# Ecological Development and Genetic Diversity of *Microcystis aeruginosa* from Artificial Reservoir in Russia

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***Microcystis aeruginosa* is a well-known *Cyanobacterium* responsible for the formation of toxic water blooms around the world. Shallow, warm, and eutrophic reservoirs provide the most favourable conditions for *M. aeruginosa* development. Numerous studies have been devoted to this species, but there still is a necessity to develop additional approaches for the monitoring of cyanobacteria in reservoirs. In this study, *M. aeruginosa* in the water column of a hypereutrophic Siberian reservoir was investigated by fluorescence, light, and electron microscopy as well as genetic analysis using a *mcy*E marker. Here, we demonstrate the genetic diversity and features of the fluorescence spectra for different ecotypes of this species. We suggest that a fluorescence approach can be used to identify *M. aeruginosa* in a natural environment in order to increase the effectiveness of ecological monitoring and water quality evaluation.**

*Keywords*: *Microcystis aeruginosa*, fluorescence method, euclidean distance, *mcy*E gene

*Microcystis aeruginosa* (Kütz.) is one representative of phylum *Cyanobacteria* included in the domain Bacteria. *M. aeruginosa* occurs as an unicellular or colonial organism in the algal-bacterial plankton communities of natural and artificial reservoirs (Kim *et al*., 2001; Lehman *et al*., 2005). *M. aeruginosa* has received special attention since it produces microcystins, group of the most dangerous hepatotoxins to animals and human beings. The occurrence of *M. aeruginosa* active strains expressing microcystin synthetase genes indicates the probability of microcystin production in any given reservoir. At the same time, the size of *Microcystis* colonies correlates positively with toxin production. Microcystins are mainly produced by large colonies (more than 100 μm), as small colonies (less than 50 μm) have a low level of micocystin production (Kurmayer *et al*., 2003).

Ecological success of *M. aeruginosa* is based on the proper regulation of cell buoyancy by gas vacuoles (Mlouka *et al*., 2004) as well as its life cycle, which includes planktonic (active) and benthic (essential for survival under unfavorable conditions) forms (Latour *et al*., 2004). Identification and exploration of the ecological form and physiological state of *M. aeruginosa* in a particular reservoir are essential in determining the mechanism of mass development and for developing practical measures aimed at preventing "water bloom" (Kolmakov, 2006).

Artificial piscicultural (a fish farm) reservoirs are one of the most favourable environments for *M. aeruginosa* mass (Morozova *et al*., 2001; Kolmakov and Gladyshev, 2003). Therefore, the reservoir-cooler of Beryozovskaya State District

Power Station No. 1 (SDPS No. 1) (Sharypovo, Krasnoyarsk Territory, Russia) was used as a model for *M. aeruginosa* exploration. Mass development of cyanobacteria and intoxication has already been reported in water bodies in Siberia (Belykh *et al*., 2010).

This work was aimed at studying the seasonal dynamics, occurrence of different ecological forms, and diversity of microcystin-producing genotypes of *M. aeruginosa* in the algal-bacterial complex of the reservoir-cooler at Beryozovskaya SDPS No. 1, and in doing so, approve fluorescence analysis as a new approach for the prediction of harmful blooms in artificial reservoirs.

## Materials and Methods

### Characteristics of reservoir and sample collection
The reservoir-cooler at Beryozovskaya SDPS No. 1 (Krasnoyarsk Territory, central station coordinates - N 55 33′44″, E 89 02′24″) is an artificial water body that was created by damming of the Beresh River flow at the point of confluence with the Bazyr and Kadat Rivers. The reservoir-coolers is used for cooling the large capacitors of the power station. After passing through the cooling system of the power station, water is discharged into the reservoir in a heated state, thereby introducing a large amount of additional heat. It is a lake-type reservoir, it has a wide-open oval area. The full volume of the reservoir-cooler is 193 mil. m$^3$ ; the water surface has an area of 35.7 km$^2$; average depth - 5.8 m; maximum depth in the central part - 15 m. The thermal conditions of the reservoir depend, to a considerable extent, on the meteorological conditions as well as the regime of the station, which provides a constant transit water flow from the discharge canal to water intake. The water of the reservoir

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belongs to the calcium-hydrocarbonate type of freshwater group with average mineralization and alkaline properties.

