatein (a biosilica-synthesising enzyme) and silicase (a catabolic enzyme) are colocalized at the surfaces of growing spicules and in axial filaments [22].

The endemic freshwater Baikal sponge *Lubomirskia baikalensis* (Pallas 1776) was the subject of our investigation. These sponges are a good model for applied studies because *L. baikalensis* is the only Baikal sponge species that can easily be identified. Lake Baikal is situated in South-Eastern Siberia (51–56° N, 104–110° E), the world's largest (23,000 square kilometres), deepest (1,643 m), and oldest (> 24 million years [MiY]) freshwater body [23]. Sponges dominate the littoral zone of Lake Baikal, covering 47% of the available surfaces [24]. A percentage cover of 47% for sponges is unusual for a freshwater ecosystem and is difficult to compare with reported sponge biomass and occurrence for freshwater ecosystems [25,26]. Habitats of Baikal sponges differ considerably from those of other freshwater sponges because of hydrological and hydrochemical peculiarities of Lake Baikal: great depths, long ice period, low water temperature (10–12°C) in the upper layers in summer, high oxygen content, and low concentration of organic matter [27,28]. Moreover, Lake Baikal contains relatively low levels of nutrients, but the nutrients are stably balanced relative to the chemical composition ratios in phytoplankton [29]. Another peculiar characteristic of Lake Baikal is relatively high level of dissolved silicic acid. It is known, that the silicon concentration in Lake Baikal is 100 μM [27], whereas in marine coastal areas the mean silicon concentration at the surface is less than 3 μM [30] gradually increasing with depth [31]. The high content of silicic acid in Lake Baikal is caused by heavy influx of silicon from the rivers [32].

The main aim of this work was to study how the concentration of silicate influenced the formation and growth of spicules in primmorphs

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from the freshwater sponge *L. baikalensis* (as model system) in both natural Baikal water (NBW) and artificial Baikal water (ABW). The *L. baikalensis* primmorphs were cultivated in a previous study in both NBW and ABW for >10 months [33]. The prior investigations have indicated that neither 1.0% or 1.5% foetal bovine serum (FBS) (HyClone Laboratories, Inc.) nor classical Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with antibiotics is necessary for the cultivation or growth of Baikal sponge primmorphs, as symbiotic green algae in cells of *L. baikalensis* and specific symbiotic microbial flora provide the freshwater sponge with necessary nutrition [34,35]. Moreover, a distinctive feature of Baikal sponges compared to other freshwater sponges is their ability to live symbiotically with various zoochlorellae and dinoflagellates [36,37]. Primmorphs are likely to grow due to photosynthetic products of their symbionts.

Materials and Methods

Sponge sampling

L. baikalensis (class Demospongiae, order Haplosclerida and family Lubomirskiidae) (Figure 1) has a branched shape and an encrusting base with erect (30–60 cm, up to 1 m high) dichotomous branches and rounded apices. Live specimens are a brilliant shade of green. The ectosomal skeleton consists of spicule tufts from the primary fibres. The skeleton consists of uniformly-spined megasclere oxeas (145–233 x 9–18 μm); microscleres are absent [38]. The *L. baikalensis* specimens were collected in Lake Baikal near Cape Listvenichny at a depth of 15 m (water temperature 3–4°C) using SCUBA in December, 2008 (minimal growth of diatoms). Apical parts of the specimens that were more than 30–40 cm high were collected. The samples were immediately placed in containers with Baikal water and ice and transported for 1.2 h at a constant water temperature (3–4°C) to Limnological Institute SB RAS (Irkutsk).

Cultivation of primmorph as a model system

Primmorphs were obtained using a classical method of mechanical dissociation of cells [39]. A clean sponge was crushed, and the cell suspension obtained was subsequently filtered through a sterile 200-, 100-, and 29-μm-mesh nylon to eliminate pieces of skeleton and spicules of the maternal sponge. The cellular suspension was diluted to a concentration of 5×10^6 cells/ml and used as the primary material for primmorph formation. The *L. baikalensis* primmorphs were cultivated as described previously [33] in both NBW and ABW at 3–6°C for >10 months, under conditions that mimicked the natural conditions as closely as possible.

NBW was taken from the depth of 500 m, passed through sterilizing filters, and treated with ultraviolet rays. ABW was prepared according to hydrochemical data on Lake Baikal water composition [27,28] excluding salts of silicon acid, the pH was adjusted to 7.9 like that in natural Baikal water [33]. All chemicals used were of reagent grade. The solution obtained was sterilized by filtration through 0.22-μm polycarbonate filters (Nalgene, Rochester, USA). Only plastic materials were used to prevent release of silicate from glassware.

Primmorphs (2112 pieces) were cultivated in 24-well plates (Nalge Nunc International) in NBW at 3–6°C and light intensity of 47 lx or 0.069 Wt with 12 h mode of day and night alteration. Real light intensity in Lake Baikal in the zone of sponge habitats is not exactly known. After 2 days, a portion (1440 pieces) of the primmorphs (1–2 mm in diameter) was transferred to separate wells of a 24-well plate with 2 ml ABW without silicate and cultivated for 6-8 weeks for adaptation to artificial conditions. The medium was replaced every day during the first week, then once a week, replacing only 50% of the medium. We tested the influence of silicate ($Na_2SiO_4\times7H_2O$) on primmorph development and spicule growth by varying the silicate concentration between 70 and 120 μM, pH was adjusted to 7.9. The primmorphs were cultured in ABW without silicate. As a control, we cultivated primmorphs *in vitro* in NBW. Silicate concentrations in the wells permanently were controlled by spectrophotometric analysis during the experiments. The protocol was adapted from Strickland & Parsons [40]. Subsequently the extinction of standards and samples was measured with a spectrophotometer (Spectronic-20 Genesys, Spectronic Instruments, Rochester, USA) at a wavelength of 810 nm. A calibration curve was made from 0 to 100 μM.

Spicule preparation and imaging

In our study, spicules totalling more than 1050 were measured in cell culture primmorphs *L. baikalensis.* Spicules were prepared on a single glass slide for each sample cultured either in ABW in the absence of silicate, ABW in the presence of 70 μM silicate and in NBW. A scalpel was used to cut 1-mm-thick sections that ran perpendicular to the surfaces of the specimens, which had been stored in 96% ethanol. Several drops of concentrated HNO_3 were applied to immerse primmorphs or sponge fragments, and the slides were heated slightly over a flame shaking to spread the liquid evenly [41]. Spicule samples were washed in distilled water (dH_2O) and air-dried. After mounting cleaned spicules on microscopic slides, we imaged them using an Axiovert 200 inverted light microscope (Zeiss, Germany). Spicule dimensions were measured by means of calibrated micrometer eyepieces with an amplification of 200× on an inverted light microscope (XDS-1B, COIC). Dynamics of formation and growth of spicules in primmorphs in different media was observed daily during a long period. We estimated a total of 10 view fields, using the methods described by Jones [42], spicule length was measured as the shortest distance between two ends, and spicule width was measured as cross-sectional thickness of the middle region of the spicule.

