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Toxic Effect of Anionic Surfactants on Freshwater Sponge *Lubomirskia baikalensis* and Its Endosymbiotic Microalgae *Chlorella* sp.

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Abstract: A number of reports on sponge diseases, including from Lake Baikal, have increased dramatically all over the world in recent years. Herewith, there are various hypotheses for sponge mortality. *Lubomirskia baikalensis* (phylum Porifera, order Spongillida) is a unique endemic freshwater sponge of Lake Baikal that contains a complex community of eukaryotic and prokaryotic endosymbiotic microorganisms. In this work, we present the first results for the effect of anionic surfactants viz. linear alkylbenzene sulphonates (LAS) at low 10 and 20 μg L^{-1} concentrations on Baikal sponge species and their symbiotic community as an experimental model. A new toxicity test protocol under conditions close to natural is proposed. It uses the sponge amoebocytes called as SA1-cells, which contain eukaryotic green microalgae *Chlorella* sp. These SA1-cells are shown to be representative indicator in assessing the impact of anionic surfactants. The acute toxic effect resulted in 97–100% sponge cell death in less than 48 h, as well as 100% symbiotic microalgae *Chlorella* sp. death over 72 h was noted under LAS solution (20 μg L^{-1}) exposure during in vivo experiments. This includes the cell membrane fatty acid changes, change in the cell sizes, cell swelling, and lysis. Long term exposure to LAS solution (10 μg L^{-1}) reflected in cellular stress (oxidative stress) and accompanied by malondialdehyde formation (0.16–2.0 μg g^{-1} of dry weight) during 14-day exposure was noted. Oxidative stress and mortality of *L. baikalensis* are associated with their low antioxidant activity. Trolox-equivalent antioxidant capacity (TEAC) found in the range from 0.00031 to 0.00077 Trolox equivalents for these freshwater sponges.

Keywords: linear alkylbenzene sulfonate toxicity; LAS toxicity; oxidative stress; antioxidant capacity; TEAC; ABTS; sponge disease; sponge amoebocytes; zoochlorella; sponge fatty acid

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1. Introduction

The freshwater Lake Baikal is the deepest lake in the world, with a maximal depth of 1642 m [1,2]. The littoral zone contributes only ~1116 km^2 (3.4%) to its total area of 32,822 km^2 [3]. This is due to geomorphological peculiarities of the Baikal basin [4]. Nevertheless, at depths of up to 20 m, the Lake Baikal littoral zone contains more than 98% of all bottom-dweller diversity because of the minimal depths and maximal light intensity sufficient for photosynthesis. At depths up to 4 m, the biomass of bottom organisms achieves 620 kg per hectare, and at depths of 20–70 m it achieves 150 kg [5].

Freshwater sponges (Phylum: Porifera, Order: Spongillida) inhabit many lake ecosystems [6] including Tanganyika [7,8], Malawi [8], Michigan [9], Baikal, and others. Baikal sponges are one of the most abundant bottom invertebrates. According to modern taxonomy, family of endemic Lubomirskiidae and family of cosmopolitan Spongillidae represent sponges in Lake Baikal [6,10,11]. Endemic sponges *Lubomirskia baikalensis* (Pallas, 1776) (class Demospongiae, order Spongillida, family Lubomirskiidae), *Baikalospongia bacillifera* (Dybowsky, 1880) (class Demospongiae, order Spongillida, family Lubomirskiidae), and *Baikalospongia intermedia* (Dybowsky, 1880) (class Demospongiae, order Spongillida, family Lubomirskiidae) are the dominant sponge species in the lake. The cortical sponge *Baikalospongia intermedia* and the globular sponge *Baikalospongia bacillifera* cover the stony nearshore bottom from 1 m of depth. The branching sponge *Lubomirskia baikalensis* covers the stones from 1 m to the maximum depths where rocks exist. Some individuals were found at 1340 m of depth with deep-submergence vehicles *Mir-1* and *Mir-2* [12], but their abundance and species diversity are concentrated mainly at depths from 5 to 30–50 m [10,13].

The *Lubomirskia baikalensis* is the only branching sponge in Lake Baikal [14]. It contains a complex symbiotic assemblage. There are exosymbionts living on the sponge's external surface (eukaryotic and prokaryotic microorganisms, protozoa, and crustacea, e.g., *Brandtia parasitica* and *Brandtia latissima* (order Amphipoda)), and endosymbiotic microorganisms inside the sponge cells and in intracellular space (microalgae, cyanobacteria, bacteria, archaea, fungi, and viruses). Photosynthetic green microalgae *Chlorella* sp. (synonyms: zoochlorellae, *Zoochlorella parasitica*) is the dominant (by biomass) symbiotic eukaryotic microorganism of *L. baikalensis*. According to recent publications, these algae might belong to genus *Choricystis* [15,16] or to genus *Mychonastes* [17]. Diatoms can occur as well [18].

Numerous picocyanobacteria developing in Lake Baikal plankton penetrate into healthy sponge body during water filtration and comprise an essential fraction of their microbiome [18]. A molecular approach based on the identification of the 16S rRNA and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit genes was applied to investigate diversity and phylogeny of bacterial phototrophs associated with four species of *Lubomirskiidae* in Lake Baikal. The phylogeny inferred from both genes represents three main clusters of *Synechococcus* associated with Baikal sponges [16]. Over 90% of the cyanobacterial molecular sequences for *L. baikalensis* revealed belonged to *Synechococcus* [18]. Sick sponge cyanobacteria fouling mainly represented by *Synechococcales*, *Chroococcales*, *Oscillatoriales*, and *Nostocales* [19]. Bacterial phyla (*Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Nitrospirae*, *OD1*, and *Chloroflexi*) were identified in *L. baikalensis* as well.

The microbes living in sponges make up to 35% of the biomass of the symbiont community [20] and up to 40–60% of the sponge's biomass [21]. Algal symbionts may comprise up to 75% of the cellular tissue in sponge-algal associations [21]. They are involved in photosynthesis which takes place even in deep cell layers of the sponge body, carbon fixation, nitrogen transformation, and anaerobic metabolism; they also perform protective functions [20].

The littoral zone is exposed to negative anthropogenic impact of biological and chemical pollutants, invasive organisms, and temperature changes to a greater extent than the pelagic zone. This can result in stress situations for different water organisms [3]. Over last years, changes in the structure of numerous benthic communities have been marked in the nearshore zone of Lake Baikal [22]. The oxidative stress of nearshore phytoplankton was revealed [23]. Widespread mortality of sponges including the endemic *L. baikalensis* along the Lake Baikal perimeter was noticed [10,24,25]. In recent years, the number of reports on sponge diseases have increased dramatically, with marine sponge populations decimated throughout the Mediterranean and Caribbean seas. The increased prevalence of marine sponge disease in Papua New Guinea, the Great Barrier Reef, and in the reefs of Cozumel, Mexico [26] has also been reported. The sponge population declines and, as a result, the filtration efficiency decreases, especially in freshwater ecosystems. This can lead

to irreversible consequences regarding health both of aquatic organisms and the ecosystem as a whole.

At present, there are various hypotheses for the mass mortality of sponges in Lake Baikal, which are associated with global warming [10,26], anthropogenic impact [11,27], methane concentration in coastal water [10,28], and changes in viral assemblages [29]. The presence of anionic surfactants at 40 μg/L concentration in Tyya River, the tributary of Lake Baikal, was fixed using the methylene blue active substances (MBAS) method in 2019 [30]. A source of these pollutants is shown to be untreated wastewater with the surfactant concentration of 170 μg L^{-1}. The structure of anionic surfactants in the samples was related to widespread linear alkylbenzene sulfonates (LAS) according to gas chromatography coupled with mass spectrometry [30]. For comparison, the concentrations of alkylbenzene sulfonates in freshwater and marine ecosystems of the world are estimated from 1–7 to 250–300 μg L^{-1} [31–34]. It has been reported that relatively low LAS concentrations (5–20 μg L^{-1}) can cause toxic effects on aquatic microorganisms including oxidative stress, extreme growth of cyanobacteria, and cell lysis [35–37]. So, the effect of anionic surfactants on sponges of Lake Baikal may be one of the reasons for sponge disease.

Unfortunately, little attention has been paid to the determination of the mentioned toxicants in the environment and their effect on water organisms. This study aims to assess the impact of LAS on *L. baikalensis* and its endosymbiotic microalgae *Chlorella* sp. as a whole organism under conditions close to natural.