Phytoplankton was sampled using 10 L plastic buckets from the surface at different sites of the reservoir during three vegetative seasons (2004-2006) from May to October. Benthic colonies of *M. aeruginosa* were collected from the discharged canal using a glass tube with a bulb. Samples were stored in dark plastic 500 ml bottles in a dark cool place and transferred to the laboratory. Phytoplankton samples were fixed in glutar aldehyde (final concentration 1%) for electron microscopy and in ethanol (final concentration 70%) for DNA extraction.

### Microscopy and imaging

To identify species of the autotrophic plankton community by light microscopy, samples (volume of 100 ml) were filtered through a membrane filter (Vladipor No.7) and fixed with Lugol's solution. Taxonomical and biovolume quantitative analysis of plankton micro-organisms was conducted in a Goryaev's chamber (volume 0.9 mm$^3$) using an Axiovert 200 (Zeiss, Germany) microscope at a magnitude of ×200, and species keys devised (Gollerbakh *et al.*, 1953; Komárek and Anagnostidis, 1999). Total cyanobacterial biomass was estimated from the volume of the individual cells or colonies. Autofluorescence of cyanobacteria was observed using a green filter (DM580). For scanning electron microscopy, samples fixed in glutar aldehyde were filtered through polycarbonate filters (pore diameter 0.22 μm; Millipore, USA) and dehydrated in an elevated concentration of ethanol. Gold coating was conducted in a CPD-030 device (Balzers, Liechtenstein), after which the specimens were observed using a Philips 525 M microscope.

### DNA extraction and sequencing

DNA was extracted from floating colonies of *M. aeruginosa* sampled in August 2006 with a RiboSorb kit (Central Scientific Investigations Institute of Epidemiology, Russia). PCR was carried out with pairs of primers complementary to the aminotransferase (AMT) domain of the *mcy*E gene (Jungblut and Neilan, 2006) encoding polyketide peptide synthetase, which is involved in the non-ribosomal synthesis of hepatotoxic cyanotoxin microcystin. AMTs play a critical role in the biosynthesis of all known microcystin congeners by mediating the transfer of an amino group ($NH_2$) to the Adda moiety (Tillett *et al.*, 2000) or from glutamate semialdehyde to glutamine (Jungblut and Neilan, 2006). The conditions for the PCR reactions were as follows: 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and an extension at 72°C for 10 min. PCR products were checked by 1% agarose gel electrophoresis, purified with a DNA and Gel Band Purification kit (Amersham Biosciences, USA), and cloned using an InsTAclone PCR Cloning kit (Fermentas, Canada). Nucleotide sequences were determined using the CEQ$^{TM}$ 8800 genetic analysis system (Beckman Coulter Inc., USA).

### Phylogenetic analysis

Sequences were aligned using BioEdit v.7.0.1 (Hall, 1999). Trees were constructed using the neighbour-joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) methods. The NJ algorithm was implemented using MEGA package v.4 (Tamura *et al.*, 2007), Kimura 2-parameter model was chosen and trees were assessed with 1,000 bootstrap replicates. ML and MP trees were constructed using PAUP beta v.4.0b10 (Swofford, 2002). For ML analysis, a HKY+G substitution model was chosen as the most appropriate according to

a hierarchical likelihood ratio test using ModelTest v.2.06 (Posada and Crandall, 1998), and trees were assessed with 1,000 bootstraps. MP analysis was carried out by the heuristic search method, and trees were assessed with 1,000 bootstrap replicates.

### Nucleotide sequence accession numbers

Sequences were deposited in the GenBank database (www.ncbi. nlm.nih.gov) under accession numbers GU817039-GU817052.

### Fluorescence analysis

Fluorescence methods allow the identification of green algae, diatoms, and cyanobacteria in phytoplankton based on their differences in fluorescence excitation spectra (Gaevsky *et al.*, 2005). Pigments from algae (chlorophylls *a*, *b*, *c*, carotenoids, including fucoxanthin, phycobilins, etc.) constitute functional groups. Only one specialized molecule, chlorophyll *a*, acts as a reaction center. Other photosynthetic pigments absorb light of different wavelengths and act as antennas that are responsive to light energy. If different spectra of light excitation are used, fluorescence emitted by algae and cyanobacteria can provide information on the taxonomic composition of phytoplankton. It has also been reported that there are five spectral groups of microalgae (Beutler *et al.*, 2002, 2004). To visualize spectral differences in fluorescence, it is necessary to compare Cartesian coordinates of the relationships between fluorescence (F) signals excited by blue (410 nm), blue-green (510 nm), and green (540 nm) spectral regions, which are predominantly absorbed by chlorophyll and carotenoids, including fucoxanthin and phycobilins. A combination of the ratios $F_{410}/F_{510}$ and $F_{540}/F_{510}$ was chosen in the present study and was used to calculate the concentration of chlorophyll *a* ($C^{Chlr}a$) using the formula ($C^{Chlr}a$) = k × $F_{510}$, as shown in our earlier work (Gaevsky *et al.*, 2005). Use of other combinations of relationships, including $F_{510}/F_{410}$, $F_{540}/F_{410}$ or $F_{410}/F_{540}$, $F_{510}/F_{540}$, showed that blue light (410 nm) was ineffective in exciting fluorescence in cyanobacteria, whereas green light (540 nm) had the same properties as those of green algae and diatoms (Golg'd *et al.*, 1986).