Figure 1: Underwater photo of *L. baikalensis in situ* from Lake Baikal at a depth of 15 m (Photo courtesy of I. Khanaev).

SEM observation

Processes of spiculogenesis in primmorphs in different media (NBW, ABW, and ABW in the presence of 70 μM silicate) were examined by means of SEM. Subsequently, aliquots of primmorph (0.3 g) were analyzed for spicule formation and silicate content. The spicules were prepared from primmorph tissue samples that were first isolated with HNO_3, H_2SO_4, as described previously [43] with slight modifications (1:4 v/v, followed by n-butanol/water/SDS). Cleaned spicules (10 mg) were washed with distilled water and then with 96% ethanol at room temperature. Spicule separation was achieved by sedimentation for 5 minutes, and final separation by filtration through a sieve (29,10-μm mesh size). SEM images of the spicules was performed with an SEM 525M (Philips, Holland) and EVO 40 microanalyser microscope (Zeiss, Germany). The samples were mounted onto aluminium stubs, then sputtered with a 20-nm coat of a gold-palladium mixture and examined. We started to study 3-week juvenile spicules during three months. Some of the spicules were used for dimension measurements and some for observation by scanning electron microscopy (SEM).

SEM X-ray microanalysis

Biosilica content and elemental composition of the cultivated spicules in ABW vs. NBW media were determined using a EVO 40 microanalyser microscope (Zeiss, Germany) for SEM X-ray microanalysis (A.V. Zhirmunsky Institute of Marine Biology FEB RAS, Vladivostok). Organic materials were cleared from the spicules, which were then mounted on platinum-coated aluminium stubs and examined using SEM with X-ray microanalysis (XMRA) to qualitatively assay the presence of Si, O, C and Na (i.e., the sum of these components was taken as 100%). SEM X-ray microanalysis uses the characteristic X-rays generated from an electron-bombarded sample to identify elemental constituents that comprise the sample [44]. This technique generates a spectrum in which the peaks correspond to specific X-ray lines, enabling the easy identification of elements present in the sample.

A 20-kV acceleration was used for the SEM-mode microanalysis. The gain rate was adjusted to 10000–20000 counts s^{-1}, and, for all of the received pulses at 20000 counts s^{-1}, the acquisition time was 100 s. The electron beam excitation was detected in a narrow window (10 mm^2). The crystal area in the detector was 10 mm^2. We used the 1.740-kV Si/K peak as a qualitative indicator of the presence of Si because it did not overlap with other peaks. Analyses were performed at 1105× magnification, producing spectra for small areas (3.0 and 1.0 μm) along the entire length of the growing spicules, including spicule tips and spines. We analyzed about 10–15 basic points in each spicule. The corresponding Si signals were examined in the spectra. Any significant differences in the Si content across the various compartments studied were assessed using the (INCA program). Moreover, peaks of the output pulses were used to map Si, O, C, and Na contents.

Statistical analysis

Data on dimensional measurements of spicules were analyzed using Microsoft Excel. For spicules that were grown in various media we calculated length and diameter of spicules for three months using Microsoft Excel. Statistical analyses (descriptive statistics; Kolmogorov-Smirnov test on normal distribution; independent t-tests) were performed using WinSTAT for Excel.

Results

Spicule formation in *L. baikalensis* primmorph cell system

The *L. baikalensis* primmorph cultures in NBW, ABW and ABW in the presence of 70 μM silicate were monitored for three months. Specimens were a brilliant shade of green. Diameter of primmorphs increased to 3–4 mm or more and continued to form spherical aggregates (Figure 2A,2B,2C). After three months in ABW with the presence of 70 μM silicate, the diameter of primmorphs increased to 4 mm or more, and we observed a smooth tissue-like surface with spicules that jutted out. In addition, we cultured primmorphs at a concentration of 120 μM silicate in ABW, the primmorphs become friable during the first 3 days and later died in culture.

The process of spicules formation appeared similar between NBW, ABW and ABW with the introduction of 70 μM silicate. Approximately on day 2, the spicules were squeezed out from the cell and into the extracellular space. Newly born spicules of usually curved shape were observed at this stage (Figure 2D,2E,2F). The spicules continued to increase in size after 12–14 days, reaching lengths of approximately 120 μm (Figure 2G,2H,2I). At the beginning of their formation, spicules were fleixble and curved and then straightened. We

