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SPECIFICS OF SAMPLE PREPARATION OF *ARTHROSPIRA (SPIRULINA) PLATENSIS* CULTURE IN THE STUDY OF ASSOCIATED MICROFLORA BY FLOW CYTOMETRY AND SCANNING ELECTRON MICROSCOPY

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In biotechnological research, it is important to control quantitative characteristics of associated microflora in algal cultures. With the aim of more complete detection and detailed study of associated microflora in *Arthrospira (Spirulina) platensis* culture, we applied methods of physical and chemical preparation of samples and subsequent investigation of a suspension by flow cytometry after cell staining with SYBR Green I fluorochrome and scanning electron microscopy. As shown, optimal sample preparation options were exposure to a reagent (sodium pyrophosphate or methanol), suspension stirring, ultrasonic treatment, centrifugation, and additional washing procedures. A mean of (27.1 ± 3.9) % of "potential" cumulative bacterial abundance was initially extracted from a cyanobacterial culture. During the following three washes, abundance of microorganisms increased significantly and averaged (88.9 ± 6.3) % (paired *t*-test; $p < 0.05$). Further, abundance of microorganisms in the sediment remained insignificant, 6–11%, and could be neglected. As shown, bacterial abundance at different stages of *A. platensis* cultivation changed from 6.7×10^6 to 1.7×10^8 cells·mL^{-1}. The morphological structure of associated microflora in *A. platensis* at the stationary phase was dominated by large rod-shaped cells (67.2%); the proportion of spiral forms was almost twice as low (30.2%); and spherical forms were even less common (2.6%). The mean bacterial cell volume was (0.16 ± 0.02) μm^3, and biomass was 0.022–0.025 g·L^{-1}. The values obtained for indicators of associated microflora are comparable to those provided in literature for *A. platensis* and other algal cultures. The proposed methods of treatment of *A. platensis* suspension boosted the efficiency of bacterial separation, facilitated removal of fragments of cyanobacterial trichomes, detritus, and other particles in samples, and provided an opportunity to study associated microflora by flow cytometry and scanning electron microscopy.

Keywords: Cyanobacteria, *Arthrospira (Spirulina) platensis*, associated microflora, bacteria, microorganisms, bacterial abundance, bacterial biomass, sample preparation methods, cell desorption, physical and chemical treatment, ultrasonic treatment, fluorescence microscopy, scanning electron microscopy, flow cytometry

In experimental setups and in open pools, associated microflora is always present when growing algacultures. Microorganisms can affect the viability and biochemical parameters of microalgae and cyanobacteria [Borisova, 1996; Kublanovskaya, 2019; Tarhova, 2005]. The relationships between heterotrophic microorganisms and algal culture are diverse: from inhibition to stimulation of bacterial growth by algae/cyanobacteria, and *vice versa* [Ashen, Goff, 2000; Ignatenko, Nemtseva, 2012;

Kublanovskaya, 2019; Ramanan et al., 2015; Sapp et al., 2007; Seymour et al., 2017]. Control of quantitative characteristics of associated microflora is an important issue in biotechnological research, since the biomass obtained can be further used for production of biologically active substances, food supplements, *etc.* [Falquet, Hurni, 2006; Jung et al., 2022; Kalenik et al., 2019].

It is known that flow cytometry, scanning electron microscopy, and fluorescence microscopy are successfully used in algological and microbiological research. For most microalgae with the size less than 30 μm (*e. g.*, *Chlorella vulgaris*, *Dunaliella salina*, *Isochrysis galbana*, *Phaeodactylum tricornutum*, *Platymonas viridis*, *Scenedesmus obliquus*, *Tetraselmis viridis*, and *Thalassiosira weissflogii*), various parameters of autotrophic microalgae (abundance, granularity, pigment fluorescence intensity, and so on) are analyzed directly in the culture suspension without any sample preparation using flow cytometry [Rauen et al., 2011; Solomonova et al., 2024; *etc.*]. In the same subsamples, after staining with fluorochromes, studying of the associated heterotrophic microflora is also possible [Danger et al., 2007; Rauen et al., 2011].

Considering that *Arthrospira (Spirulina) platensis* trichomes reach a length of 50–300 μm, the use of flow cytometry for investigating the native suspension of cyanobacteria is impossible. However, preliminary sample preparation, aimed at separating bacterial cells from *A. platensis* trichomes or non-biological particles, makes cytometric or electron microscopic studies of the associated microflora possible [Kharchuk et al., 2022].

When studying bacteria from soil samples, bottom sediments, and macrophyte thalli, preliminary physical and chemical treatment of samples is generally used [Danovaro et al., 2001; Kallmeyer et al., 2008; Lunau et al., 2005; Pugovkin, 2016; Zvyagintsev, 1970]. This methodological approach ensures desorption and release of microorganism cells from particles of various nature which greatly complicate identification of bacterial cells independently of method of their registration [Danovaro et al., 2001; Kallmeyer et al., 2008; Morono et al., 2009; Weinbauer et al., 1998].

