
ARTICLES

*In a word, I have become cognisant of many, many things...
but still I am not yet satisfied. My spirit still thirsts for further knowledge...
St. John of Kronstadt*

Determination of Free and Esterified Fatty Acids in Hydrocoles of Different Content of Polyunsaturated Fatty Acids by Gas–Liquid Chromatography

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Abstract—This paper presents a new technique of hydrocole (bacteria, blue–green algae, phytoplankton, zooplankton, sponges, fish oil and seal fat) fatty acids (FA) methyl esters determination by gas-liquid chromatography. The polyunsaturated fatty acids (PUFA) contents in these objects were different. The distinguishing features of the technique are the next ones: efficiency, lipid extraction recovery $\geq 94\%$ using the sonication during 10–15 min without sample drying, high PUFAs yield ($\geq 97\%$) by reduction of oxidation up to 40% during methylation; exhaustive extraction of fatty acid methyl esters (FAMES) including PUFA; a new approach to obtain of methyl esters of free fatty acids except using toxic reagents; accuracy of FAMES quantification including ω -3-, ω -6-, ω -9-FA using the di-*n*-decyl ether as an internal standard and accuracy of measurements using “T14165QC Fish oil” standard sample (first). The technique is appropriated for Baikal hydrocole FA determination from 20 to 2000 μg in the sample and might be used for scientific and applied tasks. The interlaboratory precision of FAME determination of phytoplankton and bacteria is $\leq 10\%$.

Keywords: gas chromatography–mass-spectrometry (GC–MS), flame-ionization, fatty acid methyl esters (FAMES), free fatty acids (FFAs), polyunsaturated fatty acids (PUFAs), Lake Baikal, phytoplankton, bacteria, blue–green algae (cyanobacteria), zooplankton, sponge, fish oil, phoca (Baikal seal)

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Fatty acids (FAs) are aliphatic monobasic carboxylic acids found in environment in oils, fats and waxes of natural origin. These acids may be both free (FFAs) and bonded or esterified (EFA). The water ecosystem organisms contain wide spectra of FAs including saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), essential, branched, cyclic fatty acids and others. For protozoan (bacteria, yeast) linear SFAs such as palmitic C16:0 and stearic C18:0, branched FAs and monounsaturated oleic C18:1 fatty acid [1–5] are dominant. Blue–green algae of some species are able to synthesize MUFA and PUFA abundantly ($>60\%$) [6–9]. There are catalogues of bacterial FAs spectra sys-

temized to carry out taxonomical investigation. The role of the free FAs for vital functions of bacteria, the mechanism of synthesise of these substances and their localization in the cells are not absolutely clean yet. At the opposite, other lipid bioactivity (for example, glycolipids antibiotic activity) is evident. The qualitative analysis of bacteria FAs may be helpful for understanding the metabolism processes of organisms of higher trophic levels. For instance, the investigation of lipid contents of bacteria and microalgae of symbiotic assemblage of the freshwater sponges of Lake Baikal make clear the causes of mortal fall of Baikal sponges. The FA composition of phytoplankton is formed by metabolic features and due to abiotic [1, 10–13] as well as biotic factors [14, 15]. Phytoplankton and ground plants are primary producers of essential FAs

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such as eicosatetraenoic acid C20:4 ω -6, eicosapentaenoic acid C20:5 ω -3 and docosahexaenoic acid C22:6 ω -3 where ω characterizes the double bond position relative to the alkyl chain of the molecule [16, 17]. These FAs have an effect on energetic cell potential and cell function and [16, 24–26] are transferred through the food chains [18–23]. Specific FAs composition of sea and freshwater hydrocoles may give information about organic matter transfer through transitional trophic levels from primary producers such as phytoplankton to consumers such as fishes. The FA composition of the human foodstuff is responsible for the quality of the production. The FA composition of the primary producers such as Lake Baikal endemic diatoms which are most sensitive to any environment condition changes may mark these environmental changes of expanded anthropogenic load. The FAs are produced by microalgae are consumed by heterotrophic bacteria. So, it is supposed that quality and quantitative concentration of phytoplankton FA have an influence on number of species and composition of bacteria of Lake Baikal.

So, the obvious composition and quantity of FAs of different objects as well as the goal of FAs determination give the analytical approach of FAs determination [17].

The direct determination of oleic and linoleic acids of vegetable oils by high performance liquid chromatography (HPLC) using the reversed phase columns and refractometer is known as the express-method of analysis [27]. The determination of acid number (Σ FFA) by alkali-, iodine- and pH-metric titration is used both for oil and fat analysis [28–33].

The FAs extraction from complicated matrices of petroleum and sediments may be carried out according to the Folch method [34] including the modified methods [35, 36] such as the Folch extraction coupled to vibromagnetic treatment which provide the better recovery of analyzed substances in the form of micelles. The following saponification of FAs by alkali solution in alcohol [37–39] and adsorption of the impurities using the ion exchanger, silica gel modified by potassium silicate and potassium hydroxide provide the selective isolation of petroleum fatty acids [34].

For the determination of the trace amounts of fatty acids in water, the method of saponification of FAs by alkali solutions with following solid-phase extraction of methyl esters on reversed phase columns is used [38, 40].

The method of extraction alkylation [41, 42] of FAs is suitable for biological fluids such as blood plasma and urine. The alkaline transesterification of esterified FAs with following alkylation of the free FAs by iodomethane with CH_3I in presence of hydroxide tetrabutylammonium catalyst $\text{N}(\text{C}_4\text{H}_9)_4\text{OH}$ at room temperature in rather soft conditions takes place. The technique allows determining free FAs for diagnostic of the FAs metabolism pathology. The weakness of the

technique is in the iodomethane toxicity and necessity of purify pigmented extracts.

The binary mixtures of polar and nonpolar solvents such as chloroform and methanol according to modified Folch method [37, 49–51] and Bligh–Dyer method [39, 49] of lipid extraction from bacteria biomass [2–8, 16, 43], phytoplankton [11–13, 17, 44, 45], zooplankton [18, 19], fishes [20, 22, 24], sponges [46–48] as well as using mixture such as propan-2-ol–cyclohexane–water under sonication and heating [17] are widely used. The Folch method as described using the chloroform-methanol mixture (2 : 1 by volume) give the best recoveries in the case of lipid content is $\geq 2\%$ [49]. They also say that there is ability to exchange the chloroform to dichloromethane with less toxicity [17, 52]. The reduction of extraction time (4–12 h and more) from bacteria which do not contain PUFAs may be carried out using the lyophilic drying of the samples [53]. Soxlet extraction is also of long-duration (4–15 h and more) and results in PUFAs destruction. Supercritical fluid extraction by carbon dioxide at 40°C and 15 MPa is nontoxic, no destructed but less effective (up to 12 h duration) [17, 54]. The sonication (19 kHz, 65 W, 1 h) at 45°C by *n*-hexane and the following microwave extraction (2.5 GHz, 0.5–1 h) [55] is more effective (12–25% recovery) compared with Soxlet extraction (2–5% recovery). There is also noted that the increasing of sonication rate from 19 to 300 kHz decreases the lipid recovery twice.

High polarity and low volatility of FAs do the direct FAs GC-determination difficult. Heating of FAs in the injection port results in their thermal and thermocatalytic dehydration and decarboxylation. It is mostly concerns ω -3 PUFAs in which bisallylic carbon atom coupled to the double bond has low activation energy for dehydration and to free radical formation [56]. Change of polar carboxylic group to less polar group through the alkylation or silylation [40] allow us to avoid FAs destruction, increase the volatility of the substances, give more useful mass-spectrometric information and increase the accuracy of peak identification, sensitivity, selectivity and do chromatographic separation using nonpolar and weak polar column phases better. Gas-chromatographic FAMES determination using flame ionization detection (FID) [57, 58] is well known from 1930s. The silylation provides determination of hydroxyl substituted acids and cyclic acids.

The methylation agents are usually added to dry extract [17, 59] or to lipid solution. There are alkaline and acidic methylation agents. Lipid esterification by methanol under presence of hard alkali [50, 51, 53, 57, 60] (saponification) is expressible (up to 2 min at normal condition) and allows us to get esters of triacylglycerides, glykolipids and phospholipids and other but results in PUFAs destruction and does not allow us to get free FAs esters.