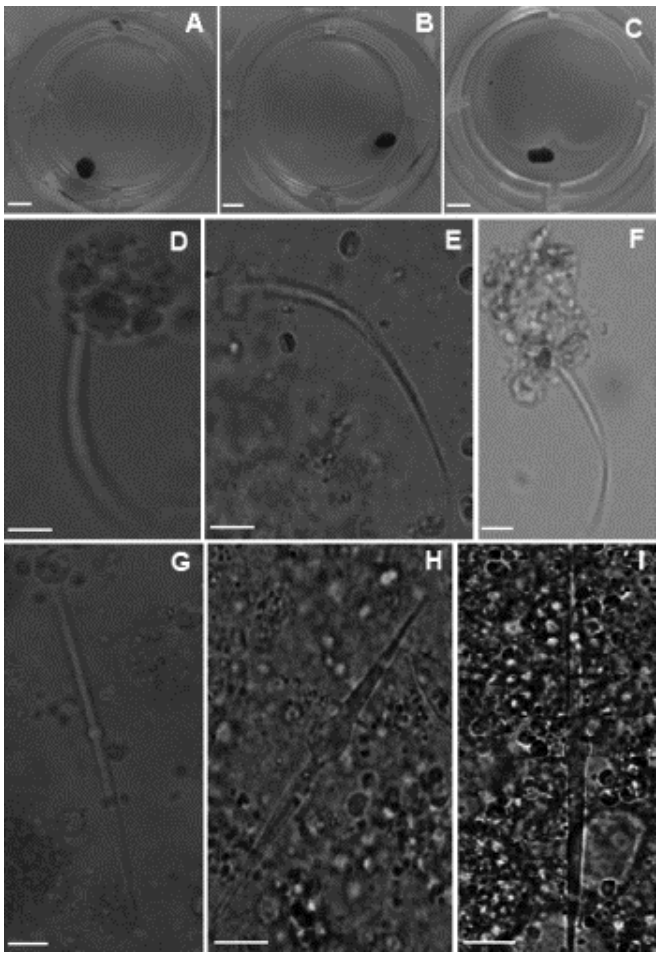

Figure 2: Light microscopy of live preparations. Dynamic formation of spicules *in vitro* in cell culture of the *L. baikalensis* primmorphs: (**A**) primmorphs cultivated for three months in NBW; (**B**) primmorphs cultivated for three months in ABW in the absence of silicate; (**C**) primmorphs cultivated for three months in ABW in the presence of 70 μM silicate; (**D**) newly formed spicule on 2 day in NBW; (**E**) formation of spicule on 2 day in ABW; (**F**) formation of spicule on 2 day in ABW in the presence of 70 μM silicate; (**G**) formation of spicule on 12 day in NBW; (**H**) formation of spicule on 12 day in ABW; (**I**) formation of spicule on 12 day in ABW in the presence of 70 μM silicate. Scale bars: (**A – C**) 0,5 mm; (**D – I**) 10 μm.

observed different stages of spicule development during three months of cultivation. Spicules, from newly born to mature, were mixed and distributed inside primmorphs for 2–3 months. During the initial stage of growth, spicules were smooth and flexible, without spines, and had an expansion bubble in the middle. Then spicules straightened, grew resembling the spicules of adult sponges. Spicules could be observed *in vivo* after 2–3 days; full-size spicules were present after 12–14 days of cultivation. At this point, growing spicules in primmorphs acquired sizes and shapes of adult spicules.

Using SEM, we found no significant differences between the spicules grown in different media. We also found no significant differences between the spicules cultivated in ABW with the introduction of 70 μM silicate (Figure 3A) and those cultivated in NBW or ABW without silicate (Figure 3B,3C). During the initial period of growth, spicules appeared smooth except for an expansion in the middle of spicules, called the "bubble".

The spicules observed in adult sponges were slightly different from those cultured from primmorphs. The spicules of adult sponge of *L. baikalensis* were slightly curved amphioxea covered with many spines (Figure 4); they were 150–210 μm in length and 8–15 μm in diameter [45,46]. High SEM magnification shows the spines at the surface of the spicules (Figure 4). In addition, the skeleton in the adult *L. baikalensis* sponges was of highly ordered arrangement of spicules.

Dynamics of spicule growth in primmorphs was observed for three months. During observations of primmorph cultivation in NBW, the number of spicules in the visual field during the first months was

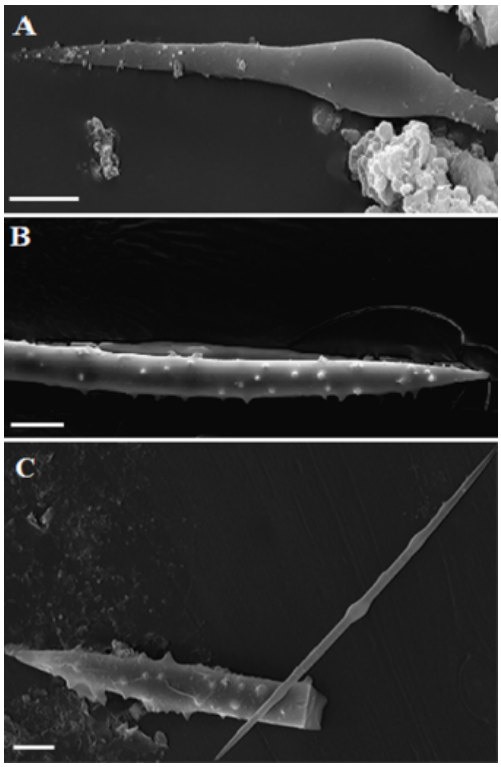

Figure 3: SEM image of spicules cultivated *in vitro* in primmorph of *L. baikalensis* during three months: (**A**) spicule cultivated in ABW in the presence of 70 μM silicate. The spicule is smooth with small spines and bubbles; (**B**) the spicule in NBW; (**C**) the spicule cultivated in ABW in the absence of silicate. Scale bars: 20 μm

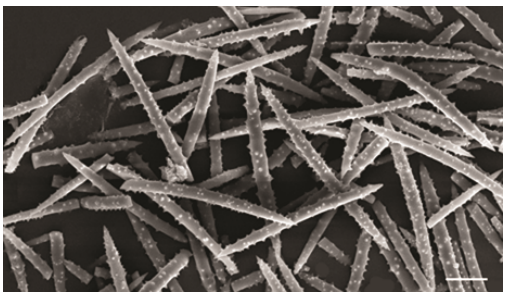

Figure 4: SEM image of adult spicules of *L. baikalensis*, this sample was used as a control. Scale bar: 20 μm.

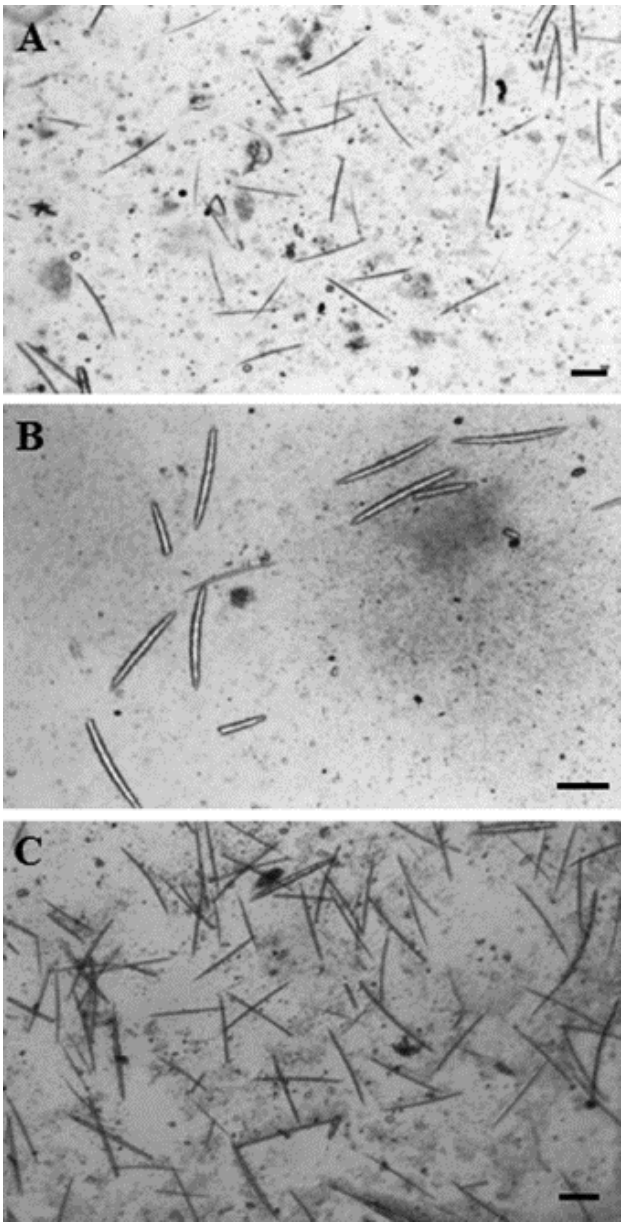

Figure 5: Light microscopy observations of spicules *L. baikalensis* primmorphs: **A** – growing spicules cultivated in NBW; **B** – growing spicules cultivated in ABW in the absence of silicate; **C** – growing spicules in ABW in the presence of 70 μM silicate. Scale bars: 60 μm.