The most commonly used chemical reagents are various surfactants: non-ionic (Tween 80) and ionic (sodium pyrophosphate) [Danovaro, Middelboe, 2010; Danovaro et al., 2001; Frischer, Danforth, 2000]. The use of methanol which destroys polysaccharide exopolymers that hold microorganism cells on organic or inorganic particles is also known [Kallmeyer et al., 2008; Lunau et al., 2005]. In addition to chemical treatment of samples, subsequent mechanical treatment is proposed: stirring, ultrasonic treatment, and centrifugation [Kallmeyer et al., 2008; Lindahl, Bakken, 1995]. It is stated that this approach allows, for example, to increase the abundance of registered bacteria in marine sediments by several orders of magnitude and to determine abundance of epiphytic bacteria on *Macrocystis integrifolia* thalli, whereas standard methods failed to do so [Velji, Albright, 1986]. In this case, it is necessary to use the correct processing mode in order to extract maximum abundance of microorganisms from a sample, not to destroy them, and not to reduce bacterial abundance during their subsequent quantitative count [Pugovkin, 2016]. In literature, we did not find any works on using a similar methodological approach in the study of microflora associated with *A. platensis* culture.

Therefore, the aim of this work was to analyze known methods of physical and chemical sample preparation for effective desorption and subsequent release of bacterial cells and to test them in the study of *Arthrospira platensis* suspension, as well as to determine quantitative indicators and morphological characteristics of microflora associated with the cyanobacteria culture using flow cytometry and scanning electron microscopy.

MATERIAL AND METHODS

Plant material. The object of the study was an algologically pure non-axenic culture of *Arthrospira (Spirulina) platensis* (Nordstedt) Gomont (strain IBSS-31) from the collection of IBSS Department of Biotechnology and Phytoresources [batch cultivation, temperature +20…+21 °C, continuous illumination, light intensity on the surface of the culture medium 5 klx (lux meter Yu-116, Russia)].

At the first stage (development of the method of preliminary sample preparation of cyanobacterial suspension for cytometry and microscopy), samples were taken from *A. platensis* collection culture daily for a week.

At the second stage, abundance of associated microflora was studied at various stages of *A. platensis* cultivation. At the stationary phase, the morphological structure of the associate was analyzed in detail: linear sizes of bacteria were determined, and their biomass was calculated.

To reduce the initial bacterial contamination during *A. platensis* preparation for the experiment and further, sterile dishes, consumables, and solutions were used: Zarrouk medium for culturing cyanobacteria [Faucher et al., 1979] and distilled water for washing. *A. platensis* trichomes (a paste) were concentrated by filtration *via* gauze (100–105 PE) and then washed three times with distilled water (in a ratio of 1 : 3) and two times with a nutrient medium. For maximum removal of associated microflora settling on mucous sheaths of cyanobacteria, the paste was placed in test tubes with a nutrient medium and centrifuged for 5 min at 3,000 rpm (centrifuge OPN-3 UKhL 42, Russia), the supernatant was removed, and the nutrient solution was again added to the sediment. This procedure was repeated three times.

The *A. platensis* paste purified in this way (2 mL) was transferred to glass flasks (volume of 0.5 dm^3, medium volume of 0.3 dm^3, and layer height of 5 cm) and grown by a batch culture technique at a temperature of +20…+25 °C on a luminostat (continuous illumination and light intensity on the culture medium surface of 10 klx). The optical density of cyanobacteria was monitored daily. Sampling for assessing the associated microflora was carried out once a week covering all stages of *A. platensis* growth. The experiment lasted for 35 days.

Preparation of *A. platensis* suspension for micoscopic and cytometric studies. When studying the associated microflora, all reagents were filtered *via* a filter with a pore size of 0.2 μm. Sterile 2-mL and 15-mL test tubes were used as glassware. An aliquot of *A. platensis* suspension (5–10 mL) was fixed with glutaraldehyde to a final concentration of 2.5% and stored in a refrigerator at +4 °C for no more than two weeks.

Preparation of *A. platensis* suspension without chemical treatment. To separate bacteria from *A. platensis* trichomes, 2 mL of the fixed suspension were thoroughly mixed and centrifuged (Microspin FV-2400, Biosan, Latvia) for 5 min at 3,000 rpm; the initial total abundance of bacteria (start) was determined in the supernatant using flow cytometry after preliminary staining with SYBR Green I fluorochrome (see below). Then, a series of washes of the entire sediment volume was performed (see below).

Treatment of *A. platensis* suspension with sodium pyrophosphate ($Na_4P_2O_7$). A 50 mM sodium pyrophosphate solution was added to a test tube with fixed *A. platensis* to a final concentration of 5 mM in a sample, and this mixture was incubated for 15 min in the dark at room temperature [Danovaro, Middelboe, 2010; Danovaro et al., 2001]. After incubation, for more complete desorption and separation of bacteria from a non-biological fraction and *A. platensis* trichomes, all samples were mixed for 10–15 s with a vortex (Microspin FV-2400) and then processed for 15 min at 0 °C

in an ultrasonic unit (Unitra Unima 01SZTYN UM-4, VA140, V220, 50 Hz). For the final release of bacteria from the sediment, samples were centrifuged (5 min at 3,000 rpm), and the initial total abundance of bacteria (start) was determined in the supernatant using flow cytometry after staining with SYBR Green I fluorochrome. The remaining supernatant was poured off, and a series of washes was performed for the entire sediment 3–10 times (see below).