There is well known way to methylation of free FAs by changing pH through adding Lewis acid, for example, boron trifluoride [45, 50, 58, 61]. Hard conditions of reaction temperature (up to 60–100°C), PUFAs destruction, formation of reaction by-products, BF₃ toxicity are serious method drawbacks. There are R'CHN₂, R'Cl, (CH₃)₄NOH, (CH₃)₃OBF₄ also known but diazomethane may be easily synthesized laboratory [62, 63]. The reagent toxicity, high duration, and laboriousness are the main disadvantages of using of these reagents. Temperature increasing or microwave exposure does the process faster due to energy activation decreasing [58]. The sodium methoxide is used for fish free fatty acid esterification [59, 64]. For fish free fatty acid esterification the sodium methoxide is used. For example the FAMES oils and fats with acid number ≤2 using sodium methoxide is State Standard for Russian Federation [64]. Also this method may be used for phytoplankton oil [65] but it is not effective because of the interaction between sodium methoxide and other sample components interaction.

Esterification and transesterification of free FAs and esterified FAs take place at 50–170°C by C1–C5 spirits treatment in acidic medium [50, 66] and it is a key moment why this method is chosen. In this case 5%-fluid HCl [2, 50], 35% HCl solution [60] and 1–5% H₂SO₄ solution [50, 52, 66, 67] are used. The duration up to 12 h, PUFAs oxidation at concentration of H₂SO₄ ≥ 10% to the reaction accelerate as well as HCl toxicity and decrease of the reaction rate when there are any water amounts in the system are major disadvantages of the method. The obtained FAMES are extract with *n*-hexane (up to 30 mL) [1, 53, 52] and are determine by gas chromatography using flame ionization detection (GC–FID) or mass-spectrometric detection (GC–MS) [1, 38, 50, 52, 65, 68, 69].

The technique proposed here has been developed for FAMES determination of complex biological Lake Baikal hydrocole samples of wide lipid concentration range from 0.7% for phytoplankton to ≥97% for Baikal seal fat, FAs content from 20 to 2000 µg, PUFAs percentage including essential ω-3-, ω-6-, ω-9-FAs from 0% (bacteria) to 60% (sponges) and free FAs percentage from 0.3% (sponge) to 40% (phytoplankton). The technique is universal for different types of hydrocoles and provides the minimal PUFAs losses during their esterification, the simplicity and the efficiency.

EXPERIMENTAL

Sampling. The *PB15/Grf7geo Geobacillus* sp. thermophilic facultatively-anaerobic strain biomass, isolated from low-temperature Lake Baikal sediment layer was taken from Lake Baikal in the Posolskaya Banka region with gaseous fluid methane source in 2016 and was obtained by the cell culture growing in modified *Widdel* growth medium for freshwater

organisms [70]. The *Bacillus* sp. 9A, 2A, 2B heterotrophic bacteria strains isolated in 2017 from Lake Baikal littoral zone (Berezovjy Cape) hard substratum biofilms were obtained by the cell culture growing in the glucose medium. The *Synechococcus* sp. *Bf2* blue-green algae strain isolated from Lake Baikal bottom biofilms (the region of the Straits of Olkhonskie Vorota) was obtained by the cell culture growing in the mineral Z-8 medium. The biomass has been centrifuged at 3000 rpm for 15 min.

The Lake Baikal phytoplankton samples were collected in 2014–2018 during May and September from different Lake Baikal basins by a vertical *Jedi* type net with 100 µm mesh size in horizons 5–50 m. Some samples were also collected by divers from under the Baikal ice using syringe sampler. All water samples passed through cellulose acetate filters (45 µm, VLADiSART, Russia) using the filter-apparatus with 1000 mL flask (Duran Group, Germany). The sample humidity was calculated according to gravity measurements. The samples containing filamentous green algae with cellulose as a major component of the cell wall were grinded in porcelain mortar. The microscopic analysis was carried out using the Carl Zeiss Axiover 200 (×400) Microscope System.

The Lake Baikal zooplankton samples (*Epishura baicalensis*, *Macrohectopus branickii* and *Daphnia*) were collected in 2014 and 2016 during September and October from different Lake Baikal basins.

The seal fat (*Phoca sibirica*, 2018) was presented by Doctor of chemical sciences Annenkov V.V. (Limnological Institute (LIN) of SB RAS). The “Fish oil” samples (salmon fish oil with ω-3-FAs content ~300 mg/g according to documentation, Israel) was presented by PhD Gorshkov A.G. (LIN SB RAS).

The Lake Baikal sponge *Lubomirskia baicalensis* samples were collected by divers in 2016 and, 2018 and also were taken from the aquarium of the Freshwater Experimental Aquarian Complex of Lake Baikal Hydrocoles of the Limnological Institute of SB RAS.

Lipid Extraction. Before lipid extraction the phytoplankton and bacterial biomass were defrosted and homogenized. Zooplankton samples were grinded in the mortar. The cell content of the sponges was wringed out. The liquid content of sponge cells, seal fat melted at 25°C and fish oil were taken with mechanical doser. The *Folch* mixture (chloroform–methanol, 2 : 1, by volume) was used to extract lipids from replicate samples in plastic microcentrifuge 2 mL tubes of Eppendorf type by shaken and sonication (1.2 mL × 3 × 5 min). Three obtained extracts were combined in glass centrifuge test-tubes with addition of 1.2 mL of distilled water, then they were emulsified and centrifuged at 3000 rpm. Before lipid extraction there 100 µL of 8% H₂SO₄ water solution was added to sponge samples (~0.02 g of wet content of sponge cells with water percentage ~97%). After the sample color changed from green to yellow (during 1–

2 min) the lipids were extracted once and then 350 μL of water were added to the extract.

Fatty acid methyl esters derived through the acid esterification of fatty acids (the general content). The chloroform extract (lower layer) was put into glass 10 mL vials. The solvent was evaporated using a stream of argon. Thereafter, 4.5 mL of 2% H_2SO_4 methanol solution immediately was added into the dry extract. Vials were capped with aluminium foil and put into the thermostat for 1.5 h at 55°C for fatty acid methanolysis. Thereafter, the solutions were cooled down to room temperature, 0.8 mL of *n*-hexane was added, and FAMES were extracted with *n*-hexane (3 mL \times 2 \times 2 min). It should be noted that 1 mL of water was added to the solutions before the second extraction. The obtained extracts were concentrated using an argon stream and then were dried with anhydrous Na_2SO_4 .

Free and esterified fatty acid methyl esters derived separately through the iodomethane treatment. Two mL of 0.4 M NaOH in MeOH were added to phytoplankton lipids extracted before (\sim 0.02 g of wet weight) and the solutions have been sonicated for 5 min. The obtained esterified fatty acid methyl esters (EFAMES) were extracted with *n*-hexane (3 mL \times 2). Then 6 mL of phosphate buffer was added to the residue solution that then was sonicated 5 min. Then 400 μL 0.2 M of tetrabutylammonium bromide (TBAB), 200 μL iodomethane and 6 mL CH_2Cl_2 were added and the solutions have been shaken during 10 min in closed flasks. The free fatty acid methyl ester extracts were selected. The EFAMES and FFAMES extracts were analyzed by GC-method.

Free and esterified fatty acid methyl esters derived separately through pH changing. Two mL of 0.4 M NaOH in MeOH was added to lipids (\sim 0.02 g of wet weight) and the solutions have been sonicated for 5 min. The obtained esterified FAMES were extracted with *n*-hexane (3 mL \times 2), rinsed with distilled water, dried with anhydrous Na_2SO_4 and concentrated using an argon stream to 1 mL volume. The EFAME extracts were analyzed by GC-method. Thereafter, 3 mL of water and \sim 0.15 g of copper (II) sulphate grinded in a mortar were added to the residue alkali solution and the mixture has been shaken for 5–10 min till changing blue color of Cu_2SO_4 to green color of $\text{Cu}(\text{OH})_2$ (pH \sim 6). It also necessary to avoid the green color of $\text{Cu}(\text{OH})_2$ change to black color of CuO that may oxidize the FAs. Free FAs were extracted with *n*-hexane (3 mL \times 2). The solvent was evaporated using a stream of argon and the esterification of free fatty acids was carried out (see above). The extracts were analyzed by GC-method.