determined to be 40–50 (Figure 5A) with a total of 10 view fields for each sample. Mean length and diameter of the spicules are given in Table 1. In the early growth the spicules lengths in the *L. baikalensis* primmorph cultures ranged from 90 to 200 μm to the end of three months. The diameter ranged from 4.0 to 7.5 μm. The mean diameter varied markedly at different developmental stages compared with the mean length. During the first month, spicules grew intensively forming new spicules.

In addition, in primmorphs that were cultivated *in vitro* in ABW without silicate, we observed insignificant spicule formation, the number of spicules in the visual field during the first months was determined to be 5–10 with a total of 10 view fields for each sample (Figure 5B). The mean spicule lengths continued to increase in size, reaching lengths of approximately 110–120 μm. The lengths of growing spicules in the *L. baikalensis* primmorph cultures ranged from 85 μm after 2 day of growth to 198.3 μm to three months (Table 1). The diameter ranged from 3.5 to 6.2 μm.

The highest number of spicules was formed in the primmorph at a concentration of 70 μM silicate in ABW (Figure 5C). The number of spicules in primmorphs was 100 or more per visual field during the initial months with a total of 10 view fields for each sample. Interestingly, that the growth of spicules is accelerates at adding concentration of 70 μM silicate in ABW. Lengths of the spicules in the *L. baikalensis* primmorph cultures ranged from 90 to 200 μm. The diameter ranged from 4.0 to 9.0 μm. In addition, we observed constant formation of new spicules during three months.

X-ray microanalysis

We investigated qualitative elemental structure of growing spicules during long-term cultivation in NBW, ABW or in ABW with the introduction of 70 μM silicate for three months. Full-size spicules formed to 3 weeks, they were flexible and brittle. Spicules that were analyzed are shown in Table 2. Mean lengths of spicules cultivated in NBW were approximately 120 μm for the first months and 193 μm to three months. The diameter ranged from 5.0 to 7.0 μm. Accumulations of silica along the lengths of the spicules were different (Figure 6A,6E,6F). General silicate contents of the spicules that were cultivated in NBW are indicated on the spectral diagram as percentages (Figure 6E). The composition of chemical elements varied along the length of spicules that were cultivated in NBW (Table 3). X-ray microanalysis indicated an increase in silica in the centers of spicules and low silica content on spines of spicules. As an example, we provide spectrum 8, which had a silicate content of up to 47.7% (Figure 6F). Oxygen content was high in spicules (Figure 6C). In addition, there was a small amount of Na (4–8%) at the ends of the spicules that decreased in the middles of spicules (1–3%).

We analyzed silica content in spicules cultured *in vitro* in ABW (Figure 7A,7E,7F). It was observed that the length and diameter of spicules were slightly smaller than of those cultured in NBW and ABW with 70 μM silicate. We recorded a more uniform distribution of silica along the spicules, but silicate contents increased towards the central part of spicules. The mean length of spicules ranged from 116.8 to 186.1 μm and the diameter ranged from 4.8–6.1 μm (Table 2). The composition of chemical elements (Si, O, C, and Na) varied along the length of spicules (Table 3). Spines of spicules contained 6.8 to 27 % silica. General silicate contents of spicules that were cultivated in ABW are given on the spectral diagram as percentages (Figure 7E). As an example, we provide spectrum 9, which had a silicate content of up to 25.9% (Figure 7F). Oxygen content was high (Figure 7C). In addition,

	Natural Baikal water			Artificial Baikal water			Artificial Baikal water with 70μM silicate		
Date (months)	1	2	3	1	2	3	1	2	3
Developmental stage	Length of spicules (μm)								
Mean length (μm)	121.7	168.1	193.7	117.3	159.9	187.4	121.1	169.7	193.5
Standard deviation	±16.7	±8.7	±6.1	±13.4	±16.6	±4.6	±16.9	±12.7	±3.6
Developmental stage	Diameter of spicules (μm)								
Mean diameter (μm)	5.2	6.4	7.1	4.7	6.0	6.2	5.9	8.4	8.9
Standard deviation	±0.63	±0.28	±0.24	±0.68	±0.07	±0.09	±0.90	±0.42	±0.10

Table 1: Dimensional characteristics of growing spicules in the *L. baikalensis* primmorphs during three months using different methods of cultivation.

	Natural Baikal water			Artificial Baikal water			Artificial Baikal water with 70μM silicate		
Date (months)	1	2	3	1	2	3	1	2	3
Dimension									
Mean length (μm)	120.5	167.8	193.0	116.8	160.5	186.2	121.8	169.0	193.5
standard deviation	±16.7	±8.7	±6.0	±13.5	±16.6	±4.63	±16.98	±12.73	±3.60
Mean diameter (μm)	5.0	6.4	7.0	4.5	5.9	6.3	6.0	8.5	9.0
standard deviation	±0.6	±0.2	±0.2	±0.68	±0.07	±0.09	±0.90	±0.42	±0.10
Number of spicules observed by SEM	28	27	28	25	24	22	28	29	28

Table 2: Mean dimensions of growing spicules in *L. baikalensis* primmorphs for X-ray microanalysis during three months using different methods of cultivation.