2. Materials and Methods

2.1. Sponge Sampling

2.1.1. Choosing the Sponge Target Species for the Experiments

The *Lubomirskia baikalensis* target species was choosing as more convenient experimental model in the contrary to *Baikalospongia bacillifera* and *Baikalospongia intermedia* due to the different structures of these sponges. The *B. bacillifera* is very hard sponge. A cell sampling from its body without cell damage is problematic. The *B. intermedia* consistency is too soft and friable. Sampling and transportation of these sponges to the laboratory are difficult. The *L. baikalensis* consistency is soft, elastic, and non-friable, which allows for taking its cells with a mechanical pipette to exclude serious sponge damage.

2.1.2. *Lubomirskia baikalensis* Sampling for In Vitro Aquarian Experiments

Branching sponges *L. baikalensis* were collected by self-contained underwater breathing apparatus (SCUBA) divers from the depths 5–20 m at nearshore stations of Lake Baikal. Sponges for in vitro toxicity test were collected in 2021 at sampling site No. 1 (N 51°52′01.2″, E 104°49′38.1″) in Listvennichnyi Bay from the depths of 10–20 m in the southern basin of Lake Baikal (Figure 1). This sampling site is located not far from Listvyanka Settlement, which is a large recreation center of the south-west coast of Lake Baikal. The *L. baikalensis* population has almost disappeared in Listvennichnyi Bay over two last decades. Nevertheless, epilithic, visually healthy, undamaged branching sponges of ≤12 cm branch length (n = 12) were taken from sampling site No. 1. This can be explained by the position of the site No. 1 with the underwater ridge in the source of the Angara River. This geomorphological peculiarity provides deep water motion from the depths up to 500 m toward the surface in this region. This water is suggested to be cleaner as opposed to surface water in Listvennichnyi Bay. After sampling sponges were put into oxygen aerated 12 L thermoses preventing the contact of sponges with atmospheric air, transported to the laboratory, and placed into 20 L volume aquariums.

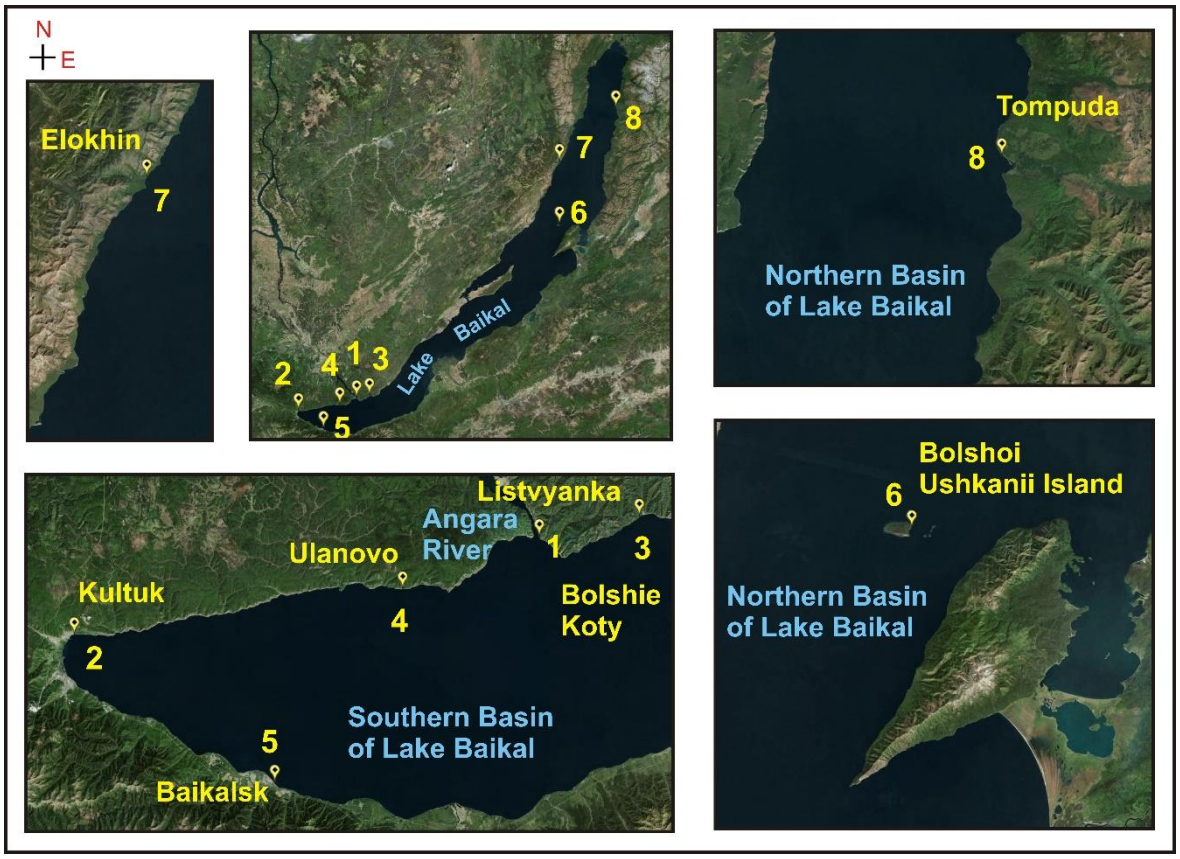

Figure 1. Scheme of Lake Baikal and the sampling sites of *Lubomirskia baikalensis* (class Demospongiae, order Spongillida, family Lubomirskiidae). Live sponges from the site No. 1 were transported into aquariums, and sponges from the sites No. 2–8 were frozen immediately.

2.1.3. *Lubomirskia baikalensis* Sampling for In Situ Biochemical Analysis

Sponge samples for in situ biochemical analysis were collected from south and north basins of the lake (Figure 1) from depths of 5–20 m. Sponges from sample site No. 2, located in the region of Kultuk Settlement (N 51°43′42.2″, E 103°44′01.7″) and No. 3 located in the region of Bolshiye Koty Settlement (N 51°54′08.7″, E 104°49′38.1″) were collected in September 2019 to reveal their oxidative stress (n = 20) (Figure 1). The stations No. 2 and 3 differed by the intensity of anthropogenic load. All samples were frozen at –70 °C.

Sponges from the sample sites No. 4 located in Ulanovo region (N 51°47′47.6″, E 104°31′34.1″), No. 5 located in the region of the inoperative Baikalsk City paper mill, No. 6 near Bolshoi Ushkanii Island, No. 7 in the region of Elokhin Cape (N 54°33′05.8″, E 108°39′55.0″), and No. 8 in Tompuda Bight (N 55°07′43.2″, E 109°43′42.6″) were collected in 2022 (June–July) from the depths of 5–20 m to estimate *L. baikalensis* antioxidant activity (n = 11, 2–3 samples were collected from each station) (Figure 1). All samples were also frozen.

2.2. Conditions for Cultivation of *L. baikalensis*

Near-natural conditions were provided for cultivation of *L. baikalensis*. The biomass of sponges in each aquarium did not exceed 300 g. The aquariums were filled with 10–12 L of bottled Lake Baikal water from a depth of 400 m and with 3–5 L of fresh pelagic surface Lake Baikal water. Pelagic water was collected from the offshore station located at 1 km from the shoreline (N 51°51.427′, E 104°50.020′) in Listvennichnyi Bay. The depth to bottom was 0.5 km. Surface water from 0.3 m depth was sampled using a plastic bottle, transported to the laboratory and poured into aquarium. The concentration of anionic surfactants in deep-bottled water did not exceed 3.0 μg L^{-1}.

Zooplankton were collected at the same pelagic station once a week to feed the sponges. The sampling was carried out at the same pelagic station using an Apstein-type net with 100 µm mesh size. Live zooplankton (~0.2 g) were put into a bottle filled with pelagic water. We changed 10–30% of aquarium water once in 2–4 days. Water temperature was kept at +8 °C, as in Lake Baikal, during the sampling period. It is known that sponges of Lake Baikal can live in a wide range of temperatures from +2 to +24 °C in the nearshore zone. The light regime was a 12-hour light–dark cycle. Light intensity (illuminance) was 2 lux at the bottom of the aquarium. The light source provided 500–600 nm range in the electromagnetic spectrum. Light conditions were close to natural at depths of 10–15 m in Lake Baikal. The pH varied from 7.66 to 8.02 at 25 °C.