Fluorescence analysis of the phytoplankton samples was carried out in the laboratory of the Station Ecology Service no later than 3 h after the sampling. FL3003 fluorometer (Krasnoyarsk, Scientific Manufacturing Enterprise Test, Russia) was used to register the fluorescence, which excites fluorescence with one wide (400-620 nm) and three narrow (410 nm, 510 nm, and 540 nm) spectral bands. The source of the background fluorescence was a water sample subjected to vacuum filtration through a membrane filter with a pore diameter of 0.65-0.75 μm (Gaevsky *et al.*, 2002, 2005).

During the vegetative seasons of 2004, 2005, and 2006, we were able to identify the dates during which the dominant cells were diatoms (May 2004, 2005), *Anabaena flos-aquae* (Bred. ex. Born) (July 2004, 2005), *Aphanizomenon flos-aquae* (L.) Ralfs) (August 2004, 2006), and *M. aeruginosa* (July 2005 and September 2005, benthic form). These dates were established corresponding statistically significant relationship of fluorescence $F_{410}/F_{510}$ and $F_{540}/F_{510}$ in diatoms and cyanobacteria. The same relationships for green algae were taken from our paper (Gaevsky *et al.*, 2005).

### Statistical analysis

One-linkage cluster analysis based on Euclidean distances was carried out according to Jeffers (1978) using STATISTICA software, v.6.0 (StatSoft, Inc.).

**Table 1.** Indicators of cyanobacteria development in June-August in the reservoir-cooler of Beryozovskaya SDPS No.1

| Part of reservoir | Chlorophyll *a* concentration (mg/m$^3$)[a] | | |
|---|---|---|---|
| | 2004[b] | 2005 | 2006 |
| River Kadat outfall | 27.7 (68.0) | 62.1 (87.5) | 27.3 (68.3) |
| Central part | 21.8 (72.6) | 77.6 (85.8) | 51.9 (75.6) |
| Area near the dam | 24.8 (83.1) | 117.3 (85.0) | 53.4 (75.3) |

[a] Weighted average values of concentration (mg/m$^3$) of chlorophyll *a* of cyanobacteria and their percentage in relation to total concentration of pigment (in brackets)
[b] years