Treatment of *A. platensis* suspension with methanol (CH_3OH). Methanol was added to a test tube with fixed *A. platensis* to a final concentration of 10% in a sample and mixed for 10–15 s with a vortex. Then, samples were kept in an ultrasonic unit for 15 min at +35 °C, mixed again, and centrifuged (5 min at 3,000 rpm) [Kallmeyer et al., 2008; Lunau et al., 2005]. Similar to the treatment of a sample with sodium pyrophosphate, an aliquot of the supernatant (start) was stained with a fluorochrome and studied in a flow cytometer; a series of washes of the sediment were performed (see below).

A series of washing procedures (both for samples without chemical treatment and samples exposed to sodium pyrophosphate or methanol). For more complete release of remaining microorganisms, 1 mL of sterile Zarrouk medium was added to the entire volume of the sediment [after aliquot sampling for the initial determination of abundance (start)], resuspended with a vortex, and centrifuged again for 5 min at 3,000 rpm. An aliquot of the supernatant was stained with SYBR Green I fluorochrome, and bacterial abundance was determined by flow cytometry (see methodology of staining with fluorochrome and cytometric measurements below). The washing procedure was repeated 3–10 times.

Cytometric studies. Bacteria were stained with SYBR Green I fluorochrome (×1,000, Maclin, China) according to the protocols [Marie et al., 1997; Noble, Fuhrman, 1998]. The fluorochrome solution was prepared at a rate of 10 μL of the stock solution *per* 1 mL of sterile Milli-Q water and stored at −20 °C. Each sample was stained by adding 10 μL of this solution *per* 1 mL of sample and incubated in the dark for 40 min right before measurements. Samples were analyzed on a MACSQuant Analyzer (Miltenyi Biotec, Germany) equipped with three lasers (405, 488, and 635 nm) at "Spectrometry and Chromatography" core facility (IBSS). Bacterial abundance was determined using cell population gating on 2-parameter forward scatter (FS) cytograms and SYBR Green I fluorescence in the green area of the spectrum (FL1 channel, 525 nm) on dimensionless logarithmic scales. FSC Express 7 Research Edition software was used for data processing.

Calculation of bacterial abundance. When calculating total bacterial abundance, volumes of solutions were taken into account: a fixative and introduced reagents. Abundances of microorganisms at the start and after a series of washes were summed up. Based on data obtained, cumulative curves were plotted; the results obtained after 10 washes were taken as 100% of cells desorbed from *A. platensis* suspension.

Microscopic studies. To prepare microscopic slides, various options were tested: only stirring and centrifugation of *A. platensis* suspension (without chemical treatment); with treatment of the suspension with sodium pyrophosphate or methanol followed by complex mechanical treatment (stirring, ultrasonic treatment, and centrifugation).

For fluorescence microscopy (hereinafter FM), 0.5–1 mL of *A. platensis* supernatant was placed in a filter unit and stained with DAPI fluorochrome (4',6-diamidino-2-phenylindole, Servicebio, China). The concentration of the working fluorochrome solution was 2 $\mu g \cdot mL^{-1}$; 50 μL of dye *per* 1 mL of the supernatant were added to a sample, and it was stained for 2 min in the dark [Hoff, 1988]. After staining, the sample was concentrated on a polycarbonate filter with a pore diameter of 0.2 μm (manufactured

by Joint Institute for Nuclear Research, Dubna, Russia); the vacuum during filtration did not exceed 0.2 atm. To reduce autofluorescence, filters were pre-stained for 24 h with irgalan black (2 g in 0.2% acetic acid) [Hobbie et al., 1977]. The material was studied under an Olympus CX43 microscope (Japan), magnification ×1,000, with excitation in the ultraviolet area of the spectrum (358 nm) and emission in the blue one (461 nm).

To prepare samples for scanning electron microscopy (hereinafter SEM), 0.5–1 mL of *A. platensis* supernatant was concentrated onto a polycarbonate filter with a pore diameter of 0.2 μm (manufactured by Joint Institute for Nuclear Research). Then, samples were dehydrated in a series of ethanol dilutions: 20, 30, 50, 75, 96, and 100% [Bratbak, 1993]. A Leica EM CPD300 critical point dryer (Germany) was used to dry the samples at the critical point (1.5–2.5 h). A Leica EM ACE200 vacuum coater (Germany) was used for sputter coating (Au/Pd, 0.5–1.0 min). Samples were viewed under a Hitachi SU3500 scanning electron microscope (Japan), magnification ×4,000.