Gas chromatography coupled with flame-ionization detector for analysis of fatty acid methyl ester extracts. All extract analyzes were carried out using the gas

chromatograph coupled to flame-ionization detector GC-2010 Plus (Shimadzu, Japan) with Optima-17MS column (30 m \times 0.25 mm, 0.25 μm , “Macherey-Nagel”, Germany). The injector temperature was 310°C; the injection volume was 2 μL in splitless mode; the detector temperature was 310°C; the hydrogen velocity was 40 mL/min; the air velocity was 400 mL/min; the carrier-gas velocity (helium) was 30 mL/min; the linear velocity was 28.5 cm/s. In the case of the simple composition bacterial extracts chromatography of the extracts was carried out by heating the column from the initial temperature of 120°C (0.5 min retention) to 260°C at the rate of 15°C/min. In the case of phytoplankton, zooplankton, fish oil and seal fat extracts, chromatography were carried out by heating the column first from 80 to 120°C at the rate of 10°C/min and then to 260°C at the rate of 2.5°C/min. In the case of most complicate sponge extracts – from 80°C (0.5 min retention) to 310°C at a heating rate 2°C/min (5 min retention).

Accuracy measuring by spiking the samples with quality control check sample. The systematic determinate errors of the FAMES analysis in phytoplankton, zooplankton, fish and seal were measured by adding the quality control check sample aliquots with known FAs contents to the biological samples before their preparation. Due to lack of quality control check FAs samples in commercial network we used the Fish Oil T14165QC (Supelco, Sigma-Aldrich Co. LLC, USA) with assigned FAs content values derived from proficiency test data which is not dependent upon one method of analysis but all of those methods are used by participants in a proficiency test itself. This Fish Oil T14165QC sample includes ω -3-, ω -6- and ω -9-FAs (oleic acid C18:1 *n*-9, linoleic acid C18:2 *n*-6, α -linolenic acid C18:3 *n*-3, γ -linolenic acid C18:3 *n*-6, stearidonic acid C18:4 *n*-3, gondoic acid C20:1 *n*-9, stearidonic acid C20:2 *n*-6, arachidonic acid C20:4 *n*-6, eicosatetraenoic acid C20:4 *n*-3, eicosapentaenoic acid C20:5 *n*-3, cetoleic acid C22:1 *n*-11, docosapentaenoic acid C22:5 *n*-3, docosahexaenoic acid C22:6 *n*-3, nervonic acid *cis*-C24:1 *n*-9). Also there was used the medical preparation of fish oil containing ω -3-FAs \sim 300 mg/g. After homogenizing the replicate samples were spiked with the known volume of the additive solution of Fish Oil T14165QC. The FAs were extracted, methylated and analyzed using the GC like as described above.

Gas chromatography–mass-spectrometry of fatty acid methyl ester extracts (qualitative analysis). The extract analyzes were carried out using the gas chromatograph coupled to mass spectrometer “6890B GC System, 7000C GC/MS Triple Quad” (Agilent, USA) with Optima-17MS column (see above). The injector temperature was 290°C; the injection volume was 2 μL

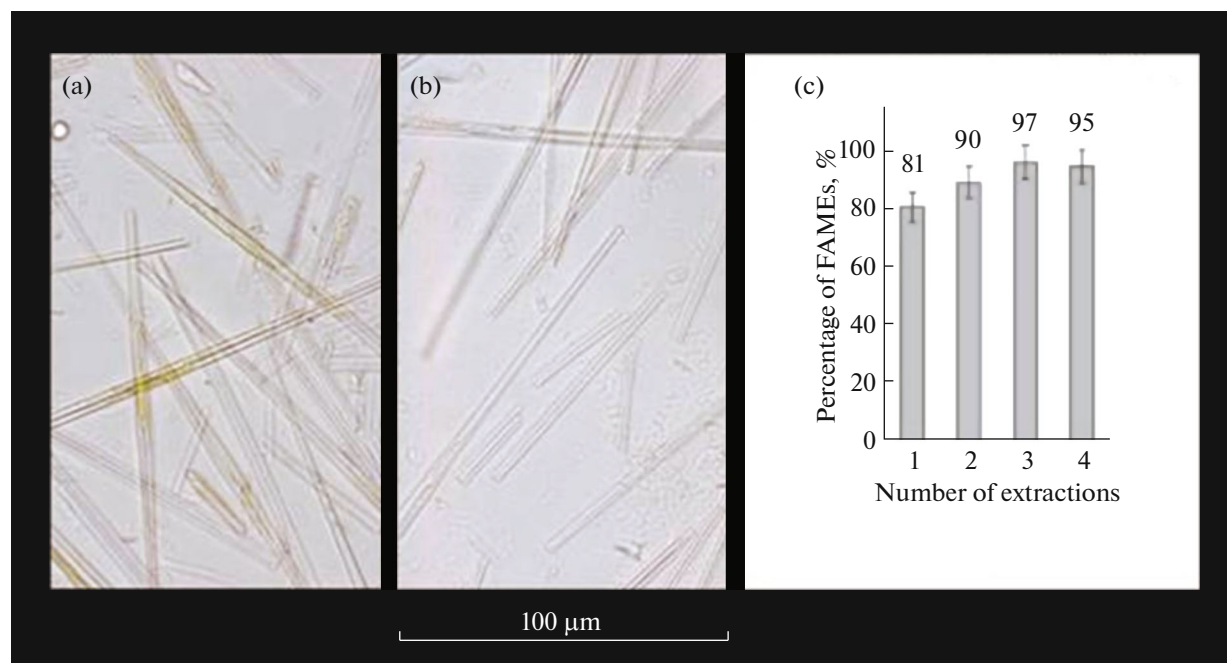


Fig. 1. Light microscopy of Baikal diatom cells: (a) before extraction; (b) empty cells after sonication; (c) lipid recovery.

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 as described above (GC–FID FAMES analysis). Chromatographic peaks were detected in the m/z 40–500 range. The mass-spectra were identified using the NIST Mass Spectral Search Program for the NIST Mass Spectral Library (V. 2.2).

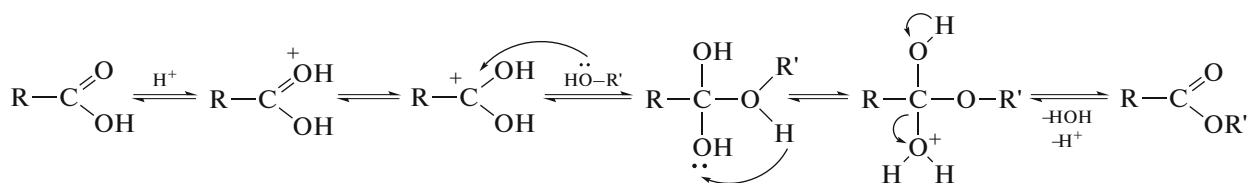
Gas chromatography coupled with flame-ionization detector for fatty acid methyl ester analysis (quantitative analysis). The FAMES quantification was carried out by adding of 50 μL of the di-*n*-decyl ether ($\text{C}_{20}\text{H}_{42}\text{O}$) internal standard solution in *n*-hexane (1 mg/mL) to the FAMES extracts. The calibration function of ΣFAMES was obtained in the range of concentrations from 40 to 540 μg in a sample using F.A.M.E. Mix, C4–C24, 100 mg neat and Methyl *cis*-4,7,10,13,16,19-Docosahexaenoic ester (10 mg/mL in heptane) (Supelco, USA). The calibration coefficients k varied from 0.2801 for docosahexaenoic acid 4,7,10,13,16,19-C22:6 methyl ester to 1.1050 for docosanoic acid C12:0. Besides the individual FAME k values saturated FAs k averages ($k = 1.0491$, $n = 14$), monounsaturated FAs k averages ($k = 0.9966$, $n = 8$), and polyunsaturated (2–6 double bonds) FAs k averages ($k = 0.6389$, $n = 10$) were calculated to determine some FAs which are absent in standard mixtures and solutions.