Chemical	Natural Baikal water			Artificial Baikal water			Artificial Baikal water with 70μM silicate		
elements	spines	ends	central	spines	ends	central	spines	ends	central
	of sp.	of sp.	part sp.	of sp.	of sp.	part sp.	of sp.	of sp.	part sp.
Silica (Si)	9-10%	22-33%	27-47%	6.8-27%	9-17%	33-35%	22-23%	30-35%	36-47%
Oxygen (O)	31-32%	43-51%	40-49%	36-47%	38-43%	50-52%	31-33%	44-57%	44-48%
Carbonate(C)	56-58%	16-35%	27-38%	24-56%	37-51%	13-14%	43-46%	20-24%	10-17%
Natrium (Na)	0.6-0.8%	6.2-7.9%	1.2-3.4%	0.5-0.4%	0.79-1.7%	0.8-1.5%	0.3-0.8%	0.3-0.6%	6.5-7.0%

sp. – spicules

Table 3: Composition of chemical elements along the length of growing spicules in primmorphs of *L baikalensis* using different methods of cultivation.

there was a small Na amount – 0.7% at the ends of the spicules which increased to 1.5% in the centers.

Table 2 demonstrates results of spicules cultivated in ABW with 70 μM silicate. The length of spicules of primmorphs *L. baikalensis* ranged from 121.5 to 192.8 μm and the diameter ranged from 6.0–9.0 μm. Relatively large amounts of silicate were also observed during long-term primmorph cultivation in ABW with 70 μM silicate, as indicated on the spectra as percentages (Figure 8A,8E,8F). The increase in silicate was recorded in the central regions of the spicules (Table 3). In the bubble, 44% silicate, 43% oxygen, and 12% carbonate were observed. The silicate content decreased towards the spicule ends and spines. However, the content of oxygen and carbonate increased in spicule spines relative to the ends (Table 3). As an example, we provide spectrum 7, in which the silicate content was 43.5% (Figure 8F). In addition, at the ends of the growing spicules there was 6.5% of Na, which decreased to 0.5% in the centers. Thus, during long-term primmorph cultivation in ABW, silicate levels gradually increased in growing spicules, resulting in 44–50% silicate, 38–47% oxygen, and 10–15% carbonate. The composition of chemical elements (Si, O, C, and Na) varied along the length of the growing spicules at cultivation in NBW, ABW and ABW with 70 μM silicate during three months.

Discussion

We developed a primmorph culture from the endemic Baikal sponge *L. baikalensis* as a model to study biosilica formation. To obtain a viable long-term primmorph culture, we aimed to create cultivation conditions that resembled natural conditions as closely as possible. Primmorphs can grow based on the sustenance of photosynthetic products of their symbionts [33,36,37]. We observed a good survival of primmorphs under different culture conditions.

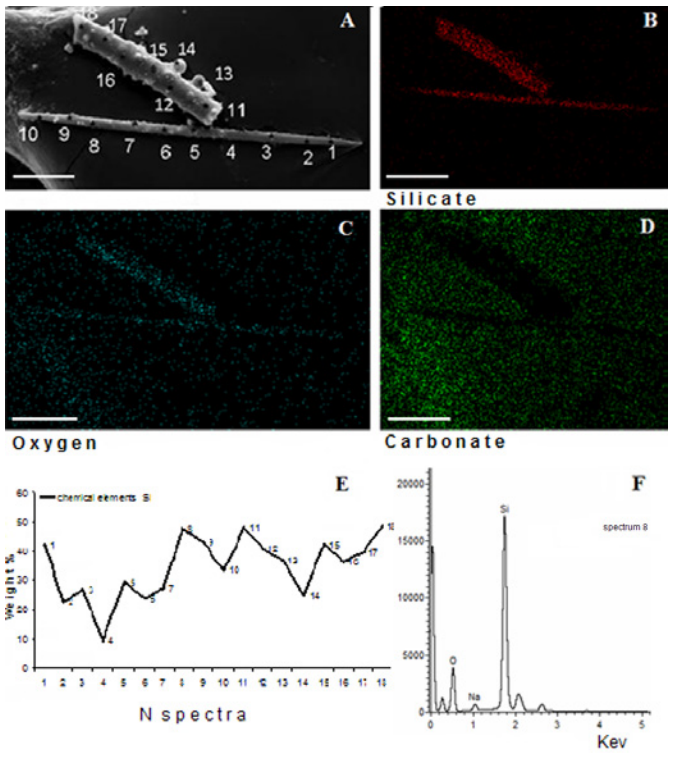

Figure 6: SEM image of spicules in primmorphs of *L. baikalensis* cultivated in NBW during three months with the points of spectra (numbers) — (**A**); (**B – D**) X-ray microanalysis (XMRA) spectra obtained for freshwater spicules. (**B**) silica contents; (**C**) oxygen contents; (**D**) carbonate contents analyzed in the wall of spicules. (**E**) general silica contents of the spicules that were cultivated in NBW are indicated on the spectral diagram as percentages; (**F**) as an example, we provide spectrum 8, which had a silica content of up to 47.7%. Scale bars: 30 μm.

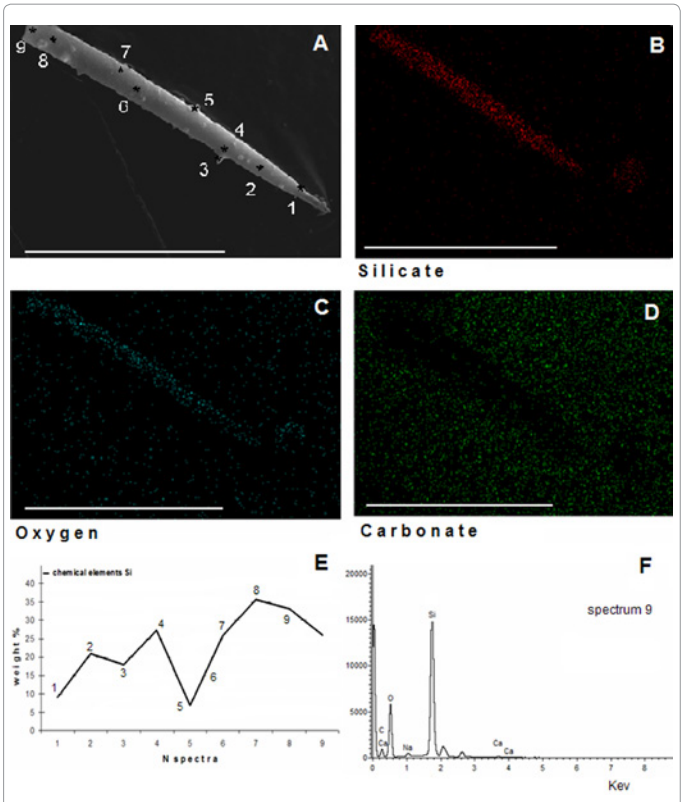

Figure 7: SEM image of spicules in primmorphs of *L. baikalensis* cultivated in ABW during three months. XMRA spectra obtained for freshwater spicules: (**A**) points spectra; (**B**) silica contents; (**C**) oxygen contents; (**D**) carbonate contents analyzed in the wall of the spicules. (**E**) general silica contents of spicules that were cultivated in ABW, as indicated on the spectra as percentages; (**F**) as an example, we provide spectrum 9, in which the silica content was 25,9%. Scale bars: 20 μm.