## Results

### Phytoplankton composition and morphological characteristics of *Microcystis*

Based on data from 2004-2006, *Anabaena flos-aquae, Aphanizomenon flos-aquae, Gomphosphaeria lacustris* (Chod.), and *Microcystis aeruginosa* were included in the phytoplankton taxonomic list of the reservoir as dominants. Cyanobacteria reached mass development (when chlorophyll *a* concentration exceeded 8 mg/m$^3$) in 2004 during the first 10-day period of July, in 2005 in the second 10-day period of June, and in 2006 in the third 10-day period of June. In 2005, the concentration of cyanobacterial chlorophyll *a* exceeded 100 mg/m$^3$ (Table 1). At the end of the vegetation period (October), *M. aeruginosa* settled to the bottom and formed mats (films) on the submerged peat tussocks. Large visible aggregations of *M. aeruginosa* cells were constantly present during spring and summer in the discharge canal of Beryozovskaya SDPS No. 1. Dynamics of cyanobacterial biomass (*Anabaena flos-aquae, Aphanizomenon flos-aquae,* and *Microcystis aeruginosa*) in the surface layer at the central site of the reservoir during the vegetative season of 2005 are shown in Fig. 1. In 2005, mass development (bloom) of *A. flos-aquae* and *Aph. flos-aquae*
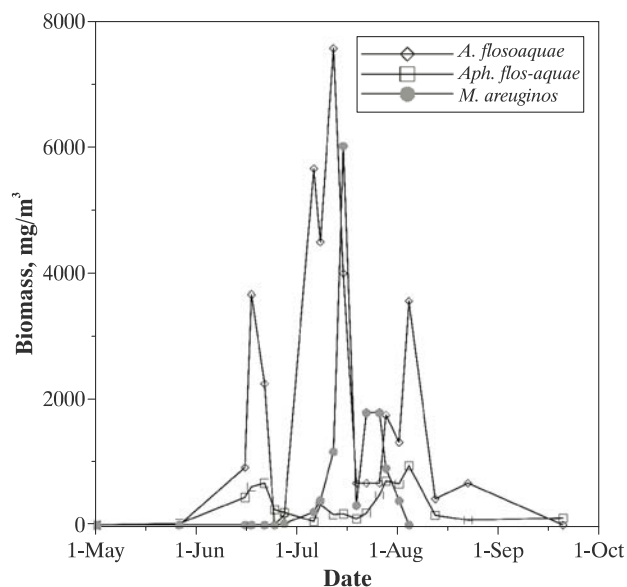
was verified on the 17th of June, 12th of July, and 4th of August. *M. aeruginosa* became the dominant species in terms of biomass on the 15th, 22th, and 26th of July 2005. The average water temperature of the surface layer reached 24.5°C (SD 1.7°C; n=8) during the intensive development (second half of June, first 10-day period of August 2005); pH, 8.9 (SD 0.3; n=8); solar radiation, 5.9 MJoule/m$^2$ a day (SD 2.2; n=24); wind speed, 5.0 m/sec (SD 2.1; n=24).

The colony size of *M. aeruginosa* varied from a few tens of microns to several millimeters. Pelagic colonies were of irregular shape and usually perforated (Figs. 2A and B). Colonial mucus was colourless and homogeneous. The cells were spherical in form with a diameter of 4.3 μm. The general view of the cells using scanning electron microscopy is shown in Figs. 2D-F, and the binary pattern of cell division is shown in Figs. 2E and F. Benthic colonies had a more regular shape, and cells were densely spaced (Fig. 2C).
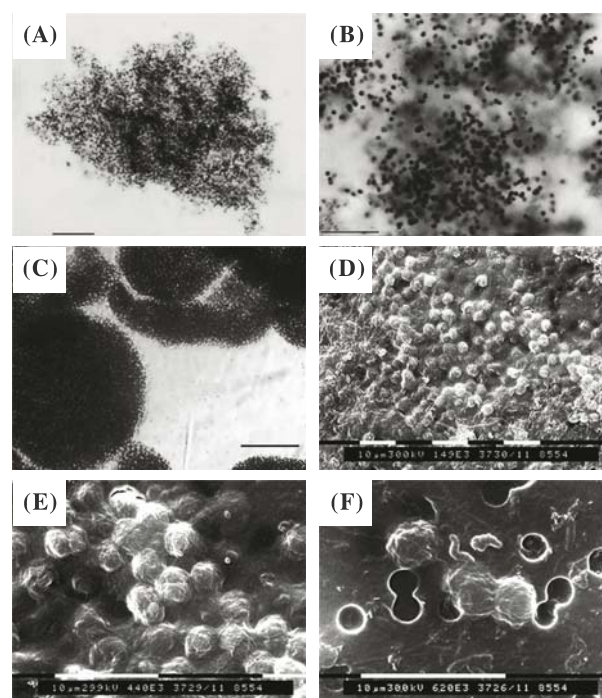


**Fig. 2.** Cyanobacterium *M. aeruginosa* from the reservoir- cooler of Beryozovskaya SDPS No. 1. (A, B) light microscopy (free floating colonies); (C) light microscopy (bentic colonies); (D-F) scanning electron microscopy (free floating colonies). Scale: (A) 100 μm; (B) 50 μm; (C) 500 μm; (D-F) 10 μm.