Morphological studies. Determination of cell sizes and calculation of bacterial biomass. Sizes of bacteria (according to SEM data) were established using ImageJ 1.50i software [National Institutes of Health, the USA, Java 1.6.0_20 (32-bit)]. A total of 475 cells were measured, their linear sizes were registered, and contribution of each morphological group of bacteria to abundance was assessed. The volume of cocci was calculated applying a formula for the volume of a sphere ($V_{sph} = 1 / 6\pi d^3$), where d is the diameter of cocci. The volume of rods and curved forms was determined by a formula for the volume of a cylinder ($V_{cyl} = 1 / 4\pi d^2 h$), where d is the width, and h is the length [Potapova, Korolevskaya, 1991; Romanenko, Dobrynin, 1973]. When calculating the volume of bacterial cells, a cell "shrinkage" conversion factor of 1.6 was introduced [Potapova, Korolevskaya, 1991; Sazhin et al., 1987]. Carbon content in bacterial cells was taken as 11% of raw biomass [Troitsky, Sorokin, 1967].

The biomass was calculated:

a) taking into account bacterial abundance (according to flow cytometry data) and the average cell volume (according to SEM data);
b) taking into account bacterial abundance (according to flow cytometry data) and a conversion factor of 2.0×10^{-14} g C·cell^{-1} [Lee, Furman, 1987].

Statistical analysis was carried out in Statistica package (Data Analysis Software System), version 10 (StatSoft, Inc., https://www.statsoft.com/). Graphs were plotted in SigmaPlot 10.0 (Systat Software, Inc.), Grapher 8 (Golden Software, Inc., https://www.goldensoftware.com/), and Surfer 11 (Golden Software, Inc.).

RESULTS

A series of preliminary experiments was carried out to determine the efficiency of bacterial separation from detritus particles and cyanobacterial cells:

- without chemical treatment (only centrifugation and stirring of *A. platensis* suspension);
- with treatment of the suspension with chemical reagents (sodium pyrophosphate or methanol) and subsequent complex mechanical treatment which involves stirring, ultrasonic treatment, and centrifugation.

In each case, a series of 10 washing procedures was additionally carried out. Bacterial abundance was registered by flow cytometry after sample staining with SYBR Green I fluorochrome.

The experiment showed as follows: both without chemical treatment and after exposure to sodium pyrophosphate or methanol, 14.8 to 45.9% of bacterial cells [(27.1 ± 9.0) % on average] were initially washed out of the culture suspension (start). After a series of 3 washes, abundance of microorganisms significantly increased (paired *t*-test; $p < 0.05$) to 82.0–94.3% [(88.9 ± 6.3) % on average] (Fig. 1A–C).

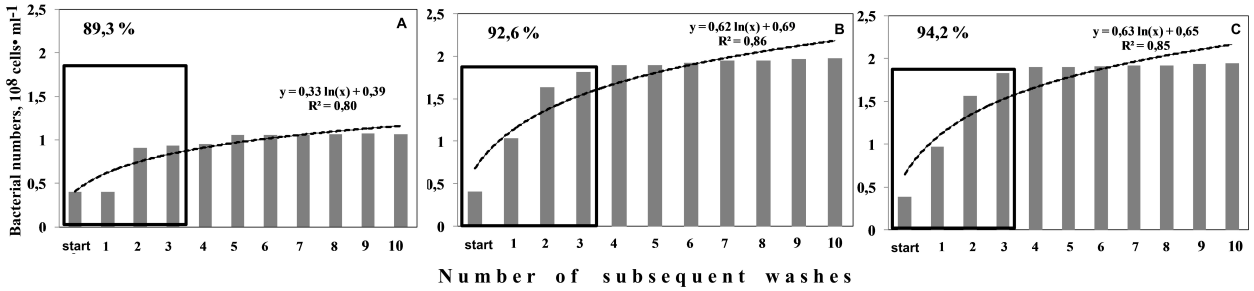

Fig. 1. Initial bacterial abundance (start) and abundance after washes (1–10) in *Arthrospira platensis* suspension after staining with SYBR Green I and cell counting by flow cytometry (*A. platensis* collection culture at the stationary phase, batch cultivation): A, without chemical treatment (stirring and centrifugation only); B, after exposure to methanol, stirring, ultrasonic treatment, and centrifugation; C, after exposure to sodium pyrophosphate, stirring, ultrasonic treatment, and centrifugation

With a gain in the number of washes to 4–10, bacteria continued to wash out of the sediment, but the difference between the "potential" cumulative cell abundance during subsequent washes was non-significant (paired *t*-test; $p > 0.05$). Thus, without using a chemical reagent (only stirring and centrifugation), another 11% of associated bacteria were extracted from *A. platensis* suspension. When using sodium pyrophosphate or methanol and carrying out subsequent complex mechanical treatment (stirring, ultrasonic treatment, and centrifugation), another 6–7% of microorganisms were washed out. Of course, even after 10 washing procedures, not all bacteria were washed out of the sediment, and some remained associated with particles. However, their abundance was insignificant and fairly constant, and this could be neglected.

In general, in this preliminary series of experiments, the total abundance of bacteria in *A. platensis* suspension without chemical treatment (centrifugation only) averaged $(1.24 \pm 0.21) \times 10^8$ cells·mL^{-1}, while when using sodium pyrophosphate or methanol, ultrasonic treatment, and centrifugation, the value increased to $(2.10 \pm 0.50) \times 10^8$ cells·mL^{-1}. Thus, with preliminary physical and chemical sample preparation, the efficiency of cell release was on average 1.8 times higher. Subsequently (at the second stage), we used a protocol prescribing exposure to a reagent (methanol), stirring, ultrasonic treatment, and centrifugation.