Here, we report spicule formation in *L. baikalensis* primmorphs that were grown in various media. Concentration of silicate influenced the formation and growth of spicules in primmorphs from the freshwater sponge *L. baikalensis*. We indicated that spicules are formed and grow in primmorphs during *in vitro* cultivation in NBW. The NBW has enough silicate for siliceous Baikal sponges, to build their spicule skeletons. Intensive formation of spicules was also observed in the primmorphs in the presence of 70 μM silicate in ABW. Similar results were observed in primmorphs of marine sponges in the presence of 60 μM silicate [7,47]. However, at a concentration of 120 μM of silicate primmorphs became friable during the first 3 days and later died in culture. Higher concentrations are likely to be toxic, e.g. 120 μM, and inhibit cultivation of primmorphs. Polymerization of silicic acid is a process which, if it is neither constrained nor controlled, is highly cytotoxic [48]. It is known that silica concentration in Lake Baikal is 100 μM [27]. Moreover, Lake Baikal contains relatively low levels of nutrients, but nutrients are stably balanced relative to chemical composition ratios in phytoplankton [29].

We indicated that spicules are formed in primmorphs and grow during *in vitro* cultivation in ABW without silicate introduction.

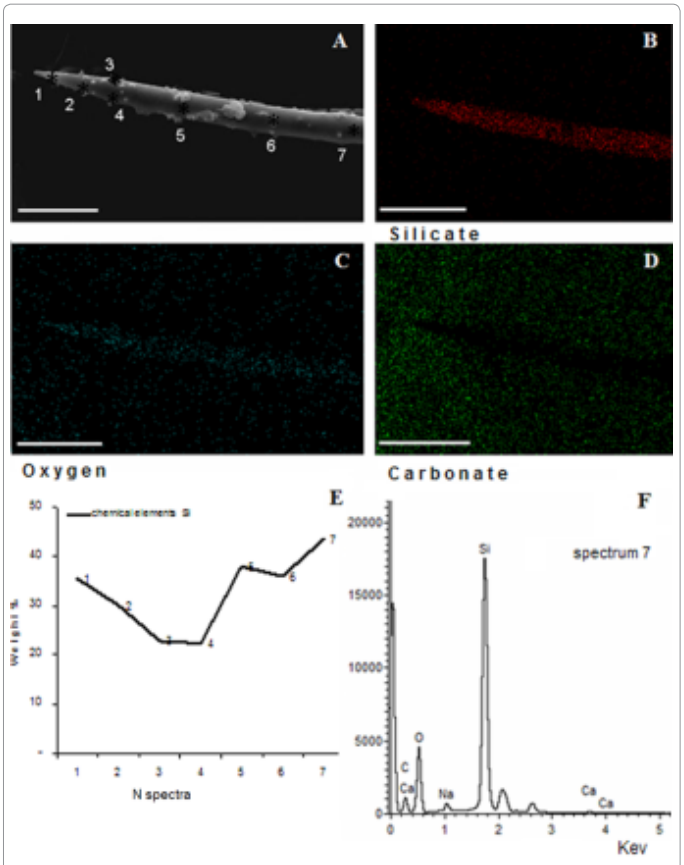

Figure 8: SEM image of spicules in primmorphs of *L. baikalensis* cultivated in ABW in the presence of 70 μM silicate during three months. XMRA spectra obtained for freshwater spicules: (**A**) points spectra; (**B**) **s**ilica contents; (**C**) oxygen contents; (**D**) carbonate contents analysed the wall of a spicules. (**E**) the general silica contents of the spicules that were cultivated in ABW in presence of 70 μM silicate, as indicated on the spectra as percentages; (**F**) as an example, we provide spectrum 7, in which the silica content was 43.5%. Scale bars: 20 μm.

Earlier, the *L. baikalensis* primmorphs were cultivated for >10 months in ABW, the composition of which was close to the Lake Baikal water [27,28] without silicate [33]. Pure primmorph culture after dissociation of sponge cells is difficult to obtain as there are traces of diatoms and spicules from adult sponge. We observed by light microscopy that cells in the primmorphs decomposed diatoms and spicules from adult sponges; it is likely to receive biogenic silica for formation of new spicules. Moreover, siliceous spicules are known to contain a definite axial filament and their synthesis is under genetic control [12,43,49]. It is known that siliceous spicules of sponges are formed intracellularly in sclerocytes and during their growth they are surrounded by a membrane called a silicalemma [3]. Spicule synthesis is rapid; for example, spicules of the freshwater sponge *Ephydatia fluviatilis* grow at a rate of 5 μm/h [50,51]. It is known that spicules of Baikal sponges are formed in two stages [39]. At the first stage of the intracellular formation a thin spicule (fiber) is formed. At the second stage a spicule goes out from of cell into the extracellular space and grows to the required thickness [39]. The production of spicules occurs inside specialized cells, the sclerocytes, where silica is deposited in an organized way [3]. Overall spiculogenesis of freshwater *L. baikalensis* primmorphs is also rapid. On the second day, they are squeezed out from sclerocytes into the intercellular space and continue to lengthen to full-size spicules on 12–14 days of cultivation. At cultivation in different media we observed intensive growth in length and diameter of spicules during three months. We also observed some (minor) differences in length and diameter of the spicules at cultivation in ABW without silicate introduction.