RESULTS AND DISCUSSION

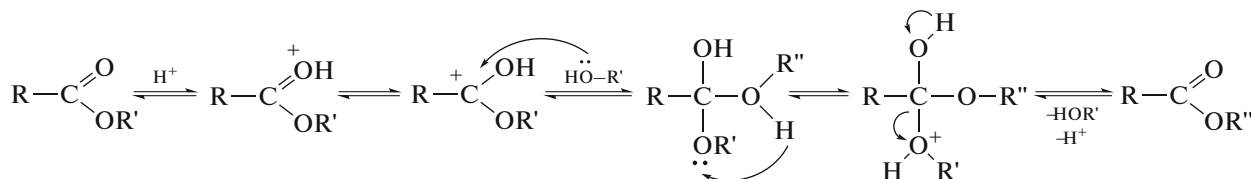
Optimization of lipid extraction process. The sampling of the environmental objects and biomass growth in vitro of Lake Baikal organisms are rather difficult processes. Therefore, the minimum quantity of the samples (0.0004–0.2 g of wet weight) was suggested to reduce the solvents consumption (3.6 mL per sample). It was shown that the lipid extraction of the wet bacterial biomass (0.008–0.02 g of dry weight), plankton (0.0003–0.02 g of dry weight), sponges (0.01–0.02 g of dry weight), fish and seal oil (0.0004–0.0008 g of the oil) excluding the drying stage reduce PUFAs and free FAs destruction and improve the accuracy. For example the free phytoplankton FAs content decreases at +24°C by more than 20% during 24 h and by more than 60% during 96 h respectively.

For the lipid extraction the *Folch* method [37] was assumed as the least destructive [39, 49]. We succeeded in achieving comprehensive lipid and PUFAs recovery (~95%) by sonication the samples under the solvent layer. In this case the duration of three extractions is 15 min that is more effective and about 100 times faster in comparison with classic *Folch* method (24–72 h at +4°C). The microscopic analysis demonstrate this result (Fig. 1).

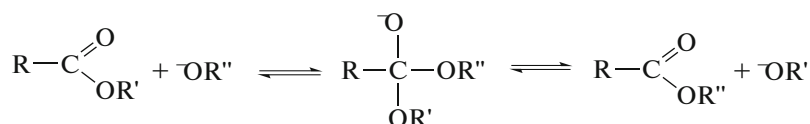
Optimization of process of fatty acid methyl esters derived through methanolysis. The free and esterified fatty acid methyl esters are derived from lipids. It is possible in terms of reactions which are catalyzed by acting acids or alkalis [50, 60] (Schemes 1–3).



Scheme 1. Acid esterification of free fatty acids.



Scheme 2. Acid transesterification of lipids.



Scheme 3. Alkali transesterification of lipids.

The diatom *S. acus* is the dominant microalgae strain in Lake Baikal. According to Vereshchagin [65] the *S. acus* contains about 50% PUFA and we suppose it contain free FAs. For free and esterified FA methylation the acid catalyst was used. It should be noted that 2% H_2SO_4 methanol solution provides both FAMES derived from FAs and chlorophyll A destruction (Fig. 2) excluding purifying extract with use of sorbate. The reduction of acid concentration does not provide the chlorophyll A destruction (Fig. 2). On the other hand high acid concentration results in by-product excess. The sponge is an exception to the rule. It is shown that 8% H_2SO_4 methanol solution additive

which should be done 1–2 min before lipid extraction with *Folch* solvent mixture provides 96% FAMES recovery. The absence of this analytical step decreases FAMES recovery by more than 40% because of multi-component sponge matrix.

The lipid percentage of the samples under investigation is estimated in wide range – from ~0.7 to 97%; the FAs content is estimated from 20 to 2000 μg in the sample; the ΣEFAMES – from 7 to 205 mg/g of dry weight (sample numerous $m = 65$, replicate sample numerous $n = 2$). In addition to that we found the essential distinctions of SFA/MUFA/PUFA percentage composition. For example, it is shown that for phytoplankton the FAs ratio is 30/30/40, for zooplankton it is 39/15/46, for blue-green algae—59/40/1, for thermophilic bacteria—92/8/0, for seal fat—13/68/19, for sponges—15/25/60. In consideration of this differences and high oxidation PUFAs tendency the optimal conditions to methylate FAs of different saturation degrees here are suggested. Using the biomass samples it was found that the minimal PUFAs and MUFAs destruction is at 40–60°C during 1–2 h in the acid catalysis conditions (Fig. 3). The temperature increasing results in PUFAs destruction and the temperature decreasing can not provide the recovery completeness. For example, the phytoplankton ΣFAMES recovery does not exceed 0.5% for methyl esters derived from FAs in strict conditions (NaOH or KOH solutions, 55–85°C, 0.2–1.5 h) because of FAs destruction. Thereby, the suggested conditions to methylate FAs (see EXPERIMENTAL) allow us to reduce PUFAs oxidation at least by 40% as compared with available methods [11, 12, 67, 71, 72].

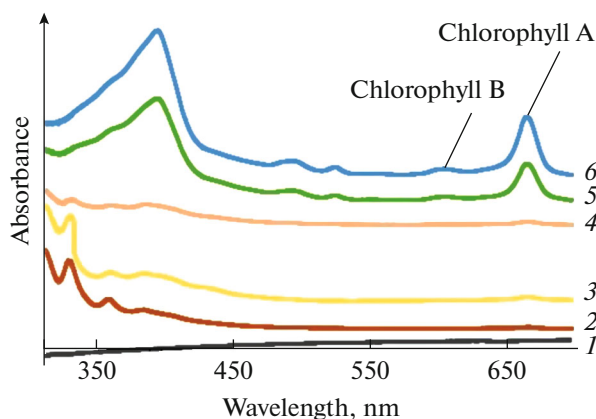


Fig. 2. Absorption spectra of fatty acid methyl ester extracts in UV and visible ranges depending on H_2SO_4 percentage in methanol solution added to lipids extracted from the samples: (1) base line. The percentage of H_2SO_4 : (2) 8, (3) 4.6, (4) 2, (5) 1, (6) 1–0.5.

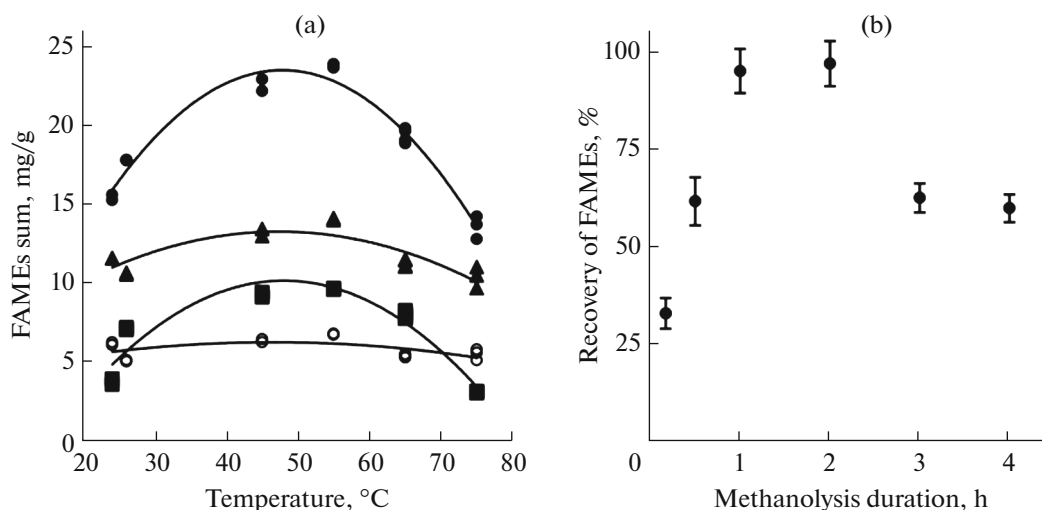


Fig. 3. (a) Recovery (mg/g of dry sample weight) depending on reaction temperature: (●) Σ MUFA, (■) PUFA, (○) SFA, (▲) SFA + MUFA; reaction duration 1.5 h. (b) Recovery (%) of fatty acid methyl ester sum of Baikal phytoplankton and bacterial culture *Geobacillus* sp. depending on the reaction duration (the averages, $n = 2-6$ for every value, 65°C).