At the second stage, we tested physical and chemical treatment (exposure to methanol, stirring, ultrasonic treatment, and centrifugation) of *A. platensis* at various stages of cultivation with determining abundance of associated microflora by flow cytometry. The number of associated bacteria at the beginning of the experiment was 6.7×10^6 cells·mL^{-1}. As the culture grew, abundance of bacteria gradually rose by 18–25 times, up to values 1.5×10^8 to 2.2×10^8 cells·mL^{-1}, averaging $(0.9 \pm 0.6) \times 10^8$ cells·mL^{-1} (Fig. 2).

Cytometric measurements were monitored by microscopic studies which pursued two goals. By FM, we checked that the supernatant (after sample preparation) contained bacteria, and that they got into a cytometer capillary. By SEM, we detailed the morphological structure of the bacterial associate. We showed as follows: without chemical treatment of the cyanobacterial suspension (in the case

of centrifugation only), fragments of *A. platensis* trichomes often got onto microscopic slides. This complicated the visualization of bacterial cells (in the case of SEM) and made it extremely difficult to view a slide (in the case of FM) due to a high autofluorescence of cyanobacteria and a rapid fading of the field of view.

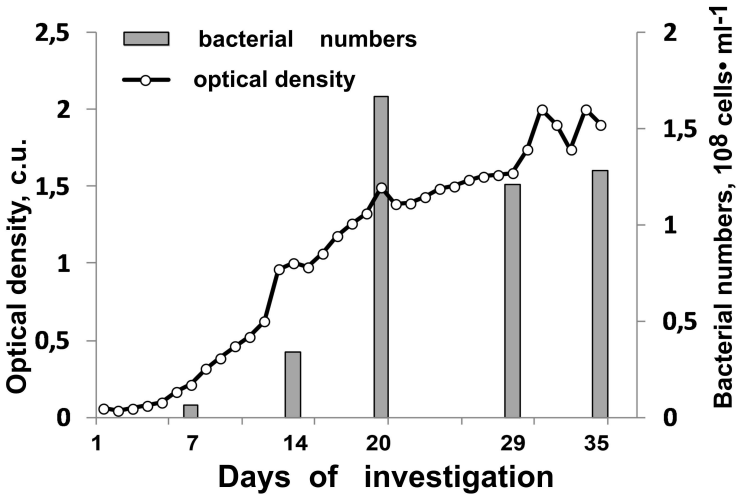

Fig. 2. Abundance of associated microflora at different stages of *Arthrospira platensis* cultivation after preliminary physical and chemical treatment of suspension (exposure to methanol, stirring, ultrasonic treatment, and centrifugation) followed by staining with SYBR Green I and cell counting by flow cytometry

Slides after preliminary complex preparation (exposure to methanol, stirring, ultrasonic treatment, and centrifugation) were the "cleanest" from cyanobacterial trichomes. Fig. 3A clearly shows that rods predominated in microflora from *A. platensis* suspension at the stationary phase. By SEM, the morphological structure of associated microflora was studied in detail, and the prevalence of rod-shaped bacteria and the presence of curved forms and cocci were confirmed [Laboratory Workbook, 2020; Schlegel, 1987] (Fig. 3B).

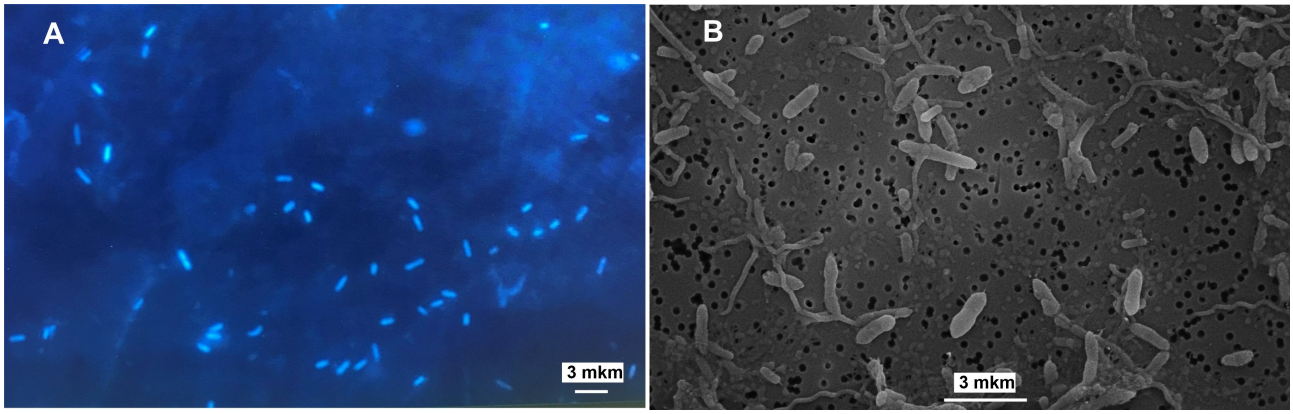

Fig. 3. Microflora associated with *Arthrospira platensis* culture (stationary phase, batch cultivation) after physical and chemical treatment (exposure to methanol, stirring, ultrasonic treatment, and centrifugation): A, according to fluorescence microscopy data after staining with DAPI fluorochrome; B, according to scanning electron microscopy data