**Fig. 1.** Dynamics of cyanobacterial biomass (*A. flos-aquae, Aph. flos-aquae,* and *M. aeruginosa*) in the surface layer at central sites of the reservoir during vegetative season of 2005.
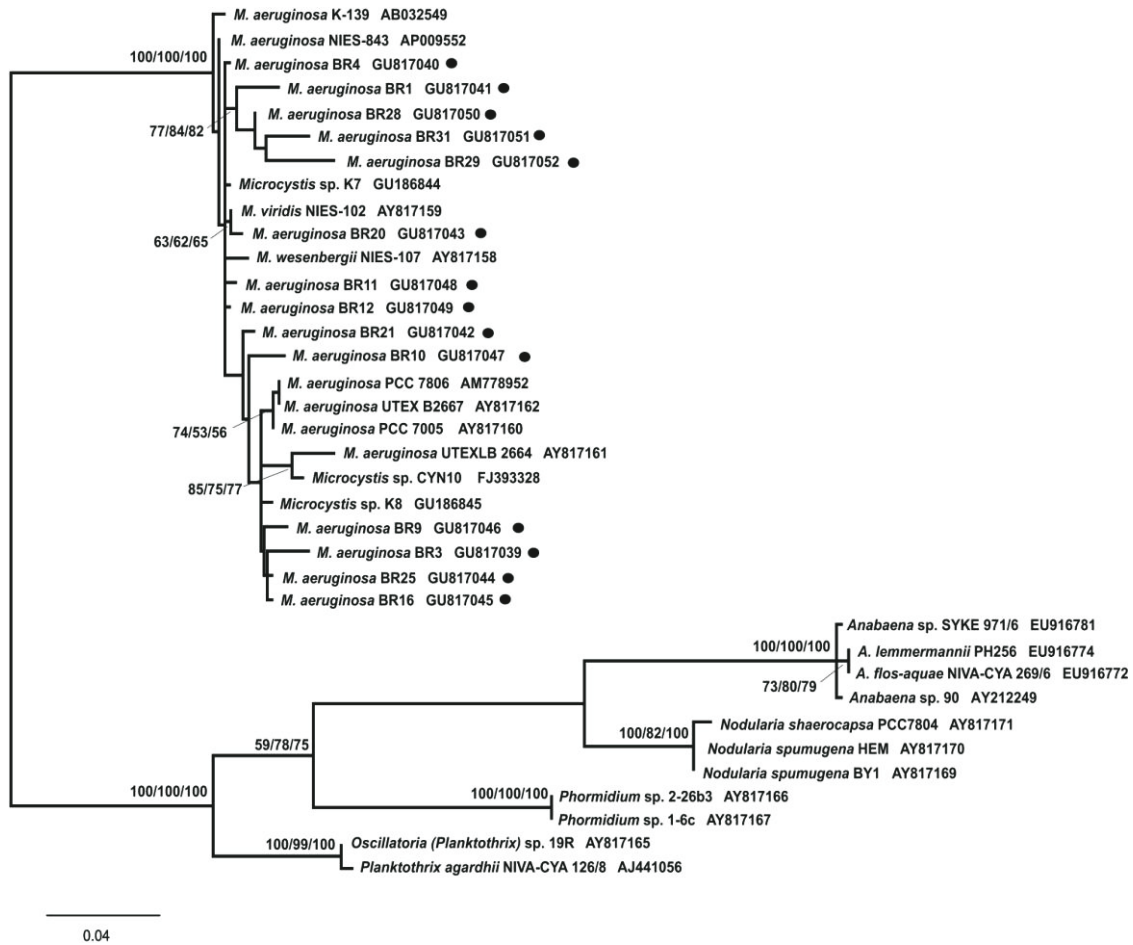
**Fig. 3.** Phylogenetic reconstruction inferred from sequence of AMT from the reservoir-cooler of Beryozovskaya SDPS No. 1 and selected sequences of cyanobacteria from GenBank. Maximum likelihood tree is shown. Sequences from our study are marked with circle (•). Distances are number of substitutions per 100 bases. Bootstrap values greater than 50% are shown at the nodes (neighbor-joining/ maximum likelihood/ maximum parsimony).

## Amplification of *mcy*E gene

Fragments of *mcy*E were amplified from floating colonies of *M. aeruginosa*. Earlier, Jungblut and Neilan showed that the presence of a AMT domain confirms the production of cyanotoxins (Jungblut and Neilan, 2006). In total, we examined 50 sequences of AMT and identified 14 different genotypes. BLAST search revealed high homology of the studied sequences with those of *Microcystis* from GenBank (95-99%) as well as more significant differences with those of other genera (not more than 83% homology). Sequences of translated amino acids were differed from each other based on not more than four amino acids per AMT fragment.