The dissolved oxygen analysis was carried out by iodometric titration according to Winkler [38]. The oxygen concentration of 10–20 mg L^{-1} in water was achieved by aeration with gaseous oxygen supplied from a high-pressure gas cylinder with a sprayer at the constant flow of ~3.2 ± 0.2 L per minute as well as with atmospheric air using an air compressor, which also saturated water with CO_2 at the constant air flow of ~5.0 ± 0.3 L per minute. The oxygen concentration in Lake Baikal water varies from 10 to 16 mg L^{-1}. The higher concentrations up to 20 mg L^{-1} are nontoxic and friendly for sponges. This can be explained by the oxygen concentration in photosynthetic organisms, which is higher than in the environment. The daily oxygen evasion into the atmosphere across the air–water interface in Lake Baikal confirms this fact [39,40]. Oxygen aeration allows us to prevent the parasitic water mold *Saprolegnia* sp.'s growth (order Saprolegniales, family Saprolegniaceae) and to provide better adaptation and survival for sponges and its internal and external symbionts. For example, it was shown that aeration with atmospheric air results only in gradual or mass mortality of symbiotic *Brandtia parasitica*, which reflects the unusual conditions for these organisms.

2.3. Cell Viability Test Procedure

Cell suspension from the sponge body of ~50 µL volume was taken under the water with a mechanical pipette. This allowed us to obtain sponge cells without damaging them. Suspension was placed into a plastic Eppendorf and stored in a thermos at +8 °C. Microscopy was carried out immediately after sampling. For this purpose, 10 µL of cell suspension was placed onto a microscope slide. Methylene blue (tetramethylthionine chloride, $C_{16}H_{18}ClN_3S$) aqueous solution (3 µL, C = 0.007 g L^{-1}, C_M = 0.02 mM) was added to the sample. The cover glasses of 0.17 mm thickness and 15 × 15 mm size were used. The microscopic slides with samples were cooled to +8–12 °C during microscopy.

Live and dead cells of sponges and symbiotic *Chlorella* sp. were counted using an Axiovert inverted microscope (Carl Zeiss, Göttingen, Germany) at 1000× magnification or Olympus CX23 Binocular Microscope (Olympus, Tokyo, Japan) with 100× (oil) infinity plan objectives, 10× focusable eyepieces with 20 mm field of view, and the LED-transmitted illumination. Each sample was analyzed in 10–12 replicates (10–12 fields of view) and 1–70 cells in each field of view were analyzed in every replicate. The sum number of screened cells was up to 230. The number of live unstained cells and dead, blue-stained ones was counted within 1–3 min and percentages of live and dead cells were calculated.

2.4. Blank Samples–The "Control Sponges"

The blank samples were analyzed before the anionic surfactants toxicity assessment. To select sponge individuals that would reflect representatively the viability of *L. baikalensis* under LAS impact, we sampled visible, healthy, adult sponges in the lake. These sponges were cultivated for 10 days in clean water after sampling. Two parameters were controlled: the percentages of live/dead sponge amoebocytes containing endosymbiotic microalgae *Clorella* sp. (Division: Chlorophyta) and the presence of lipid peroxidation [41] and oxidation stress marker vis. MalonDiAldehyde (MDA) in sponge biomass. Sponges were considered to be "control sponges" if the percentages of live cells was ≥80% and MDA was

not revealed. Fatty acid content and profiles were investigated before toxicity testing as well. Control sponges were used in the subsequent experiments.

2.5. Linear Alkylbenzene Sulfonate Toxicity Studies

The toxic effect of linear alkylbenzene sulfonates (LAS) on sponge cells was evaluated under near-natural conditions. The standard solution of sodium linear alkylbenzene sulfonates (GSO 8578-2004, C = 100 mg mL^{-1}, Analytik-Him, Russia) containing 20 prevailing isomers related to C10, C11, C12, and C13 homologue groups was used in toxicity test experiments. To prepare LAS stock solution, 10 µL of standard solution were diluted in 10 mL of bi-distilled water to obtain 100 µg mL^{-1} concentration. The stock solution was added into aquariums in which the control sponges were cultivated. The LAS concentrations in aquarian water after LAS addition were 10 or 20 µg L^{-1} and were stable during the experiments. It should be said that two mentioned concentrations were chosen according to concentration range of LAS in Lake Baikal surface water from <10 µg L^{-1} (n = 70) to 10–20 µg L^{-1} (n = 32) [23]. The toxicity test protocol aimed to establish the LAS toxicity to freshwater sponge *L. baikalensis* and included live/dead cell counting, fatty acid and MDA analysis. Mentioned analyses were realized in the same time intervals of 12 h duration.

2.6. Fatty Acid Qualitative and Quantitative Analysis

2.6.1. Lipid Extraction and Fatty Acid Derivatization

The brilliant green sponge cell biomass was wrung out into glass. Biomass was mixed with a glass stick. To calculate the biomass humidity according to gravity measurements, ~0.4 g of sponge biomass was placed into a 1.5 mL plastic microcentrifuge tube and dried at 50 °C to achieve the constant weight. To extract lipids, ~0.2 g of the biomass was placed into a 2 mL plastic microcentrifuge tube and sonicated for 5 min with 1.2 mL of Folch mixture (chloroform–methanol, 2:1, by volume). Distilled water (~0.2 mL) was added to the extracts, shaken for 10 s and centrifuged at 13,000 rpm for 3 min. The extract (lower layer) was put into glass 10 mL vial. The solvent was evaporated using argon gas stream at 30 °C. Thereafter 4.5 mL of 2% H_2SO_4 solution in methanol was added immediately to dry extract. The obtained solution was heated during 1.5 h at 55 °C. Fatty acid methyl esters (FAMEs) were extracted with *n*-hexane (3 mL × 2 × 2 min). The extract was dried with anhydrous Na_2SO_4 and concentrated to ~0.5 mL using an argon stream. Note: 1 mL of water was added to the solutions before the second extraction. The di-*n*-decyl ether ($C_{20}H_{42}O$) solution in *n*-hexane was used as an internal standard for quantitative analysis.

2.6.2. Fatty Acid Analysis by Gas Chromatography Coupled with Mass-Spectrometry

The gas chromatography coupled with mass spectrometry (GC-MS) method was used to analyze FA composition of *L. baikalensis* samples. The extracts were analyzed using the gas chromatograph coupled to mass spectrometric detector "6890B GC System, 7000C GC/MS Triple Quad" (Agilent Technologies Inc., Wilmington, DE, USA) with Optima-17MS column (30 m × 0.25 mm, 0.25 µm, Macherey-Nagel GmbH and Co. KG., Düren, Germany). The injector temperature was 290 °C; the injection volume was 2 µL in splitless mode; the quadrupole temperature was 150 °C; the ion source temperature was 230 °C; the ionization energy was 70 eV. Chromatography of the extracts was carried out by heating the column from the initial temperature of 80 °C (0.5 min retention) up to 310 °C at a heating rate 2 °C/min (5 min retention). Chromatographic peaks were detected in the m/z range of 40–500.

The mass spectra of fatty acid methyl esters were identified using the NIST Mass Spectral Library. The calibration function was obtained in the range of concentrations of fatty acid sum from 40 to 540 µg in a sample using "F.A.M.E. Mix C4–C24" (Supelco, Bellefonte, PA, USA) and "Methyl cis-4,7,10,13,16,19-Docosahexaenoic ester" (10 mg mL^{-1} in heptane) (Supelco, St. Louis, MO, USA). The calibration coefficients for individual substances and for the groups of saturated fatty acids (SFA), monounsaturated fatty acids

(MUFA), polyunsaturated fatty acids (PUFA), and demospongic fatty acids (DSFA) were calculated to quantitate FA that were absent in standard mixtures [42].

2.7. Malondialdehyde Content Determination

To reveal the lipid peroxidation processes in collected sponges, the malondialdehyde content in sponge biomass was analyzed. Sample preparation was carried out according to Al-Rashed et al.'s method [43] with modifications. Sponge biomass (~0.2 g at ~95–98% humidity) was placed into a 2 mL round-bottom plastic Eppendorf taste-tube. Trichloroacetic acid (TCA) 10% solution (1 mL) was added. Samples were sonicated for 5 min in 40 GHz ultrasonic bath and centrifuged at 13,000 rpm for 3 min. The supernatant was placed into a 1.5 mL conic-bottom Eppendorf taste-tube, centrifuged at 13,000 rpm for 3 min, and moved into a 5 mL glass vial with 2 mL of 0.5% thiobarbituric acid (TBA) solution in 10% TCA. The vial was capped with a plastic screw cap and kept for 20 min at 95 °C. Then, the vial was opened, and the solution was kept for 10 min at 95 °C. The solution volume was normalized to the initial volume. The obtained solutions were cooled and centrifuged at 13,000 rpm for 3 min. Absorbance of pink MDA-TBA complex was measured using a double-beam UV-Vis Cintra-20 spectrophotometer (GBC Scientific Equipment, Braeside, Australia) and standard quartz cuvettes of 1 cm path length. The MDA absorption was measured at 532 nm. Baseline absorption was measured at 532 nm using the solution obtained by mixing of 1 mL of 10% TCA with 2 mL of 0.5% TBA in 10% TCA.