In associated microbial community of *A. platensis* at the stationary phase, large rod-shaped cells dominated (67.2% of the total abundance of counted cells), with the volume of (0.12 ± 0.01) μm^3. The cell length varied within 0.46–7.18 μm, and the width, within 0.11–0.94 μm. The contribution of curved forms (spirilla and vibrio) accounted for 30.2%. Their volume was (0.07 ± 0.01) μm^3; length was 0.11–11.04 μm; and width was 0.05–0.33 μm. Even less common (2.6% of cases) were rounded forms, with the diameter of 0.19–0.55 μm and volume of (0.03 ± 0.01) μm^3 (Table 1, Fig. 3A, B). On average, the volume of a bacterial cell calculated from measurements of 475 cells was (0.10 ± 0.01) μm^3 and varied 0.001 to 1.03 μm^3.

Table 1. Morphological parameters of microflora associated with *Arthrospira platensis* culture (stationary phase, batch cultivation) according to scanning electron microscopy data

Morphotype	% of occurrence	Dimensions, μm		Volume, μm^3
		length/diameter	width	
Rods	67.2	1.35 ± 0.07 0.46–7.18	0.31 ± 0.01 0.11–0.94	0.12 ± 0.01 0.04–1.03
Curved forms	30.2	4.76 ± 0.39 0.11–11.04	0.13 ± 0.009 0.05–0.33	0.07 ± 0.01 0.004–0.35
Cocci	2.6	0.36 ± 0.05 0.19–0.55	–	0.03 ± 0.01 0.0004–0.09

Note: above the line, mean ± standard deviation; under the line, range (min–max).

Considering the fact that sample preparation for SEM was quite “rigid” (treatment with fixative, dehydration in ethanol, drying at the critical point, and sputter coating), we used the cell “shrinkage” conversion factor of 1.6 [Potapova, Korolevskaya, 1991; Sazhin et al., 1987], as mentioned above. Thus, the obtained average volume of a bacterial cell in the cyanobacterial culture, (0.10 ± 0.009) μm^3, was corrected to (0.16 ± 0.02) μm^3. Taking into account carbon content in bacterial cells (11% of raw biomass) [Troitsky, Sorokin, 1967] and the average bacterial abundance determined by flow cytometry [(1.39 ± 0.28) × 10^8 cells·mL^{-1}], biomass of microflora associated with *A. platensis* culture was 0.022 g·L^{-1}. Also, biomass was calculated using the conversion factor of 2.0×10^{-14} g C·cell^{-1} [Lee, Furman, 1987] and the same bacterial abundance. In this case, biomass of bacteria associated with *A. platensis* culture was 0.025 g·L^{-1}.

DISCUSSION

According to various manuals on *A. platensis* growing, non-axenic cultures usually contain 3×10^4 to 1.6×10^6 cells·mL^{-1} (Table 2) [Falquet, Hurni, 2006; Khadad et al., 1990; Tarhova, 2005; Wu, Pond, 1981]. However, those studies were carried out using classical microbiological methods of bacteria seeding on nutrient media, but it is known that not all bacteria are capable of growing on media [ZoBell, 1943].

Therefore, in this study, a more accurate and up-to-date technique was used for recording abundance of microorganisms: flow cytometry after staining with bright SYBR Green I fluorochrome. Notably, the efficiency of bacterial release was governed by preliminary physical and chemical sample preparation and a series of washes. This is consistent with the opinion of authors who use similar methods when analyzing abundance of microorganisms in samples containing a large amount of particles of various nature. Thus, for bottom sediments, the efficiency of bacterial release from the sediment

at the start and after three washes was 60–95% of the "potential" cumulative abundance of microorganisms [Danovaro, Middelboe, 2010; Rylkova et al., 2019; Siem-Jørgensen et al., 2008]. For microflora associated with *A. platensis* culture (this study), similar values were obtained: 82.0–94.3%. Washing procedures slightly increased the sample treatment time but ensured the most accurate cell counting.

Table 2. Abundance of bacteria associated with different non-axenic algal cultures (mean values are given with confidence intervals)

Algal culture	Bacterial abundance, cells·mL^{-1}	Method	References
Arthrospira platensis	from 3×10^4 to 6×10^5	plating on nutrient media	Tarhova, 2005; Khadad et al., 1990; Falquet, Hurni, 2006; Wu, Pond, 1981
Various strains of *Arthrospira platensis*	from 2.1×10^6 to 1.3×10^7	staining with DAPI, FM	Shiraishi, 2015
Scenedesmus obliquus	from 1.1×10^7 to 1.5×10^7	staining with DAPI, FM	Danger et al., 2007
Pavlova lutheri, *Platymonas suecica*	$(5.1 \pm 3.6) \times 10^6$, $(1.1 \pm 0.4) \times 10^7$	staining with acridine orange, FM	Nicolas et al., 1989
Chlorella vulgaris, *Platymonas viridis*, *Dunaliella salina*, *Isochrysis galbana*, *Thalassiosira weissflogii*	from 10^5 to 10^7	staining with SYBR Green I, FC	Rauen et al., 2011
Arthrospira platensis at various stages of cultivation	from 6.7×10^6 to 1.7×10^8, $(0.9 \pm 0.6) \times 10^8$	exposure to methanol, US, CF; staining with SYBR Green I, FC, SEM	this study

Note: FM, fluorescence microscopy; FC, flow cytometry; US, ultrasonic treatment; CF, centrifugation; and SEM, scanning electron microscopy.