Here, we also suggest determining the free and esterified FAs through two steps (see EXPERIMENTAL):

the first step—alkali transesterification of lipids and the next EFAs extraction with *n*-hexane;

the second step—neutralization of the residual solution using the copper(II) sulphate and the next acid FAs esterification and SFAs extraction with *n*-hexane.

The method proposed is simple, effective and supports the Σ FAMES recovery $\sim 85-90\%$ (Table 1) excluding using of toxic reagent. For example, we determined the phytoplankton esterified and free FAs contents using the TBAB as catalyst and iodomethane as methylating agent according to Ukolova and Orlova et al. [41, 42] method for FAs determination in human biological fluids. The results of FAs methylation by two different methods were tabulated (Table 2). As one can see, the FAs methylation in acid catalysis conditions give the best FFAs, EFAs, PUFAs recovery are higher by 30%, SFAs and MUFAs recovery are higher by 20% in comparison with methylation in iodomethane catalysis conditions. The proposed method also excludes using of toxic reagent (iodomethane). The FAMES recovery is depends on double bond quantity (Fig. 4a) and different solubility in water (Fig. 4b) [73, 74]. So, two or more extractions are need for exhaustive PUFAs recovery ($\geq 97\%$). At the same time one extraction is enough for the comprehensive SFAs recovery.

It is known [50] that the odd-branched C17:0, C19:0 FAs surrogate standards can be used to compensate FAs loss during the sample preparation. The disadvantages of this method are the presence of these saturated acids in some hydrocoles [75] as well as significant differences of FID responses to saturated and

polyunsaturated acids. For instance, the calibration coefficient (k) when determining SFAs is 1.0491 ($R^2 = 0.9928$), MUFAs – 0.9966 ($R^2 = 0.9939$), PUFAs – 0.6389 ($R^2 = 0.9832-0.9989$). Because of good recovery (see above) of the method we spiked the sample extracts with di-*n*-decyl ester ($C_{20}H_{42}O$) solution before GC directly to quantify FAs. The retention time t_R of the di-*n*-decyl ester peak is in the middle of the environmental sample FAMES interval of the retention times. The FID and MS response to this substance are intensive. The peak of this internal standard is not merged with any peaks. Di-*n*-decyl ester is stable to long-term extract storage (≥ 12 months), commercially available, nontoxic and simple in use.

Precision of fatty acid methyl ester sum values in Lake Baikal bacteria and phytoplankton samples. With the use of archival data of current sample analyze values [55] we calculated the point estimated repeatability of FAME sum, SFAs, MUFAs, PUFAs and individual FA substances determination in biological samples. It was shown that the FAME sum values ranged from 6 to 60 mg/g of dry weight for bacteria and from 10 to 60 mg/g of dry weight for phytoplankton. We grouped all sample values to two subranges because of the concentration range widths (Table 3). The \bar{x}_j values differ 3–4 times within the scope of subranges. Because of this we calculated the relative deviation $\delta_{ji} = (x_{ji} - \bar{x}_j) / \bar{x}_j$ to estimate the dispersion. The sample dispersions V_i^2 of every subrange are tabulated (Table 3). Comparing the r -criteria to the tabulated values we estimate the belonging the uncertain results to any selection [55].

We established also the homogeneity of the random dispersions of the neighbor subranges of bacteria biomass and phytoplankton FAME sum values using

Table 1. Diatom *S. acus* free and esterified fatty acid methyl esters are separately derived through the fatty acid alkylation with iodomethane in tetrabutylammonium catalysis conditions and through the fatty acid alkylation with methanol in acid catalysis conditions

The way of deriving FAMES	FAMES	FAME content, mg/g of dry sample weight*			
		sum	SFAs	MUFAs	PUFAs
Alkali transesterification of EFAs and TBAB catalysis of the methylation reaction with iodomethane	$\Sigma(\text{EFAMES, FFAMES})$	11.2 ± 1.0	3.3 ± 0.3	3.2 ± 0.3	4.6 ± 0.4
	$\Sigma(\text{EFAMES}),$	3.6 ± 0.3	1.25 ± 0.11	0.96 ± 0.07	1.34 ± 0.12
	$\Sigma(\text{FFAMES})$	7.6 ± 0.7	2.07 ± 0.19	2.24 ± 0.20	3.29 ± 0.29
Acid catalysis of EFAs and FFAs of the methylation reaction (see Experimental)	$\Sigma(\text{EFAMES, FFAMES})$	14.6 ± 1.3	3.8 ± 0.3	4.1 ± 0.4	6.8 ± 0.6

* The number of significant digits of Δx values in the tables was presented according to Smagunova et al. [55]. If the first significant digit of Δx value was more than 2, we approximate the value to one digit. If the first digit of Δx value was 1 or 2, we wrote two or three digits. Shortening the number of significant digits to one in our case increase the uncertainty up to 30%.

Table 2. Diatom *S. acus* fatty acid methyl ester sums are derived through the fatty acid alkylation with methanol in acid catalysis conditions and esterified fatty acid methyl esters are separately derived through the alkylation with the using copper(II) sulphate

The way of deriving FAMES	FAMES	FAME content, mg/g of dry sample weight			
		sum	SFAs	MUFAs	PUFAs
Acid catalysis of EFAs and FFAs of the methylation reaction with methanol	$\Sigma(\text{EFAMES and FFAMES})$	15.6 ± 1.4	5.7 ± 0.5	4.7 ± 0.4	4.7 ± 0.4
Alkali EFA transesterification, neutralization by CuSO_4 adding and acid catalysis of the methylation reaction of FFA by methanol	$\Sigma(\text{EFAMES},$	14.2 ± 1.3	5.7 ± 0.5	4.0 ± 0.4	4.0 ± 0.4
	$\text{FFAMES}),$	4.9 ± 0.4	2.20 ± 0.20	1.42 ± 0.13	1.42 ± 0.13
	$\Sigma(\text{EFAMES}),$	9.3 ± 0.8	3.5 ± 0.3	2.53 ± 0.22	2.53 ± 0.22

the F -criteria that allowed us to merge the estimation values [55]. The average dispersions \bar{V}_i^2 , which characterize the repeatability of FAMES sum values in phytoplankton and bacteria are the similar. Therefore, we established the homogeneity using the *Bartlett* criteria [59]. Comparing the calculated B -criteria to the tabulated values using χ^2 -criterion showed that $\chi^2(0.01, 51) =$

$76 > B = 29 < \chi^2(0.05, 51) = 68$. Therefore, the dispersions of the FAME determination results are homogeneous. The average dispersion $\bar{V}_i^2 = 0.0060$ ($f_\Sigma = 52$) was calculated using the degrees of freedom of every dispersion. The estimated variation coefficient V_r is 7.7%. The interlaboratory precision (reproducibility) Σ FAME determination values in bacteria and phyto-

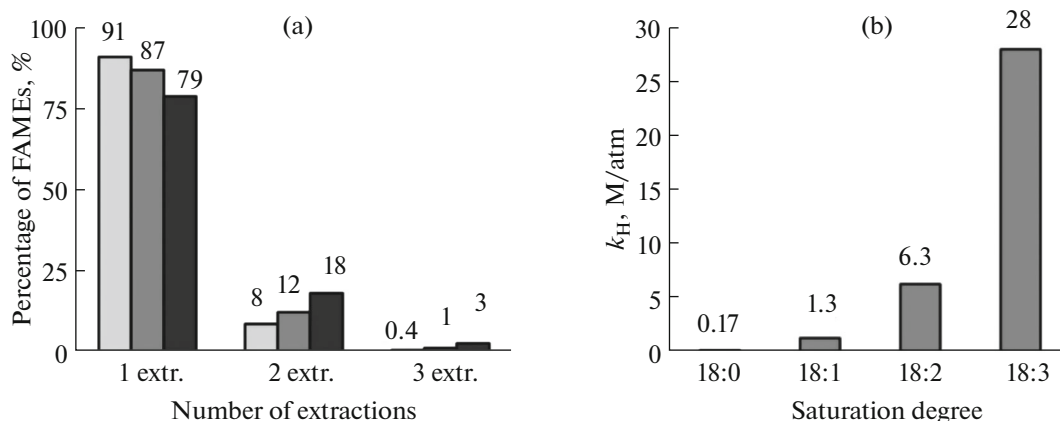
**Fig. 4.** (a) Recovery (%) of methyl esters extracted from spirit solutions with *n*-hexane: □—SFA, ■—MUFA, ■—PUFA. (b) Water solubility of fatty acid methyl esters [73, 74].