The SEM X-ray microanalysis data provided some information on the process of biosilica deposition during the cell cultivation of *L. baikalensis* primmorphs. Distributions of silica along the lengths of the spicules were significantly different in all of the specimens. The highest concentration of silica was observed at the bubble (the central parts of the young spicules contained up to 50% silica), and minimal amount of silica was observed at the spicule ends and spines (Table 3). We also found a great number of spicules with the "bubble" in the primmorphs cultured in ABW and ABW with the introduction of 70 μM silicate. It is likely that silica is synthesized in the middle of the spicule, where the thickening occurs. However, at cultivation in ABW without silicate introduction we observed a more uniform distribution of silica on the spicules. Probably the lack of silicon influences the growth of spicules. Moreover, accumulation of silica in spicules occurs on the intracellular molecular level [43,49]. For example, in the marine sponge *Crambe crambe*, TEM X-ray microanalysis has indicated that the extracellular space between sclerocyte and growing spicule contains 50–65% Si [52]. This intracellular process is related to polycondensation of silicic acid that is mediated by enzyme silicatein [43,49]. Moreover, we observed robust maintenance of O (41%) and C (36%) and slight maintenance of Na (8%) at the ends of spicules in the samples. These concentrations are reduced to 0.5–1% in the central region of spicules, confirming the presence of biogenic silica in growing spicules of *L. baikalensis* primmorphs. This distribution of elements is probably related to metabolism of biosilica in the primmorph cells. Based on the data discussed here, Si, O, C, and Na are the most important elements for construction of spicules. All of these elements were involved in the basic structure of siliceous spicules in primmorphs of Baikal sponges. SEM X-ray microanalysis allowed us to provide X-ray spectra and to demonstrate accumulation of biogenic silica in cultured freshwater spicules in various media. Silica plays an important role in formation of spicules, the overabundance of silica leads to destruction of cell culture primmorphs. A tendency for gradually increasing Si-levels is observed during long-term spicule growth in cultures. The fact that the model culture of *L. baikalensis* primmorphs lives for a long time under these conditions makes it possible to study genes and proteins participating in the formation of silicon spicules. In addition, the study adds important knowledge to understanding of silica deposition in spicules of primmorphs.

Overall, the Lubomirskia primmorph system is shown to be a good model for studying silica biomineralization due to biomass growth independent of external nutrients based on photosynthetic symbionts that present in primmorphs. Primmorphs cultivation *in vitro* for a long period of time will allow the creation of a live controlled model system under experimentally controlled conditions in the absence of any additional organic component and are a good model system that offers controlled experimental conditions to understand a mechanism of silica transport from the environment into sponge cells of Baikal sponges (i.e., the mechanism of spicule biosynthesis). Primmorph system described in this work can be considered as a powerful novel model system to study basic mechanisms of silicatein expression (an enzyme responsible for spicule silicification) for the identification and genetic determination of the proteins involved in the process biomineralization, and dimensional changes of spicules during developmental process of individual primmorphs. On the basis of this work we will be able to carry out further research of some

processes and mechanisms occurring in primmorphs and their cells. In addition, these methods will allow the investigation of the early stages of intracellular spicule formation.

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References

1. Love GD, Grosjean E, Stalvies C, Fike DA, Grotzinger JP, et al. (2009) Fossil steroids record the appearance of Demospongiae during the Cryogenian period. Nature 457: 718-721.
2. Uriz MJ (2006) Mineral skeletogenesis in sponges. Can J Zool 84: 322-356.
3. Simpson TL (1984) The Cell Biology of sponges. Springer-Verlag, New York, NY.
4. De La Rocha CL (2003) Silicon isotope fractionation by marine sponges and the reconstruction of the silicon isotope composition of ancient deep water. Geology 31: 423-426.
5. Uriz MJ, Turon X, Becerro MA, Agell G (2003) Siliceous spicules and skeleton frameworks in sponges: origin, diversity, ultrastructural patterns, and biological functions. Microsc Res Tech 62: 279-299.
6. Morse DE (1999) Silicon biotechnology: harnessing biological silica production to construct new materials. Trends Biotechnology 17: 230-232.
7. Krasko A, Lorenz B, Batel R, Schröder HC, Müller IM, et al. (2000) Expression of silicatein and collagen genes in the marine sponge Suberites domuncula is controlled by silicate and myotrophin. Eur J Biochem 267: 4878-4887.
8. Müller WEG, Krasko A, Pennec GL, Schröder HC (2003) Biochemistry and cell biology of silica formation in sponges. Microsc Res Tech 62: 368-377.
9. Mohri K, Nakatsukasa M, Masuda Y, Agata K, Funayama N (2008) Toward Understanding the morphogenesis of siliceous spicules in freshwater sponge: differential mRNA expression of spicule-type-specific silicatein genes in Ephydatia fluviatilis. Dev Dyn 237: 3024-3039.
10. Kozhemyako VB, Veremeichik GN, Shkryl YN, Kovalchuk SN, Krasokhin VB, et al. (2010) Silicatein Genes in Spicule-Forming and Nonspicule-forming Pacific Demosponges. Mar Biotechnol 12: 403-409.
11. Cha JN, Shimizu K, Zhou Y, Christianssen SC, Chmelka BF, et al. (1999) Silicatein filaments and subunits from a marine sponge direct the polymerization of silica and silicones in vitro. Proc Natl Acad Sci U S A 96: 361-365.
12. Müller WEG, Krasko A, Pennec GL, Steffen R, Ammar MSA, et al. (2003b) Molecular mechanism of spicule formation in the demosponge Suberites domuncula: silicatein - collagen - myotrophin. Prog Mol Subcell Biol 33: 195-221.
13. Ehrlich H, Worch H (2007) Sponges as natural composites: from biomimetic potential to development of new biomaterials. Museu National, Rio de Janeiro, Brasil 303-312.
14. Custodio MR, Prokic I, Steffen R, Koziol C, Borojevic R, et al. (1998) Primmorphs generated from dissociated cells of the sponge Suberites domuncula: a model system for studies of cell proliferation and cell death. Mech Ageing Dev 105: 45-59.
15. Koziol C, Borojevic R, Steffen R, Müller WE (1998) Sponges (Porifera) model systems to study the shift from immortal to senescent somatic cells: the telomerase activity in somatic cells. Mech Ageing Dev 100: 107-120.
16. Müller WE, Wiens M, Batel R, Steffen R, Schroder HC, et al. (1999) Establishment of a primary cell culture from a sponge: primmorphs from Suberites domuncula. Mar Ecol Prog Ser 178: 205-219.
17. Müller WEG, Rothenberger M, Boreiko A, Tremel W, Reiber A, et al. (2005) Formation of siliceous spicules in the marine demosponge Suberites domuncula. Cell Tissue Res 321: 285-297.
18. Zhang W, Zhang X, Cao X, Xu J, Zhao Q, et al. (2003) Optimizing the formation of in vitro sponge primmorph from Chinese sponge Stylotella agminata (Ridley). J Biotechnol 100: 161-168.
19. Zhang X, Cao X, Zhang W, Yu X, Jin M (2003) Primmorphs from archaeocytes - dominant cell population of the sponge hymeniacidon perleve: improved cell proliferation and spiculogensis. Biotechnol Bioeng 84: 583-590.
20. Cao X, Fu W, Yu X, Zhang W (2007) Dynamics of spicule evolution in marine sponge Hymeniacidon perlevis during in vitro cell culture and the seasonal development in field. Cell Tissue Res 329: 595-608.
21. Cao X, Yu X, Zhang W (2007) Comparison of Spiculogenesis in in Vitro ADCP-Primmorph and Explants Culture of Marine Sponge Hymeniacidon perleve with 3-TMOSPU Supplementation. Biotechnol Prog 23: 707-714.
22. Eckert C, Schroder HC, Brandt D, Perovic-Ottstadt S, Müller WE (2006) Histochemical and electron microscopic analysis of spiculogenesis in the Demosponge Suberites domuncula. J Histochem Cytochem 54: 1031–1040.
23. Belikov SI, Kaluzhnaya OV, Schröder HC, Krasko A, Müller IM, et al. (2005) Expression of silicatein in spicules from the Baikalian sponge Lubomirskia baicalensis. Cell Biol Int 29: 943-951.
24. Pile AJ, Patterson M.R, Savarese M, Chernykh VI, Fialkov VA (1997) Trophic effects of sponge feeding within Lake Baikal's littoral zone. Sponge abundance, diet, feeding efficiency, and carbon flux. Limnol Oceanogr 42: 178-184.
25. Frost TM, De Nagy GS, Gilbert JJ (1982) Population dynamics and standing biomass of the freshwater sponge Spongilla lacustris. Ecology 63: 1203-1210.
26. Bailey RC, Day KE, Norris RH, Reynoldson TB (1995) Macroinvertebrate community structure and sediment bioassy results from nearshore areas of North American Great Lakes. J Great Lakes Res 21: 42-52.
27. Falkner KK, Measures CI, Herbelin SE, Edmond JM (1991) The major and minor element geochemistry of Lake Baikal. Limnol Oceanogr 36: 413-423.
28. Grachev MA (2002) About a modern condition of ecological system of Lake Baikal. Publishing House The Siberian Branch of the Russian Academy of Science, Novosibirsk.
29. Genkai-Kato M, Sekino T, Yoshida T, Miyasaka H, Khodzher TV, et al. (2002) Nutritional diagnosis of phytoplankton in Lake Baikal. Ecological Research 17: 135–142.
30. Maldonado M, Carmona MC, Uriz MJ, Cruzado A (1999) Decline in Mesozoic reef-building sponges explained by silicon limitations. Nature 401: 785-788.
31. Tréguer P, Nelson DM, van Bennekom AJ, DeMaster DJ, Leynaert A, et al. (1995) The silica balance in the world ocean: a reestimate. Science 268: 375-379.
32. Bukharov AA, Fialkov VA (2001) Baikal in numbers. Baikal Museum, Irkutsk 72.
33. Chernogor LI, Denikina NN, Belikov SI, Ereskovsky AV (2011) Long-term cultivation of primmorphs from freshwater Baikal sponges Lubomirskia baikalensis. Mar Biotechnol 13: 782-792.
34. Efremova SM (1981) The structure and embryogenesis of the Baikal sponge Lubomirskia baikalensis (Pallas) and relations of Lubomirskiidae with other sponges. In: Morphogenesis in sponges. Korotkhova, G. P. (eds), Leningrad University, Leningrad. 33: 93-107.
35. Latyshev NA, Zhukova NV, Efremova SM, Imbs AB, Glysina OI (1992) Effect of habitat on participation of symbionts in formation of the fatty acid pool of freshwater sponges of Lake Baikal. Comparative Biochemistry & Physiology 102: 961-965.
36. Sand-Jensen K, Pedersen MF (1994) Photosynthesis by symbiotic algae in the freshwater sponge, Spongilla lacustris. Limnol Oceanogr 39: 551-561.
37. Bil K, Titlyanov E, Berner T, Fomina I, Muscatine L (1999) Some Aspects of the Physiology and Biochemistry of Lubomirska baikalensis, a Sponge from Lake Baikal Containing Symbiotic Algae. Symbiosis 26: 179-191.
38. Manconi R, Pronzato R (2002) Suborder Spongillina subord. nov.: Freshwater Sponges. In: Hooper JNA, Van Soest RWM (eds), Systema Porifera: A Guide to the Classification of Sponges. New York, Kluwer Academic /Plenum Publishers 1: 921-1020.
39. Rezvoj PD (1936) Freshwater sponges Fam. Spongillidae & Lubomirskiidae, Vol. 2. Fauna URSS Academie Sciences URSS, Moscow.
40. Strickland JDH, Parsons TR (1972) A practical handbook of seawater analysis. Queen's Printer, Ottawa, Canada.