The quantitative characteristics of associated microflora obtained at various stages of the cyanobacteria cultivation, 6.7×10^6 to 1.7×10^8 cells·mL^{-1}, are consistent with data provided in literature for algal cultures when registering bacterial cells under a fluorescence microscope or in a flow cytometer (Table 2). It is known that for various strains of *A. platensis*, abundance of associated bacteria was 2.1×10^6 to 1.3×10^7 cells·mL^{-1} (when studied by FM after staining with DAPI) [Shiraishi, 2015]. For *S. obliquus* (FM, staining with DAPI), abundance of microorganisms varied 1.1×10^7 to 1.5×10^7 cells·mL^{-1} [Danger et al., 2007]. In *Pavlova lutheri* and *Platymonas suecica* cultures, the values were $(5.1 \pm 3.6) \times 10^6$ and $(1.1 \pm 0.4) \times 10^7$ cells·mL^{-1}, respectively (FM, staining with acridine orange) [Nicolas et al., 1989]. Based on flow cytometry data obtained after staining with SYBR Green I, T. Rauen *et al.* [2011] reported as follows: abundance of microflora associated with cultures of *C. vulgaris*, *P. viridis*, *D. salina*, *I. galbana*, and *T. weissflogii* was 10^5 to 10^7 cells·mL^{-1}.

Thus, the used approach of preliminary physical and chemical preparation of suspension (exposure to methanol, stirring, ultrasonic treatment, and centrifugation) is quite applicable for studying microflora associated with *A. platensis* when recording bacterial abundance by flow cytometry after staining with SYBR Green I.

As known, to calculate biomass of microflora, in addition to abundance of microorganisms, it is necessary to know their volume (therefore, linear sizes of cells) or to use conversion factors frequently proposed in literature, for example, those within 1.7×10^{-14} to 2.0×10^{-14} g C·cell^{-1} [Alongi, 1988; Lee, Furman, 1987].

It turned out that used methods of preliminary sample preparation (exposure to methanol, stirring, ultrasonic treatment, and centrifugation) of the cyanobacterial suspension significantly improved the quality of microscopic slides. However, it should be taken into account as follows: for any microscopy, bacterial cells undergo additional processing procedures. In the case of light microscopy and FM, in addition to fixation, several procedures are carried out (sample staining, its filtration, and drying), and each stage can lead to deformation and compression of cells. As known, after fixation with formalin and staining with erythrosine, bacterial cells "shrink" by 1.3–2.5 times (1.6 times on average) [Potapova, Korolevskaya, 1991; Sazhin et al., 1987; Troitsky, Sorokin, 1967]. The use of fluorochrome dyes and FM (when there is no stage of a slide drying) minimized the distortion of linear sizes, but there was a disadvantage: the rapid fading of the background (in the case of staining with acridine orange). Besides, the volume of bacteria stained with DAPI was 59% of the volume of cells stained with acridine orange [Posch et al., 2001; Suzuki et al., 1993]. It should be noted that measurements and determination of the morphological affiliation of bacteria are often at the limit of the resolving power of optical microscopes.

SEM that can magnify images more than 1,000 times noticeably reduces the errors related to cell measurements and determination of their morphotypes. However, a rather more "rigid" sample preparation for a scanning electron microscope (dehydration in ethanol/acetone, subsequent drying at the critical point, and sputter coating) can also lead to cell deformation. When studied by SEM, "true" linear sizes of bacteria are known to be underestimated by 11–37% (by 24% on average) compared to those analyzed by FM [Fuhrman, 1981]. In this case, the average volume of a bacterial cell should be corrected – increased by 2.5 times: it is the upper limit of the "shrinkage" conversion factor proposed by Russian researchers [Potapova, Korolevskaya, 1991; Sazhin et al., 1987; Troitsky, Sorokin, 1967].

In this study, when calculating cell volume and biomass of microorganisms, we used the average value of the "shrinkage" conversion factor of 1.6 [Potapova, Korolevskaya, 1991; Sazhin et al., 1987] and the value of carbon content in bacterial cells of 11% of raw biomass [Troitsky, Sorokin, 1967]. Interestingly, biomass of microflora associated with *A. platensis* culture established in this way (0.022 $g \cdot L^{-1}$) was close to biomass determined using the conversion factor of 2.0×10^{-14} g C·$cell^{-1}$ (0.025 $g \cdot L^{-1}$) [Lee, Furman, 1987].