2.8. Total Antioxidant Activity (AOA) of Lubomirskia baikalensis

2.8.1. Extraction of Antioxidants

To estimate *L. baikalensis* antioxidant capacity, the frozen sponges were used. Frozen sponges were cut to separate pieces. Sponge biomass was not wrung out and the whole sponge including the skeleton was prepared. Masses of wet samples varied from 0.4 to 2.0 g. The humidity was estimated gravimetrically, with values from 83.9 to 93.3%. Fractions of sponge antioxidants were extracted for 2 min with 1:1 by volume ethanol:acetone mixture (1.5 mL) using a mortar. The second extraction was carried out for 1 min with acetone (1.0 mL). The extracts were combined, centrifuged, and kept in the dark until the analysis. Each sample was analyzed in two replicates. The analysis of obtained crude extracts was carried out immediately.

2.8.2. Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The Trolox-equivalent antioxidant capacity (TEAC) assay was used to determine the total antioxidant capacity (TAC) of freshwater sponge *Lubomirskia baikalensis* and its endosymbionts as a whole. TEAC assay is based on the ability of antioxidants to quench activity of long-lived stable radical cation 2,2′-Azino-Bis(3-ethylbenzoThiazoline-6-Sulfonic acid) ($ABTS^{\bullet+}$)-containing chromophore group responsible for its intensive blue color. To prepare the stable stock solution of $ABTS^{\bullet+}$ 14.5 mg of ammonia persulfate $(NH_4)_2S_2O_8$ were diluted in 25 mL of bi-distilled water to obtain 0.0025 M solution. Then, 10.6 mg of $ABTS^{\bullet+}$ diammonium salt (HPLC grade, Sigma-Aldrich, USA) was diluted in 2.6 mL of bi-distilled water and 2.6 mL of $(NH_4)_2S_2O_8$ solution was added. The stock solution was kept in the dark at room temperature for 12–16 h before use. At the beginning of the analysis day, the $ABTS^{\bullet+}$ working solution was obtained by the dilution in ethanol of the stock solution to an absorbance of 0.70 ± 0.02 AU at 730 nm [44]. The absorbance was measured using an SF-2000 spectrophotometer (OKB-Spectr, St. Petersburg, Russia) and PE-5300V spectrophotometer (ECROSKHIM Co. Ltd, St. Petersburg, Russia) at 37 °C and standard quartz cuvettes of 1 cm path length. Two mL of $ABTS^{\bullet+}$ working solution was placed into a cuvette. Then, 20 µL of sponge extract with a known concentration of dry substance was added to $ABTS^{\bullet+}$ working solution and the decreased value of absorbance was measured in 4 min. The next portions of the sponge extract were added into a cuvette every 4 min till absorbance value was $\leq$0.3 AU. The calibration function was obtained for

$ABTS^{\bullet+}$ solutions. Baseline absorption was measured using distilled water in the parallel cuvette. Results of the measurements were recalculated to the sponge's dry weight (d.w.).

2.8.3. TEAC Data Presentation

The obtained data were compared with that of Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic) (Sigma-Aldrich, St. Louis, MO, USA), a water-soluble vitamin E analog. Antioxidant capacity of Trolox was stated as 1.0. The values of $ABTS^{\bullet+}$ scavenging by the obtained crude extracts of *L. baikalensis* were expressed as Trolox-equivalent antioxidant capacity (TEAC) units. This abbreviation is the analogous with Trolox equivalents (TE). The inhibition was estimated according to the formula I (%) = $(A_0 - A_i)/A_0 \cdot 100\%$, where A_0 and A_i are the initial optical absorbance of $ABTS^{\bullet+}$ working solution and the subsequent decreased absorbance value, correspondingly. The values of $ABTS^{\bullet+}$ inhibition (I) were presented as $ABTS^{\bullet+}$ percentages that were scavenged by 1 μg of Trolox standard solution (μg Trolox mL^{-1} $ABTS^{\bullet+}$) or 1 μg of dry sponge sample extract per mL $ABTS^{\bullet+}$ solution. The half maximal inhibitory concentration IC_{50} (μg mL^{-1}) was determined as the concentration of Trolox or dry sponge extract, which scavenges 50% of $ABTS^{\bullet+}$ activity.

3. Results

3.1. Classification of L. baikalensis Dominant Cell Types

It was already noted that unicellular symbiotic microorganisms are located within the organelles viz. vacuoles of mesenchymal cells (archeocytes) from freshwater sponges [45] or located directly in sponge amoebocyte cytosol [21]. In this study, we classified *Lubomirskia baikalensis* (Figure 2A) amoebocytes into four types depending on the domain of endosymbiotic microorganisms inside. Microalgae of two domains of Chlorophyta and Cyanophyta were easily seen with a light microscope and were localized within colorless amoebocytes (Figure 2B–E). The amoebocytes containing eukaryotic symbiotic green microalgae *Chlorella* sp. are found to be the largest and dominant cells of the investigated sponges (Figure 2A). The list below illustrates a description of different amoebocytic cell types.

1. Sponge Amoebocytes of the first type named as SA1-cells (Figure 2B1) contained predominantly eukaryotic Chlorophyta (Figure 2B4). These spherical sponge cells were characterized by relatively large size from 10 to 25 μm and large central globular nuclei with prominent nucleoli of ~2.8 μm. The flexible cell membranes of *L. baikalensis* SA1-cells provide structural strength, regular spherical shape, as well as counteracting the osmotic pressure of the cytoplasm. Ectoplasm and endoplasm of different viscosity were found within the plasma membrane. The outside membrane was characterized by the singular and branching strands (Figure 2B) of different lengths. The cells of the similar structure are known for marine sponge *Microciona prolifera* (Ellis and Solander, 1786) (phylum Porifera, class Demospongiae, order Poecilosclerida, family Microcionidae) [46]. We suppose that the SA1-cells present nucleolar archeocytes, also called sponge stem-like cells or an intermediate cell type between the archeocytes and gray cells [46]. These cells might have different functions, including the function of phagocytes or amoebocytes [17], immunocytes [46], as well as potentially representing evolutionary precursors to a true nervous system [47,48], and allow the sponge cells to communicate with one another by passing electrical or chemical signals.
2. Amoebocytes of the second type (SA2-cells) (Figure 2D2) contained prokaryotic Cyanophyta (Figure 2C5,D5).
3. Amoebocytic single cells of the third type contained both prokaryotic symbionts and small amount of eukaryotic symbionts (SA3-cells) (Figure 2E8).
4. Amoebocytic cells with moving flagella containing predominantly prokaryotic symbionts were also noticed (SA4-cells) (Figure 2E3).