Table 3. Comparison of sample dispersions V_r^2 of the results of the fatty acid methyl ester sum determination in hydrocole samples with the use of the Fisher (F) criteria

Object	Range	Σ FAME, mg/g	$V_i^2 (f_i)^*$	$F(\alpha, f_1, f_2) = F(0.05, 10, 9)$	$\bar{V}_i^2 (f_i)^*$
Bacteria	I	6–20	0.1011 ($f = 11$)	$F_{I-II} = 1.01 \leq F(0.05, 10, 9) =$	0.0978 ($f = 20$)
	II	20–60	0.0993 ($f = 10$)	3.14	
Phytoplankton	I	10–20	0.0744 ($f = 15$)	$F_{I-II} = 1.53 \leq F(0.05, 15, 15) =$	0.0619 ($f = 31$)
	II	20–60	0.0488 ($f = 15$)	2.40	

* $f_i = m(n - 1)$ – number of degrees of freedom for which we calculate V_i^2 , where m – sample number, n – number of single replicas.

plankton are 6 and 10% respectively. The reproducibility of individual FAMES determination values $\leq 15\%$.

Accuracy of the results of the fatty acid methyl ester determination. We did not find in available literature the information concerning the accuracy measuring of FAs in the environmental samples. This may be due to lack of quality control check FAs samples in commercial network. Here we suggest the method of FAMES accuracy measuring through the estimation of determinate systematic constituent of the error by spiking the samples with known quantity of the quality control check sample Fish Oil T14165QC (further called “Fish Oil”). The individual FAs concentration values in the Fish Oil sample are found within the confidence diapason by different analytical methods in 46 independent laboratories according to FAPAS (Food Chemistry Proficiency Test Report 14165. Omega-3, Omega-6 and Omega-9 Fatty Acids in Fish Oil) (further called “Test Report”) but they are not certified values [76]. First, we determined the FAs content of Fish Oil according to State Standard method [64] of FAMES deriving of oils and fats. The volume of 0.1 mL of freshly made sodium methylate CH_3ONa was added to 0.03–0.04 g of Fish Oil material. The obtained FAMES were extracted with 1.9 mL of *n*-hexane (2×5 min), rinsed with distilled water, dried with anhydrous Na_2SO_4 , centrifuged, spiked the extract with an internal standard and analyzed by GC. The obtained FAMES averages ($n = 2$) were converted to FAs averages and compared to those according to Test Report. The averages obtained did not leave the confidence interval calculated for Fish Oil and were assumed as a basis values when the same quantity of Fish Oil was added to the sample material (\bar{x}) (Table 4). This approach have been tested for the first time. The results of FAs methylation of Fish Oil with the use of CH_3ONa (State Standard method [64]) and with the use of CH_3OH (see EXPERIMENTAL) are comparable. The FAMES sum averages are 172 ± 6 and 177 ± 7 g/100 g of the sample respectively. It should be notice that the State Standard method is unsuitable for complex matrices such as phytoplankton, sponge and others because of low FAMES recovery. It is shown (Table 4) that the found Fish Oil addi-

tive value corresponds to introduced one. The determinate permanent constituent of the systematic constituent error was not found for most FAs of phytoplankton. The C20:5 and C22:6 ω -3-FAs are exceptions. Their content in Fish Oil sample is much lower than their content in Baikal phytoplankton. Therefore in this case we used the medical preparation of fish oil (salmon fish oil with ω -3-FAs content ~ 300 mg/g according to documentation, Israel). The C20:5 and C22:6 ω -3-FAs contents for this medical preparation were estimated and corresponded to C20:5 and C22:6 ω -3-FAs contents for Baikal phytoplankton. In this case the determinate permanent constituent of the systematic constituent error of C20:5 and C22:6 ω -3-FAs determination was not revealed.

Results of FAs analysis of Lake Baikal hydrocoles.

We could identified 15–24 FAs for different bacterial and blue-green algae strains. It is known that FAs with the chain of C13–C21 are responsible with cell membrane plasticity [77]. For seal fat 44 FAs were identified. Percentages of MUFAs and PUFAs are ~ 80 and $\sim 20\%$ correspondently. Percentage of PUFAs for laboratory culture of diatom microalgae *Synedra acus* (2/3 of exponential growth stage) which inhabits many freshwater ecosystems is $\sim 25\%$ (our results). Percentage of PUFAs for laboratory culture of *Synedra acus* sp. Ehrenberg is $\sim 30\%$ [75]. Percentage of PUFAs for Lake Baikal *Synedra acus* is ~ 30 – 50% [75]. The minimal PUFAs contents are typical for littoral phytoplankton and the maximal PUFAs contents are typical for pelagial phytoplankton samples probably because of physicochemical properties of the environment. High PUFAs content in Lake Baikal diatom *Melosira baicalensis* ~ 40 – 50% is correspond to that in arctic diatom *Melosira arctica* (Barents Sea) ~ 40 – 60% [78]. High content of free FAs (up to 25%) of *Melosira arctica* is lower than that for Lake Baikal *S. acus* (4–40%). Maybe, the free FAs of phytoplankton play antibacterial protective role because of FFAs toxicity. This assumption can explain the seasonal differences between bloom extremums of phytoplankton and bacterioplankton. Small number of publications in this field should be noted. They say [79] about high FFAs content in the micellar fungi and about probability of FFAs direct synthesis as opposed to opinion about FFAs derived due to cell destruction. Also they say

Table 4. Accuracy of the results of determination of individual fatty acid methyl esters and their sums in Baikal phytoplankton

FAME*	\bar{x} , μg	Were added, μg	\bar{x}' , μg	Were found, μg
C18:1(n-9)	2.4 ± 0.3	118 ± 15	120 ± 15	118 ± 15
C18:2(n-9,12)	4.9 ± 0.6	32 ± 4	35 ± 4	30 ± 4
C18:4(n-6,9,12,15)	0.37 ± 0.03	0.54 ± 0.05	1.01 ± 0.09	0.64 ± 0.06
C18:3(n-9,12,15)	0.43 ± 0.04	12.4 ± 1.3	13.3 ± 1.4	12.9 ± 1.3
C20:1(n-11)	0.107 ± 0.019	5.9 ± 0.9	6.0 ± 1.0	6.0 ± 1.0
20:2 (n-11,14)	0.27 ± 0.04	0.85 ± 0.12	1.13 ± 0.16	0.86 ± 0.12
C20:4(n-5,8,11,14)	0.075 ± 0.007	0.096 ± 0.009	0.182 ± 0.018	0.108 ± 0.011
C20:5(n-5,8,11,14,17)	31 ± 5	1.11 ± 0.16	34 ± 5	3.3 ± 0.5
C20:5(n-5,8,11,14,17)**	31 ± 5	21 ± 3	56 ± 8	25 ± 4
C20:4(n-8,11,14,17)	0.04 ± 0.01	0.19 ± 0.04	0.20 ± 0.04	0.16 ± 0.04
C22:1(n-11)	0.20 ± 0.05	3.6 ± 0.8	3.74 ± 0.88	3.5 ± 0.8
C22:1(n-13)	0.60 ± 0.09	2.7 ± 0.4	3.12 ± 0.49	2.5 ± 0.4
C22:6(n-4,7,10,13,16,19)	1.875 ± 0.202	0.42 ± 0.05	2.41 ± 0.26	0.53 ± 0.06
C22:6(n-4,7,10,13,16,19)**	1.875 ± 0.202	4.0 ± 0.4	6.5 ± 0.7	4.5 ± 0.5
C22:5(n-7,10,13,16,19)	0.101 ± 0.011	0.146 ± 0.016	0.218 ± 0.023	0.119 ± 0.013
ΣFAME	40 ± 4	177 ± 16	220 ± 20	180 ± 16

* The *n*-character point at the double-bond position counting from the carboxylic group of FA. ** The medical preparation of fish oil additive to estimate the determinate systematic constituent of the error of C20:5 and C22:6 FAs analysis instead of Fish Oil T14165QC check sample additive.

about the FFAs role in medical diagnostics [80]. In addition we noticed free FAs concentration increasing in opposite to free PUFA concentration decreasing in the case of the organic pollutant concentration increasing in the phytoplankton and water samples which were collected in the littoral zone near the towns and townships. Thereby, the free FAs of the phytoplankton might be a biomarker of water ecosystem state of the Lake. It was shown [81] that when spiking feed for rats with diethylhexylphthalate the free FAs concentration in plasma increases in opposite to essential PUFA concentration decreasing [82]. The influence of pollution and other environmental factors on FAs phytoplankton composition should be analyzed properly. We may talk about obvious influence of pollution on Lake Baikal phytoplankton well-being of which results in well-being of all Lake ecosystem.