As we have previously indicated for marine bacterioplankton [Rylkova, Polikarpov, 2021], a detailed study of the morphological structure of the microbial community requires determination of linear sizes of bacterial cells. However, in the absence of data on direct cell measurements, when calculating biomass, conversion factors can be used [Alongi, 1988; Lee, Furman, 1987].

Conclusions:

1. When determining abundance of microflora associated with *Arthrospira (Spirulina) platensis*, it is proposed to use complex physical and chemical sample preparation of the culture suspension (methanol treatment, stirring, ultrasonic treatment, and centrifugation). On average, (27.1 ± 3.9) % of "potential" cumulative bacterial abundance was initially extracted from the cyanobacterial culture. During the following three washes, abundance of microorganisms significantly increased to an average of (88.9 ± 6.3) % (paired *t*-test; $p < 0.05$). Then, in the sediment consisting of *A. platensis* trichomes, abundance of microorganisms remained insignificant, 6–7%, and could be neglected.
2. An optimal registration method for determining abundance of bacteria associated with *A. platensis* culture was flow cytometry after sample staining with bright SYBR Green I fluorochrome. Abundance of bacterial associates at different stages of *A. platensis* growth varied 6.7×10^6 to 1.7×10^8 cells·mL^{-1} and averaged (0.9 ± 0.6) × 10^8 cells·mL^{-1}. These values are comparable with abundance of bacteria associated with other non-axenic algal cultures.

3. According to scanning electron microscopy data, the community of associated microflora of *A. platensis* at the stationary phase was dominated by large rod-shaped cells (67.2%); the contribution of spiral forms was 30.2%; and spherical forms accounted for 2.6%. The mean volume of a bacterial cell, with a cell "shrinkage" conversion factor taken into account, was (0.16 ± 0.02) μm^3.
4. Biomass of microorganisms in *A. platensis* culture based on "true" cell measurements and using a conversion factor of 2.0×10^{-14} g C·cell^{-1} was 0.02 and 0.025 g·L^{-1}, respectively. To calculate biomass of bacteria, in the absence of data on linear sizes of cells, it is entirely acceptable to use known conversion factors.

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ОСОБЕННОСТИ ПРЕДВАРИТЕЛЬНОЙ ПРОБОПОДГОТОВКИ КУЛЬТУРЫ *ARTHROSPIRA (SPIRULINA) PLATENSIS* ПРИ ИССЛЕДОВАНИИ АССОЦИИРОВАННОЙ С НЕЙ МИКРОФЛОРЫ МЕТОДАМИ ПРОТОЧНОЙ ЦИТОМЕТРИИ И СКАНИРУЮЩЕЙ ЭЛЕКТРОННОЙ МИКРОСКОПИИ

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Контроль количественных характеристик ассоциированной с альгокультурами микрофлоры важен в биотехнологических исследованиях. С целью более полного выявления и детального анализа сопутствующей микрофлоры в культуре *Arthrospira (Spirulina) platensis* использованы методы физико-химической пробоподготовки образцов для последующего изучения суспензии с помощью проточной цитометрии после окраски клеток флуорохромом SYBR Green I и сканирующей электронной микроскопии. Показано, что оптимальными вариантами пробоподготовки

являлись применение химического реагента (пирофосфата натрия или метанола), перемешивание суспензии, ультразвуковая обработка, центрифугирование и дополнительные промывочные процедуры. При этом из культуры цианобактерий изначально извлекалось в среднем (27,1 ± 3,9) % «потенциальной» кумулятивной численности бактерий. При последующих трёх промывках количество микроорганизмов достоверно увеличивалось в среднем до (88,9 ± 6,3) % (парный *t*-тест; $p < 0,05$). Далее в осадке количество микроорганизмов оставалось незначительным, 6–11 %, и этим можно было пренебречь. Показано, что численность бактерий на различных этапах культивирования *A. platensis* изменялась от $6,7\times10^6$ до $1,7\times10^8$ кл.·мл$^{-1}$. В морфологической структуре ассоциированной микрофлоры (на стационарной стадии роста *A. platensis*) доминировали крупные палочковидные клетки (67,2 %), доля извитых форм была почти вдвое ниже (30,2 %), ещё реже встречались округлые формы (2,6 %). Средний объём бактериальной клетки составлял (0,16 ± 0,02) мкм^3, биомасса — 0,022–0,025 г·л$^{-1}$. Полученные величины показателей сопутствующей микрофлоры сопоставимы с данными, приведёнными в литературе для *A. platensis* и других альгокультур. Предложенные методы обработки суспензии *A. platensis* существенно повышали эффективность высвобождения бактерий, способствовали очищению пробы от фрагментов трихом цианобактерий, детрита и прочих частиц и обеспечивали возможность исследования ассоциированной микрофлоры с использованием проточной цитометрии и сканирующей электронной микроскопии.

Ключевые слова: цианобактерии, *Arthrospira (Spirulina) platensis*, ассоциированная микрофлора, бактерии, микроорганизмы, численность бактерий, биомасса бактерий, методы пробоподготовки, десорбция клеток, физико-химическая обработка, ультразвуковая обработка, люминесцентная микроскопия, сканирующая электронная микроскопия, проточная цитометрия