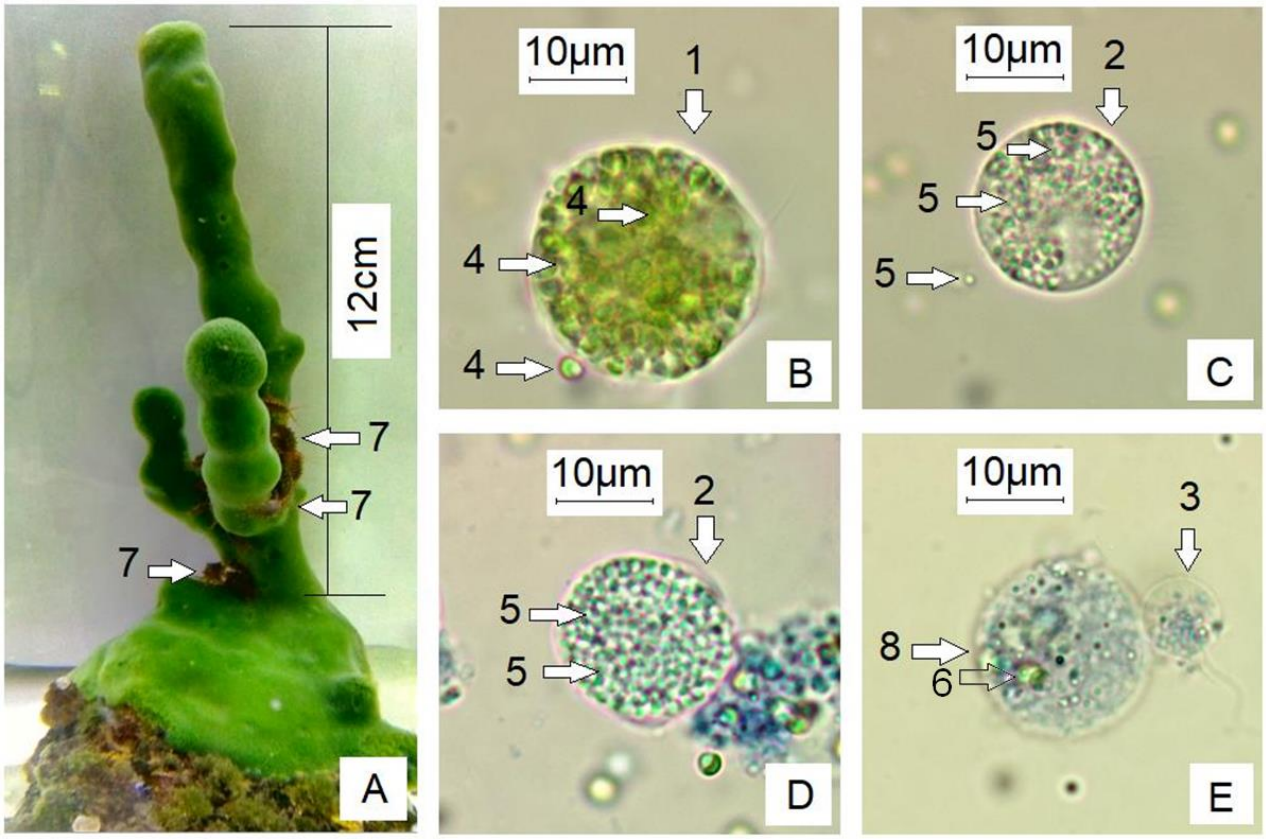

Figure 2. Photo of *Lubomirskia Baikalensis* (class Demospongiae, order Spongillida, family Lubomirskiidae) in aquarium and the sponge dominant cell light microscope images. (**A**)–underwater image of epilithic sponge with exosymbionts (7) *Brandtia parasitica* (class malacostraca, order Amphipoda, family Acanthogammaridae); (**B**)–healthy sponge amoebocytes of the first type (SA1-cells) (1) and its eukaryotic endosymbionts *Chlorella* sp. (4); (**C**)—healthy amoebocytes of the second type (SA2-cells) (2) and its prokaryotic endosymbionts; (5); (**D**)–healthy amoebocytes (SA2-cells) (2) and its prokaryotic endosymbionts (5), stained with methylene blue; (**E**)–amoebocytes containing symbiont assemblage (SA3-cells) (8) including green algae (6) and a cell with flagellum (SA4-cell) containing prokaryotic symbionts (3).

3.2. Lubomirskia baikalensis SA1-Cells as an Indicator of LAS Pollution of Water

In this investigation, we demonstrate staining effect of methylene blue dye on live and dead amoebocytes of *L. baikalensis*. Staining with methylene blue of SA1-cells is shown to depend on cell viability and the presence of LAS in aquarium water. Staining of SA2-, SA3-, and SA4-cells is nonselective and useless to revealing LAS pollution. The following variants of sponge cell staining took place:

1. The SA1-cells of the healthy control sponges *L. baikalensis* did not dye with methylene blue. Cells of symbiotic microalgae *Chlorella* sp. were not stained with methylene blue either. On the contrary, the amoebocytic cells of other types as well as endosymbiotic prokaryotes Cyanophyta stained with methylene blue. Instant staining of SA2-, SA3-, SA4-, and Cyanophyta cells occurs due to absence of enzymes that suppress the methylene blue effect.
2. The SA1-cells and *Chlorella* sp. cells exposed to 10 μg L^{-1} LAS solution for two weeks also did not stain as well as the control sponge cells. On the contrary, the amoebocytes of other types and blue–green algae cells stained immediately.
3. The SA1-cells and endosymbiotic *Chlorella* sp. cells exposed to 20 μg L^{-1} LAS solution for 48 and 72 h, accordingly, became blue in color. The SA2-, SA3-, SA4-, and Cyanophyta cells stained immediately. Flagellum motility of SA4-cells and endosymbiotic flagellates was noted during the first minutes after LAS being added into the aquariums.

Thus, the *L. baikalensis* SA1-cells, containing endosymbiotic green algae *Chlorella* sp. were selected as the indicator of LAS pollution of water and used to carry out the toxicity testing of synthetic anionic surfactants to evaluate their effect on the sponge.

3.3. Control Sponges Cell Viability

Using light microscopy, it was shown that healthy SA1-cells of the control sponges were not dyed with methylene blue (n = 12). Healthy symbiotic algae *Chlorella* sp. did not stain with methylene blue either. On the contrary, the amoebocytic cells of other types vis. SA2-, SA3-, SA4-cells as well as endosymbiotic prokaryotes Cyanophyta stained with methylene blue instantly.

The percentage of unstained live SA1-cells was estimated in the range from 82 to 95 (n = 3) and from 95 to 100 (n = 9) and did not decrease after 10-day cultivation in clean water. Control sponges were used for further experiments.

3.4. Cellular Stress as a Response to Toxic Effect of Linear Alkylbenzene Sulfonates

The acute toxicity of alkylbenzene sulfonates at 10 μg L^{-1} concentration on *L. baikalensis* was not revealed. The percentages of the live cells were the same in the beginning of the experiment and in its ending. The SA1-cells and *Chlorella* sp. cells stained with methylene blue like the control sponges in 1, 2, 3, 7, and 14 days of exposure. No significant changes in FA composition were noticed after two-week-term 10 μg L^{-1} LAS impact on *L. baikalensis*. The malondialdehyde was not revealed in *L. baikalensis* samples after 1, 2, 3, and 7 days of 10 μg L^{-1} LAS exposure.

Cellular stress as a response to the toxic effect of alkylbenzene sulfonates at 10 μg L^{-1} concentration on *L. baikalensis* was revealed after 14 days of anionic surfactant exposure. The oxidation stress was accompanied by malondialdehyde formation. Its content was rated from 0.16 ± 0.06 to 2.0 ± 0.7 μg g^{-1} of dry weight (d.w).

3.5. Acute Toxicity of Linear Alkylbenzene Sulfonates to Lubomirskia baikalensis

3.5.1. Cell Size and Shape Changes: Cell Viability

We revealed acute toxicity of LAS to *Lubomirskia baikalensis* at 20 μg L^{-1} concentration, which already appeared in the first hours of the influence. This toxic effect accompanied the drastic biochemical and physiological changes in *L. baikalensis* as a whole.

It was shown that healthy control sponge SA1-cell sizes (diameters) varied from 10 to 25 μm. These SA1-cell sizes increased up to 1.5 times after 48–72 h LAS exposure. Their values ranged from 15 to 35 μm. A lot of lysed SA1-cells–up to 97%–were found (Table 1). The increased number of larger symbiotic algae cells was found after LAS treatment. Their percentage increased from 17.5 to 32.1 (Table 1).

Table 1. Sponges and symbiotic algae cell sizes before and after anionic surfactant exposure.

Organisms					
***L. baikalensis* SA-1 Cells**			**Endosymbiotic Algae *Chlorella* sp. Cells**		
Cell Size, μm	**Cell Percentages (of the Sum)**		**Cell Size, μm**	**Cell Percentages (of the Sum)**	
	Control	**48 h LAS Exposure**		**Control**	**48 h LAS Exposure**
10.0–15.0	17.9	0.0	2.0–2.5	27.5	3.6
15.0–20.0	38.5	0.5	2.5–3.0	20.0	35.7
20.0–25.0	38.5	1.0	3.0–3.5	30.0	28.6
25.0–30.0	5.1	0.8	3.5–4.0	17.5	32.1
30.0–35.0	0.0	0.5	4.0–4.5	5.0	0.0
35.0–40.0	0.0	0.2	4.5–5.0	0.0	0.0
Lysed cells	0.0	97	Lysed cells	0.0	0.0

Changes in the shape of the cells from round to elongated were fixed (Figure 3C,D). The swelling and the subsequent lysis and death were observed. During 10 h of exposure, ~60% of the SA1-cells died and were stained with methylene blue. During 48–72 h of exposure ~97–100% of SA1-cells died (number of sponges n = 5) (Figure 3C,D and Figure 4A). When host cells disrupted, the symbiotic organisms moved out (see Figure 3D). Therefore, mass mortality of green algae was noted. During 72 h of exposure, 100% *Chlorella* sp. cells died (number of sponges n = 3) (Figure 4B). Death algae stained with methylene blue (Figure 4C).