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REFERENCES

1. Kulakovskaya, E.V. and Mironov, A.A., *Appl. Biochem. Microbiol.*, 2016, vol. 52, no. 6, p. 615.
2. Suzuki, K.I. and Komagata, K., *Int. J. Syst. Bacteriol.*, 1983, vol. 83, no. 2, p. 188.
3. O'Leary, W.M., *Bacteriol. Rev.*, 1962, vol. 26, no. 4, p. 421.

4. Takai, K., Inoue, A., and Horikoshi, K., *Int. J. Syst. Bacteriol.*, 1999, vol. 49, p. 619.
5. Spanevello, M., Yamamoto, H., and Patel, B.K.C., *Int. J. Syst. Evol. Microbiol.*, 2002, vol. 52, p. 795.
6. Řezanka, T., Dor, I., Prell, A., and Dembitsky, V.M., *Folia Microbiol. (Dordrecht, Neth.)*, 2003, vol. 48, no. 1, p. 71.
7. Řezanka, T., Viden, I., Go, J.V., Dor, I., and Dembitsky, V.M., *Folia Microbiol. (Dordrecht, Neth.)*, 2003, vol. 48, no. 6, p. 781.
8. Li, R., Yokota, A., Sugiyama, J., Watanabe, M., Hiroki, M., and Watanabe, M.M., *Phycol. Res.*, 1998, vol. 46, no. 1, p. 21.
9. Guides, A.C., Amaro, H.M., Barbosa, C.R., Pereira, R.D., and Malcata, F.X., *Food Res. Int.*, 2001, vol. 44, p. 2721.
10. Younsi, M., Ramanandraibe, E., Bonaly, R., Donner, M., and Coulon, J., *Antimicrob. Agents Chemother.*, 2000, vol. 44, no. 7, p. 1911.
11. Zhila, N.O. and Kalacheva, G.S., *J. Appl. Phycol.*, 2011, vol. 23, p. 47.
12. Wagenen, J.V., Miller, T.W., Hobbs, S., Hook, P., Crowe, B., and Huesemann, M., *Energies*, 2012, vol. 5, p. 731.
13. *Temperature Adaptation in a Changing Climate: Nature at Risk (Climate & Weather)*, Storey, K.B. and Tanino, K.K., Eds., CAB Int., 2012, p. 238.
14. Kirpenko, N.I., Usenko, O.M., and Musii, T.O., *Gidrobiol. Zh.* 2016, vol. 52, no. 6, p. 74.
15. Braun, A.D. and Mozhenok, T.P., *Nespetsificheskii adaptatsionnyi sindrom kletochnoi sistemy* (Nonspecific Adaptation Syndrome of a Cellular System), Leningrad: Nauka, 1987.
16. Abedi, E. and Sahari, A.M., *Food Sci. Nutr.*, 2014, vol. 2, no. 5, p. 443.
17. Li, Y., Naghdi, F.G., Garg, S., Adarme-Vega, T.C., Thurecht, K.J., Ghafor, W.A., Tannock, S., and Schenk, M., *Microb. Cell Fact.*, 2014, vol. 13, no. 14, p. 1. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3926349/>
<https://doi.org/10.1186/1475-2859-13-14>
18. Hiltunen, M., The role of zooplankton in the trophic transfer of fatty acids in boreal lakes food web, *PhD Thesis*, Joensuu, Finland: Univ. Eastern Finland, 2015; Publ. Univ. Eastern Finland Dissertations in Forestry and Natural Sciences, 2015, no. 210, p. 66.
19. Bazarsadueva, S.V. and Radnaeva, L.D., *Khim. Interesakh Ustoich. Razvit.*, 2013, no. 21, p. 533.
20. Murzina, S.A., Nefedova, Z.A., and Nemova, N.N., *Tr. Karelsk. Nauchn. Tsentra*, 2012, no. 2, p. 18.
21. Perhap, G.M., Arhonditsis, G.B., and Brett, M.T., *Environ. Rev.*, 2012, vol. 20, no. 3, p. 155.
22. Glyzina, O.Yu., Dzyuba, E.V., Smirnova-Zalumi, N.S., Basharina, T.N., Smirnov, V.V., and Glyzin, A.V., *Khim. Interesakh Ustoich. Razvit.*, 2010, no. 18, p. 139.
23. Aveina, E.S., Pintaeva, E.Ts., and Radnaeva, L.D., *Vestn. Buryat. Gos. Univ.*, 2009, no. 3, p. 61.
24. Kozlova, T.A. and Khotimchenko, S.V., *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.*, 2000, vol. 126, no. 4, p. 477.
25. Basova, M.M., *Al'gologiya*, 2005, vol. 15, no. 4, p. 415.
26. Sushchik, N.N., *Zh. Obshch. Biol.*, 2008, vol. 69, no. 4, p. 299.
27. Deineka, V.I., Gabruk, N.G., Fofanov, G.M., Deineka, L.A., Manokhina, L.A., and Sidel'nikova, N.A., *J. Anal. Chem.*, 2003, vol. 58, no. 12, p. 1160.
28. Kulichenko, S.A. and Shevchenko, A.M., *J. Anal. Chem.*, 2003, vol. 58, no. 1, p. 58.
29. *GOST (State Standard) 5476-80 (ST-SEV 4715-84): Vegetable oils. Methods for Determination of Acid Value*, Moscow: Izd. Standartov, 1998.
30. Lapshina, T.M., Sharudina, S.Ya., and Tur'yan, Ya.I., USSR Patent 1825423, 1993.
31. Lapshina, T.M., Tur'yan, Ya.I., and Danil'chuk, S.I., *Zh. Anal. Khim.*, 1991, vol. 46, no. 6, p. 1150.
32. Ashworth, M.R.F., *Titrimetric Organic Analysis, vol. 2: Indirect Methods*, New York: Interscience, 1965.
33. Ruvinskii, O.E., Vyskubova, E.N., and Sharudina, S.Ya., *Izv. Vyssh. Uchebn. Zaved., Pishch. Tekhnol.*, 2000, no. 4, p. 108.
34. Strel'nikova, E.B., Stakhina, L.D., and Petrenko, T.V., *J. Anal. Chem.*, 2009, vol. 64, no. 1, p. 8.
35. Ivanova, L.V., Koshelev, V.N., Sokova, N.A., Burov, E.A., and Primerova, O.V., *Tr.—Mosk. Inst. Neftekhim. Gazov. Prom-sti. im. I. M. Gubkina*, 2013, no. 1(270), p. 68.
36. Rakhman'ko, E.M., Zhilko, V.V., and Egorov, V.V., *J. Anal. Chem.*, 2005, vol. 60, no. 1, p. 16.
37. Folch, J., Lees, M., and Stanley, G.H.S., *J. Biol. Chem.*, 1957, vol. 226, no. 1, p. 497.
38. Ryzhova, G.L., Tyunina, M.A., and Dychko, K.A., *J. Anal. Chem.*, 2003, vol. 68, no. 8, p. 736.
39. Blight, E.G. and Dyer, W.J., *Can. J. Biochem. Physiol.*, 1959, vol. 37, no. 8, p. 911.
40. Khasanov, V.V., Makarycheva, A.I., and Slizhov, Yu.G., *J. Anal. Chem.*, 2003, vol. 68, no. 8, p. 1028.
41. Ukolov, A.I., Orlova, T.I., Savel'eva, V.I., and Radilov, A.S., *J. Anal. Chem.*, 2015, vol. 70, no. 9, p. 1123.
42. Orlova, T.I., Ukolov, A.I., Savel'eva, V.I., and Radilov, A.S., *Analitika Kontrol'*, 2015, vol. 19, no. 2, p. 183.
43. Kornprobst, J.M. and Barnathan, G., *Mar. Drugs*, 2010, vol. 8, no. 10, p. 2569.
44. Chiu, S.Y., Kao, C.Y., Chen, C.H., Kuan, T.C., Ong, S.C., and Lin, C.S., *Bioresour. Technol.*, 2008, vol. 99, no. 9, p. 3389.
45. Tsai, H.P., Chuang, L.T., and Chen, C.N.N., *Food Chem.*, 2016, vol. 1, no. 192, p. 682.
46. Latyshev, N.A., Zhukova, N.V., Efremova, S.M., Imbs, A.B., and Glyzina, O.Y., *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.*, 1992, vol. 102, no. 4, p. 961.
47. Glyzina, O.Yu., Bazarsadueva, S.V., Glyzin, A.V., and Radnaeva, L.D., *Ekologiya*, 2016, no. 2, p. 152.
48. Rod'kina, S.A., *Izv. Tikhookean. Nauchno-Issled. Inst. Rybn. Khoz. Okeanogr.*, 2003, vol. 135, p. 327.
49. Iverson, S.J., Lang, S.L., and Cooper, M.H., *Lipids*, 2001, vol. 36, no. 11, p. 1283.