41. Kelly-Borges M, Pomponi S (1992) The simple fool's guide to sponge taxonomy. Harbor Branch Oceanographic Institution, Fort Pierce, Florida.

42. Jones WC (1984) Spicule dimensions as taxonomic criteria in the identification of haplosclerid sponges from the shores of Anglesey. Zoological Journal of the Linnean Society 80: 239-259.

43. Shimizu K, Cha J, Stucky GD, Morse DE (1998) Silicatein alpha: cathepsin L-like protein in sponge biosilica. Proceeding of the National Academy of Sciences USA 95: 6234-6238.

44. Goldstein JI, Newbury DE, Echlin PE, Joy DC (1992) Scanning Electron Microscopy and X-ray Microanalysis, 2nd ed., Plenum Press, New York.

45. Dybowski W (1880) Studien über die Spongien des russischen Reiches mit besonderer Berücksichtigung der Spongien-Fauna des Baikal-Sees. Mém Acad Sci St. Petersburg sér. 27: 1-71.

46. Masuda Y, (2009) Studies on the taxonomy and distribution of freshwater sponges in the Lake Baikal. Prog Mol Subcell Biol 47: 81-110.

47. Krasko A, Schröder HC, Batel R, Grebenjuk VA, Steffen R, et al. (2002) Iron induces proliferation and morphogenesis in primmorphs from the marine sponge Suberites domuncula. DNA Cell Biol 21: 67-80.

48. Iler RK (1979) The chemistry of silica. Wiley, New York.

49. Weaver JC, Pietrasanta LI, Hedin N, Chmelka BF, Hansma PK, et al. (2003) Nanostructural features of demosponge biosilica. J Struct Biol 144: 271–281.

50. Weissenfels N, Landschoff HW (1977) Bau und Funktion des Süßwasserschwammes E. fluviatilis L. (Porifera). IV. Die Entwicklung der monaxialen SiO2-Nadeln in Sandwich-Kulturen. Zool Jahrb Abt Anat 98: 355-371.

51. Weissenfels N (1989) Biologie und Mikroskopische Anatomie der Süßwasserschwämme (Spongillidae). Gustav Fischer Verlag, Stuttgart.

52. Uriz MJ, Turon X, Becerro MA (2000) Silica deposition in Demospongiae: spiculogenesis in Crambe crambe. Cell Tissue Res 301: 299-309.