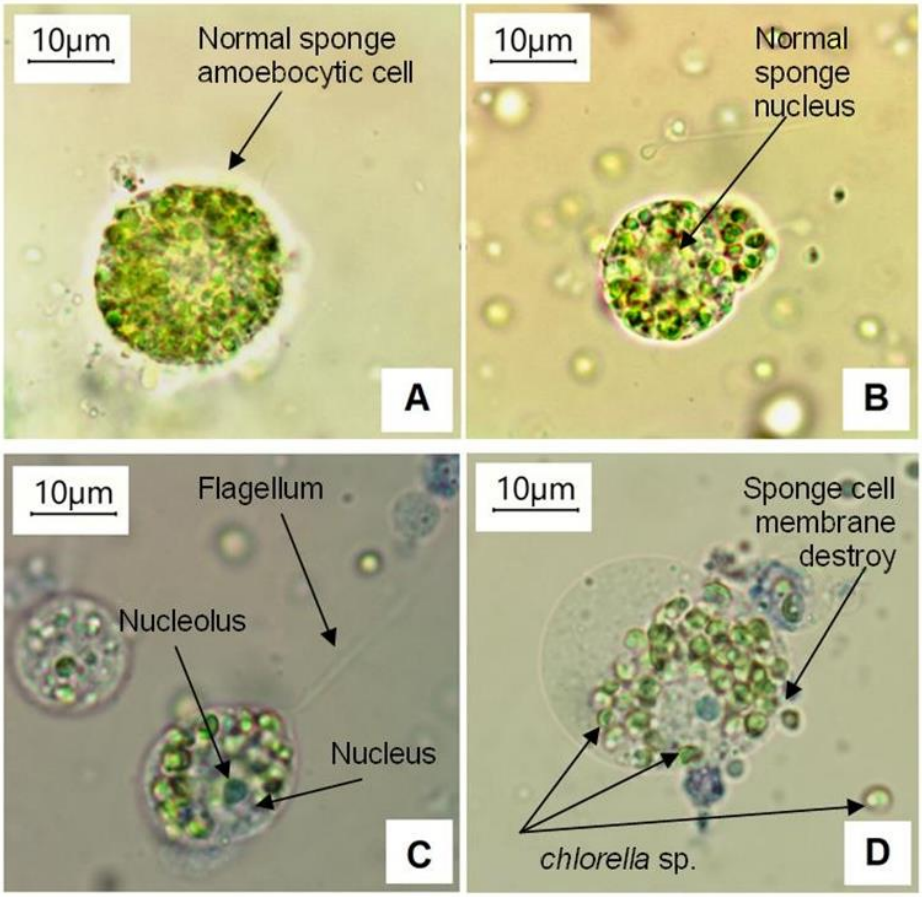

Figure 3. *Lubomirskia baikalensis* cells. (**A**)–normal SA1-cell of 21.7 µm size; (**B**)–normal SA1-cell of 16.1 µm size; (**C**)–swelling of SA4-cell with flagellum; a cell stained with methylene blue; (**D**)–SA1-cell of 27.8 × 23.9 µm size swelled and lysed due to linear alkybenzene sulfonate 72 h exposure (20 µg L^{-1}); the cell stained with methylene blue; symbionts *Chlorella* sp. moving out of the host cell.

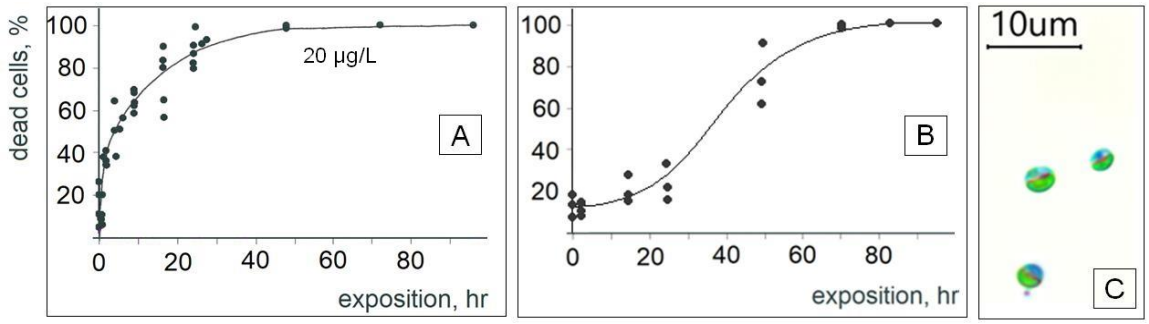

Figure 4. Cell death curves. (**A**)–*Lubomirskia baikalensis* dead SA1-cell percentages increase in dependence on duration of 20 µg L^{-1} linear alkylbenzene sulfonates exposure; (**B**)–*Chlorella* sp. dead cell percentages increase in dependence on duration of 20 µg L^{-1} LAS exposure; (**C**)–dead symbiotic *Chlorella* sp. cells stained with methylene blue for 72 h exposure.

3.5.2. *Lubomirskia baikalensis* Fatty Acid Composition and Content Changes

Nineteen dominant FAs were identified for all individuals of control sponges. Percentages of saturated fatty acids (SFA) ranged from 24 to 25. Percentages of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFAs) including demospongic fatty acids (DSFA) ranged from 32 to 34% and from 43 to 44%, respectively (Figure 5A, left). These values are similar to the literature data for healthy individuals of *L. baikalensis* sampled in 1990 [49]. Total FA content of control sponge was ~90 ± 9 μg g^{-1} of the dry weight (of d.w.) (Figure 5B, left).

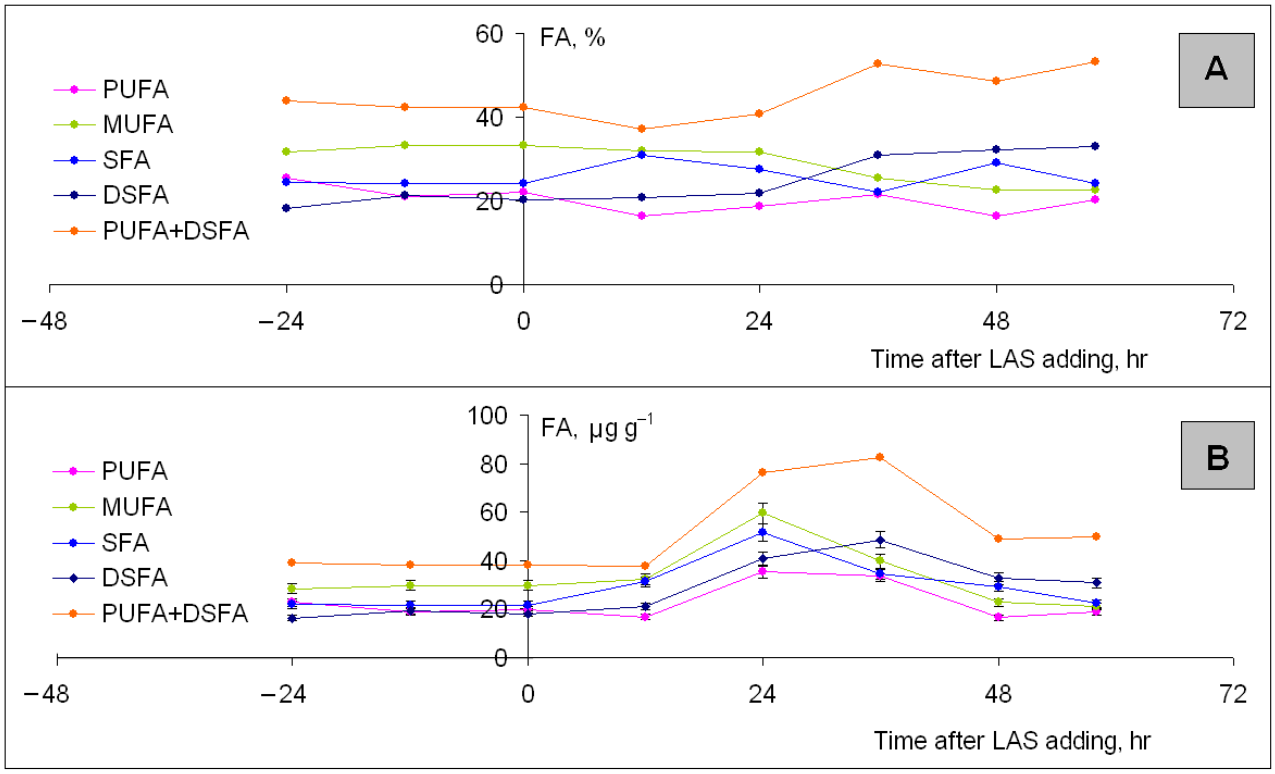

Figure 5. *Lubomirskia baikalensis* fatty acid composition and content changes. (**A**)—fatty acid composition (%) including saturated, monounsaturated, polyunsaturated, and demospongic acids before anionic surfactant exposure (in the left of the vertical axis) and after linear alkylbenzene sulfonate (LAS) addition into aquarian water (in the right of the vertical axis) to achieve an LAS concentration of 20 μg L^{-1}; (**B**)—fatty acid content (μg g^{-1} of d.w.) before and after LAS addition.