50. Christie, W.W., *Adv. Lipid Methodol.*, 1993, vol. 2, p. 69.
51. Leveille, J.-C., Amblard, C., and Bourdier, G., *J. Plankton Res.*, 1997, vol. 19, no. 4, p. 469.
52. Ivankin, A.N., Oliferenko, G.L., Kulikovskii, A.V., Chernukha, I.M., Semenova, A.A., Spiridonov, K.I., and Nasonova, V.V., *J. Anal. Chem.*, 2016, vol. 71, no. 11, p. 1131.
53. Taipale, S., Strandberg, U., Peltomaa, E., Galloway, A., Ojala, A., and Brett, M.T., *Aquat. Microbiol. Ecol.*, 2013, vol. 71, p. 165.
54. Li, Y., Naghdi, F.G., GArg, S., Adarme-Vega, T.C., Thurecht, K.J., Ghafor, W.A., Tannock, S., and Schenk, P.M., *Microb. Cell Fact.*, 2014, vol. 13, no. 14. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3926349/>. <https://doi.org/10.1186/1475-2859-13-14>
55. Smagunova, A.N., *Metody matematicheskoi statistiki v analiticheskoi khimii* (Methods of Mathematical Statistics in Analytical Chemistry), Irkutsk: Irkutsk. Gos. Univ., 2008.
56. Albert, B.B., Cameron-Smith, D., Hofman, P.L., and Cutfield, W.S., *BioMed Res.*, vol. 2013, 464921. <https://doi.org/10.1155/2013/464921>
57. James, A.T. and Martin, A.G.P., *Biochem. J.*, 1956, vol. 63, no. 1, p. 144.
58. Brunton, N.P., Mason, C., and Collins, M.J.J., *J. Anal. Chem.*, 2015, vol. 70, p. 1218.
59. Fournier, V., Destailats, F., Juaneda, P., Dionisi, F., Lambelet, P., Sebedio, J.-L., and Berdeaux, O., *Eur. J. Lipid Sci. Technol.*, 2006, no. 108, p. 33.
60. Ichihara, K. and Fukubayashy, Y., *J. Lipid Res.*, 2010, vol. 51, p. 635.
61. Sicko-Goad, L., Lazinsky, D., Hall, J., and Simmons, M.S., *Arch. Environ. Contam. Toxicol.*, 1989, vol. 18, p. 629.
62. Zaikin, V. and Halket, J.M., *A Handbook of Derivatives for Mass Spectrometry*, Chichester: IM, 2009.
63. Rosenfeld, J.M., *Anal. Chim. Acta*, 2002, vol. 465, p. 93.
64. *GOST (State Standard) R 51486-99: Vegetable Oils and Animal Fats. Preparation of Methyl Esters of Fatty Acids*, Moscow: Izd. Standartov, 2001.
65. Vereshchagin, A.L., Glyzina, O.Yu., Basharina, T.N., Safonova, T.A., Latyshev, N.A., Lyubochko, S.A., Korneva, E.S., Petrova, D.P., Annenkov, V.V., Danilovtseva, E.N., Chebykin, E.P., Volokitina, N.A., and Grachev, M.A., *Biotekhnologiya*, 2008, no. 4, p. 55.
66. Leu, E., Daase, M., Schulz, K.G., Stuhr, A., and Riebesell, U., *Biogeosciences*, 2013, vol. 10, p. 1143.
67. Maghraby, D.M.E. and Fakhry, E.M., *Oceanologia*, 2015, vol. 57, p. 86.
68. Christie, W.W., *Gas Chromatography and Lipids*, Ayr, Scotland: The Oily Press, 1989, p. 307.
69. Shishlyannikov, S.M., Nikonova, A.A., Bukin, U.S., and Gorshkov, A.G., *Ecol. Indic.*, 2018, vol. 85, p. 878.
70. Widdel, F. and Back, F., in *The Prokaryotes*, Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E., Eds., New York: Springer, 1992, 3rd ed., vol. 4, p. 787.
71. Gladyshev, M.I., Emelianova, A.Y., and Kalachova, G.S., *Hydrobiologia*, 2000, vol. 431, p. 155.
72. Koussoroplis, A.-M., Bec, A., Perga, M.-E., Koutrakis, E., Desvillettes, C., and Bourdier, G., *Mar. Ecol.: Prog. Ser.*, 2010, vol. 404, p. 207.
73. Krop, H.B., Velzen, J.M., Parsons, J.R., and Govers, H.A.J., *J. Am. Oil Chem. Soc.*, 1997, vol. 74, no. 3, p. 309.
74. Sander, R., *Compilation of Henry's Law Constants for Inorganic and Organic Species of Potential Importance in Environmental Chemistry*, Mainz: Max-Planck Inst. Chem., 1999.
75. Jiménez-Valera, S. and Sánchez-Saavedra, M.P., *Lat. Am. J. Aquat. Res.*, 2016, vol. 44, no. 4, p. 689.
76. Knaggs, M., *FAPAS—Food Chemistry Proficiency Test Report 14165, Omega-3, Omega-6 and Omega-9 Fatty Acids in Fish Oil*, September–November 2016, Sand Hutton, UK: FAPAS, 2016.
77. Zakharova, Yu.V. and Sukhikh, A.S., *Sorbtsionnye Khromatogr. Protsessy*, 2015, vol. 15, no. 6, p. 776.
78. Falk-Petersen, S., Sargent, J.R., Henderson, J., Hegseth, E.N., Hop, H., and Okolodkov, Y.B., *Polar Biol.*, 1998, vol. 20, p. 41.
79. Babitskaya, V.G., Chernook, T.V., Shcherba, V.V., Puchkova, T.A., Filimonova, T.V., and Osadchaya, O.V., *Vestn. Belorus. Gos. Univ. Ser. 2: Khim. Biol. Geogr.*, 2009, vol. 4, part 1, p. 101.
80. Vel'kov, V.V., *Lab. Zh. Vrachei*, 2008, no. 1, p. 16.
81. Sakurai, T., Miyazawa, S., and Hashimoto, T., *J. Biochem.*, 1978, vol. 83, no. 1, p. 313.
82. Xu, Y., Agrawal, S., Cook, T.J., and Knipp, G.T., *Placenta*, 2008, vol. 29, no. 11, p. 962.