Significant changes in FA composition within 24–48 h after LAS addition (Figure 5A, right) into aquarian water were fixed. Percentages of SFA, MUFA, and the sum of PUFA and DSFA ranged within 22–30, 23–32, and 37–53, respectively (Figure 5A).

Quick increases in SFA, MUFA, and PUFA production including DSFA were also noticed (Figure 5B). The total FA content increased up to 187 ± 13. The sum content of PUFA and DSFA increased from 40 to 80 μg g^{-1} of d.w. within 24 and 36 h. Thereafter, it decreased to a level somewhat higher than the initial one. An increase in PUFA percentages was fixed within 24, 36, 48, and 58 h as well. An increase in SFA and MUFA percentages was fixed within 24 h.

3.6. In Situ Experiments: Analysis of the Environmental Samples

3.6.1. Oxidative Stress of *Lubomirskia baikalensis* in Lake Baikal

Two lipid peroxidation markers were analyzed viz. MDA concentration (C_{MDA}) and PUFA percentages to reveal oxidative stress of frozen sponges collected at the sampling sites No. 2 and No. 3 in Lake Baikal (Table 2). The MDA was not revealed in a number of sponges (n = 9). Percentages of PUFA in these sponges were 42–43 (Table 2).

Table 2. Lipid peroxidation in *Lubomirskia baikalensis* collected in Lake Baikal from two sampling sites of different anthropogenic load in 2019.

Sample Site No.	Depth of Sampling in Lake Baikal, m	Lipid Peroxidation Markers	
		C_{MDA}, μg g^{-1} of d.w.	PUFA, %
2	5	from 0.05 to 0.53 average 0.18 ± 0.06	33 (*n* = 7)
	20	not detected	43 (*n* = 3)
3	5–15	from 0.01 to 0.41 average 0.20 ± 0.07	32 (*n* = 4)
	5–15	not detected	42 (*n* = 6)
Control sponges after 10 days cultivation in clean water	5–15	not detected	42–55 (*n* = 12)

Concentration of MDA rated from 0.010 ± 0.004 to 0.53 ± 0.19 μg g^{-1} of d.w. in other sponges (*n* = 11) collected at the same stations (Table 2). The PUFA percentages for that were 1.3 times lower (Table 2).

MDA was not found in control sponges (*n* = 12) after 10 days cultivation in clean water. Percentages of PUFA in these individuals were the highest and achieved 55.

3.6.2. Antioxidant Capacity of *Lubomirskia baikalensis*

Low values of $ABTS^{\bullet+}$ inhibition (I) by crude extracts of frozen sponges collected at the sampling sites No. 4, 5, 6, 7, and 8 in southern and northern basins of Lake Baikal were noted. High IC_{50} values indicated low antioxidant capacity (AOC) as well (Table 3). The AOC values were ranged from 0.00031 to 0.00077 Trolox equivalents (TE). The lowest values of 0.00031 and 0.00040 were found in sponges collected at the site No. 4.

Table 3. Inhibition (I), half maximal inhibitory concentration (IC_{50}), and antioxidant activity (AOA) of *L. baikalensis* crude extracts and standard (Trolox).

Sample Site No.	Sampling Sites	Sample (Sponge) No.	I, %	IC_{50}, mg L^{-1}	AOC, TE
4	Ulanovo Cape	1	0.0064 ± 0.0003	7808 ± 390	0.00040
		2	0.0059 ± 0.0003	8542 ± 427	0.00031
5	Region of inoperative Baikalsk City paper mill	3	0.0140 ± 0.0007	3574 ± 179	0.00075
		4	0.0133 ± 0.0007	3770 ± 189	0.00064
		5	0.0084 ± 0.0004	5972 ± 299	0.00044
6	Bolshoi Ushkanii Island	6	0.0129 ± 0.0006	3889 ± 194	0.00071
		7	0.0110 ± 0.0006	4529 ± 226	0.00065
7	Elokhin Cape	8	0.0149 ± 0.0007	3360 ± 168	0.00077
		9	0.0111 ±0.0006	4491 ± 225	0.00062
8	Tompuda Bight	10	0.0140 ± 0.0007	3580 ± 179	0.00071
		11	0.0089 ±0.0004	5628 ± 281	0.00046
2	Control healthy sponge after 6 months exposure in clean aquarian water	12	0.0160 ± 0.0008	3125 ± 156	0.00062
	The same sponge treated with 20 μg L^{-1} LAS for 72 h	12	0.0122 ± 0.0006	4098 ± 205	0.00081
	Trolox (standard solution)		19.7 ± 1.0	2.53 ± 0.13	1.00000

Low values of $ABTS^{\bullet+}$ inhibition and AOC by crude extracts of live control sponge that lived in the aquarium for six months at the moment of AOC analysis were also fixed (Table 2). Mentioned low values were close to that for the same sponge treated with 20 μg L^{-1} LAS for 72 h.

3.7. Statistical Data

To determine sponge humidity, each sample was analyzed in two replicates. To assess the ability of the method to generate similar results for multiple preparations, the repeatability was measured and characterized by variation coefficient V = 2%.

To determine the total fatty acid content, each sample was analyzed in two replicates as well. The repeatability of the technique was characterized by V = 6%.

To assess the antioxidant capacity, two pieces were cut from the sponge top and from the sponge foot. Pieces were extracted and analyzed. The coefficient of variation was V = 5%.

To determine MDA content, each sponge sample was analyzed in three replicates. The variation coefficient was characterized by the value of 25%.

To conduct the cell viability test and to count the number of live/dead cells, each sample of sponge cell suspension was analyzed in two–four replicates and 10–12 fields of view were screened. The total number of blue stained SA1-cells and symbiotic *Chlorella* sp. cells as well as unstained green cells achieved 230 for SA1-cells and 500 for symbiotic algae cells. The repeatability was characterized by V = 15%.

4. Discussion

4.1. Toxicity Test Protocol

In this study, we propose the toxicity test protocol to evaluate adverse effect of short-term exposure of linear alkylbenzene sulfonates at low concentrations (10 and 20 μg L^{-1}) on freshwater sponge *Lubomirskia baikalensis*. The experiments were carried out with the use of live multicellular invertebrates exposed in aquariums under conditions close to natural in Lake Baikal. Symbiotic microalgae *Chlorella* sp. were studied, excluding their extraction from the host organism and cultivation in vitro. This allowed us to reflect the LAS effect on a whole sponge organism.

Sampling cell suspension of ~50 μL volume from live *L. baikalensis* was proposed to exclude serious damage to the target animal. This made multiple replicate analysis in short time intervals to evaluate the cell viability possible. Low-temperature (~8–12 °C) inverted microscopy with light-emitting diodes (LED)-transmitted illumination of live cells allowed us to elongate the duration of microscopy of a sample by up to 3 min. Incandescent lamps, on the contrary, increased the temperature with the following cell swelling during the first minute of analysis that led to errors of up to 60%.

The sponge cell classification suggested by the authors allows us to assign the nucleolar sponge amoebocytes of the first type (SA1-cells) containing symbiotic microalgae *Chlorella* sp. as indicator cells to evaluate the toxic effects of surfactants. Enzymes of live eukaryotic cells containing nuclei with nucleoli are known to oxidize the methylene blue and suppress its staining effect [50]. This explains the absence of staining of live *L. baikalensis* SA1-cells and symbiotic *Chlorella* sp. (see Figure 3A,B). In turn, the absence of methylene blue oxidizing enzymes in prokaryotic cells results in the blue staining of live sponge SA2-, SA3-, SA4-cells and symbiotic Cyanophyta cells. Therefore, live SA1-cells were not stained with methylene blue, and the dead ones were stained, which reflects the acute toxic LAS effect on *L. baikalensis*.

The large size of SA1-type-cells makes the viability test procedure easy, repeatable, and effective. Thus, *L. baikalensis* and symbiotic *Chlorella* sp. can be used as bioindicators or biomonitors to evaluate the ecosystem state of Lake Baikal. The proposed toxicity test protocol includes a cell viability test, fatty acid analysis, and probable oxidation stress estimation. It can be used to evaluate anthropogenic impact level at regional and global scales in the interest of ecological health and biodiversity conservation.

4.2. Linear Alkylbenzene Sulfonate Acute Toxicity Effect

This study presents the results of acute toxic 20 μg L^{-1} LAS effect on organisms of two different kingdoms: Animalia and Plantae. This effect caused 97–100% sponge *L. baikalensis* cell death in less than 48 h (see Figure 4A) and 100% symbiotic microalgae *Chlorella* sp. cell death over 72 h (see Figure 4B). Changes in sizes of the sponge amoebocytes (SA1-cells) containing the eucaryotic symbionts *Chlorella* sp. as well as symbiotic cells have been revealed. It was noted that SA1-cells' volume increased up to 1.5 times and more. Therefore, cell lysis occurred as a result of cell swelling and osmotic imbalance at a critical volume (see Table 1).

A close toxic effect of 20 μg L^{-1} LAS has been found [51] on different marine microalgae such as *Tetraselmis chuii* (division Chlorophyta, class Chlorodendrophyceae, family Chlorodendroceae), *Rhodomonas salina* (division Cryptophyta, class Cryptophyceae, family Pyrenomonadaceae), *Chaetoceros* sp. (division Ochrophyta, class Bacillariophyceae, family Chaetocerotaceae), *Isochrysis galbana* (division Haptophyta, class Prymnesiophyceae, family Isochrysidaceae), and *Nannochloropsis gaditana* (division Ochrophyta, class Eustigmatophyceae, family Monodopsidaceae). The inhibition of cell growth and the general tendency to increasing cell volume/size were revealed [51].

A report on acute toxic influence of LAS solutions at the same concentration of 20 μg L^{-1} on *Chlorella ellipsoidea* (division Chlorophyta, class Trebouxiophyceae, family Chlorellaceae) was published [52]. The strongest ability of LAS to decrease lipid content in the thylakoid membranes was shown. Changes in cell membrane permeability, metabolism, and growth interruption were found [52]. The literature's data testify that 20–100 μg L^{-1} LAS concentrations are the most harmful to aquatic organisms [36,37,51–54].

According to the results (see Figure 4A), the SA1-cells' mortality curve exhibits steep slope due to cell death at the beginning of the experiment. The small plateau and the gentle slope on the microalgae mortality curve (see Figure 4B) until 24 h of LAS exposure corresponds to the moment of sponge SA1-cell death (see Figure 4A). The plateau and the gentle slope at the start of the experiment till 24 h reflects viability of microalgae inside the host cells, which protect them from a hazardous impact (see Figure 4B).

Nonspecific adaptation response of the cell and the whole organism is the response to a stress factor that is common for different organisms. This response aims to restore the homeostasis of a live system. A normal live cell surrounded by a liquid cell membrane with a lipid bilayer ~4–7 nm thick tends to its state of minimum free energy and minimum surface according to Le Chatelier's principle. Aggregation of LAS on a cell surface disrupts the bilayer reducing surface tension. An imbalance occurs with the environment [3,55–58]. A prolonged, intensive, or recurrent effect results in adaptation malfunction and potential exhaustion of a cell membrane.

A visual example of a nonspecific adaptation response of *L. baikalensis* to LAS impact is the sharp change in fatty acid content within 24 h of exposure (see Figure 5B). Let us compare Figures 4B and 5B. One can see the correlation between the beginning of symbiotic algae death (Figure 4B) and increase in FA content up to two times (Figure 5B). We assume that this increase is caused by *Chlorella* sp.'s ability to trigger the adaptive mechanism and to produce FA more intensively. As supposed, SFA and MUFA production decreases within 36 h of exposure due to nutrient deficit. The cell membrane potential is exhausted. PUFA production decreases within 48 h due to lack of SFA and MUFA as PUFA synthesis raw materials. Thus, 20 μg L^{-1} LAS concentrations found in water of the nearshore zone can cause the mass morbidity effect on Porifera in Lake Baikal.

4.3. Oxidative Stress as a Response to LAS Toxic Effect: In Situ and In Vitro Investigations

For the first time, we found the oxidative stress of some individuals of *Lubomirskia baikalensis*, collected in situ in Lake Baikal (see Table, Figure 1, sample sites No. 2 and 3), but for some others, the stress was not revealed. The pollution of aquatic ecosystems by xenobiotics and the absence of adaptation of water dwellers to their impact are acute problems for the 21st century. Some of the most persistent micropollutants in aquatic

ecosystems such as polycyclic aromatic hydrocarbons (PAH) [59], heavy metals [60], and anionic surfactants including alkylbenzene sulfonates [35,36] are able to induce oxidative stress and hypoxia of the cell and organism as a whole. The oxidative stress of freshwater organisms can occur due to species sensitivity to these toxicants.

Our first results of in vitro experiments have shown the oxidative stress of *L. baikalensis* as a response to toxic effect of alkylbenzene sulfonates at 10 μg L^{-1} concentration after 14-day exposure. The oxidation stress is accompanied by malondialdehyde formation. Alkylbenzene sulfonates are the most widespread pollutants for water ecosystems. The mechanism of the effect of these substances is associated with the induction of a negative charge of the cell membrane. When redistributing the charge, the process of lipid peroxidation under free radical attack is activated [3,53]. One of the final products of the attack is MDA, which is a marker of oxidative stress [55,61,62]. The cell wall of *L. baikalensis*, as well as many other organisms of cold water oligotrophic ecosystems, contains a high percentage of unsaturated fatty acids (see Figure 3, suppl. Table S1), which are the most suitable target for the attack of free radicals. We observed the correlation of MDA presence in stressed sponges with the decrease of PUFA content (see Table 2). The same situation for Lake Baikal phytoplankton was found in previous work [23].

The high sensitivity of *L. Baikalensis* to the hazardous LAS exposure can be related to its very low antioxidant capacity (TEAC) from 0.00031 to 0.00077 Trolox equivalents (see Table 3) and low ability to adapt. These values are up to 10–110 times lower than the TEAC for marine species (see Table 4). The antioxidant capacity of *L. Baikalensis* dry crude extract is responsible for the sponge ability to scavenge free radical attack. The high values of IC_{50} in *L. Baikalensis* also confirm the low sponge ability to resist the oxidation stress factor and to adapt to the oxidant impact. Inattention to the problem of anionic surfactants in freshwater ecosystems and the incorrect choice of toxicity test protocol with the use of testing animals that have not highest responsiveness to the hazardous impact of toxicants can lead to dramatic situations for sponge populations and their associated flora and fauna in particular.

Table 4. $ABTS^{\bullet+}$ antioxidant activity for some marine sponges in comparison with freshwater Lake Baikal sponge *Lubomirskia baikalensis* and with trolox.

Sponge [Ref.]	Type: Marine/Freshwater	IC_{50}, μg mL^{-1}	I (%)	TEAC
Lubomirskia baikalensis with maximal TEAC (present investigation)	freshwater	3125	0.0160	very low
Lubomirskia baikalensis with minimal TEAC (present investigation)	freshwater	8542	0.0059	very low
Tedania ignis [63]	marine	no data	0.135 *	moderate
Niphates erecta [63]	marine	no data	0.184 *	moderate
Callyspongia vaginalis [63]	marine	no data	0.265 *	moderate
Lissodendoryx carolinensis [63]	marine	no data	0.325 *	moderate
Tetilla rodriguesi [64]	marine	297	0.327 *	moderate
Amorphinopsis atlantica [63]	marine	88	0.531 *	high
Ircinia felix [63]	marine	89	0.608 *	high
Mycale microsigmatosa [63]	marine	60	0.669 *	high
Standard (Trolox)		2.5	19.733 *	very high

* The data recalculated from 100 ppm to 1 ppm to universe them for different investigations.

5. Conclusions

A new toxicity test protocol under conditions close to natural was proposed. It has good potential for reliable estimation of the anionic surfactant toxicity because of it using an in vivo assay and testing sponges of high responsibility, and it is applicable to a wide variety of water pollution problems. The sponge amoebocytes named SA1-cells that contain eukaryotic microalgae *Chlorella* sp. are shown to be the representative indicator in assessing the impact of anionic surfactants. Toxic effects of linear alkylbenzene sulfonates on *L. baikalensis* and its symbiotic microalgae *Chlorella* sp. was found at low concentrations in in vitro experiments. The acute toxic effect of LAS was noticed at 20 μg L^{-1}. Oxidation stress of the sponges in response to the LAS effect at 10 μg L^{-1} was revealed. For the first time, we revealed the oxidative stress of *L. baikalensis* in situ in Lake Baikal. The stress may be a result of long-term pollutant effects coupled with the low-antioxidant activity of *L. baikalensis*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15010077/s1, Table S1: Fatty acid (FA) profiles of controlled individual of Lubomirskia baikalensis as well as of the same sponge individual treated with 20 μg L^{-1} LAS solution.

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