## Phylogenetic characterization

Phylogenetic trees constructed by NJ, ML, and MP analyses were congruent in terms of topology. The ML tree is shown in Fig. 3. Phylogenetic analysis showed that five genera, *Anabaena*, *Planktothrix (Oscillatoria)*, *Nodularia*, *Phormidium*, and *Microcystis* formed confident and separate monophyletic clades with the highest bootstrap-support (100%). These ancestral clusters are very stable and are always stand out as for
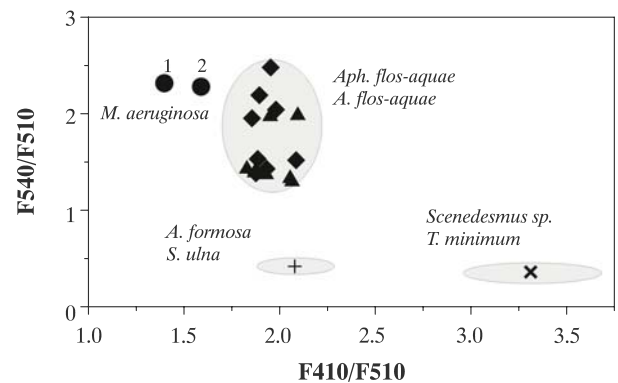


**Fig. 4.** Visualization of differences in fluorescence relationships between *M. aeruginosa* (•), planktonic form (1), benthic form (2), *Aph. flos-aquae* (▲), and *A. flos-aquae* (♦) cell colonies. Average fluorescence relationship values of green algae *Scenedesmus* sp., *Tetraedron minimum* (⊠) and diatoms *Asterionella formosa*, *Synedra ulna* (+) registered in plankton samples from the reservoir-cooler of Beryozovskaya SDPS No. 1. Gray highlighted area±SD.

**Table 2.** Euclidean distance characterizing the difference in fluorescence excitation spectra between *M. aeruginosa* and other planktonic microorganisms in the reservoir-cooler of Beryozovskaya SDPS No. 1

|  | *Aph. flos-aqua* | *A. flos-aqua* | Chlorophyta | Bacillariophyta |
|---|---|---|---|---|
| *M. aeruginosa* planktonic form | 0.94±0.11 | 0.81±0.12 | 2.56 | 1.91 |
| *M. aeruginosa* benthic form | 1.08±0.10 | 0.94±0.07 | 2.72 | 2.00 |

AMT domains, as well as for other genetic markers (Jungblut and Neilan, 2006; Rantala *et al.*, 2006; Hotto *et al.*, 2007; Allender *et al.*, 2009). Strains isolated from the reservoir-cooler at Beryozovskaya SDPS No. 1 did not form a separate clade on the tree. Sequences BR1, BR29, BR30, and BR31 formed a subgroup on the tree, and BR3, BR9, BR16, and BR25 clustered together. The remaining sequences were heterogeneously distributed within the *Microcystis* clade. In addition, the species *M. aeruginosa*, *M. viridis* NIES-102, and *M. wesenbergii* NIES-107 were analysed. On the tree, they did not form separate branches, and strain BR20 was clustered together with *M. viridis* NIES-102.

### Fluorescence analysis

Visualization (Fig. 4) allows comparison of the spectral differences between the planktonic and benthic forms of *M. aeruginosa*, other cyanobacteria (*Aph. flos-aqua, A. flos-aqua*), green algae, and diatoms (average values established for a reservoir-cooler). The diagram shows that the points belonging to *M. aeruginosa* (benthic and floating forms) were outside the area that bounds *Aph. flos-aqua* and *A. flos-aqua* points (Fig. 4). Distinction of *M. aeruginosa* from other representatives of the algal-bacterial community was based on the Euclidean distance (ED) between two chosen points in the coordinate space, $X = F_{410}/F_{510}$ and $Y = F_{540}/F_{510}$ (Table 2). ED values formed a descending series in a comparison of *M. aeruginosa* with green algae, diatoms, and the cyanobacteria *Aph. flos-aquae* and *A. flos-aquae*. The lowest values were recorded between the planktonic and benthic forms of *M. aeruginosa*. There was considerable variation in the relationships $F_{410}/F_{510}$ and $F_{540}/F_{510}$ of cyanobacteria compared to those of green algae and diatoms. This can be explained by the fact that the composition of phykobilines was less constant than that of chlorophyll pigments in the antenna of photosystem 2 in green algae and diatoms. In addition, allophycocyanin and phycobilines were capable of emitting fluorescence at the same wavelength as chlorophyll *a*.

It was possible to find *M. aeruginosa* cells in phytoplankton of the reservoir with ED between two points: the point of *M. aeruginosa* location in the coordinate space $X = F_{410}/F_{510}$ and $Y = F_{540}/F_{510}$ and the point obtained from the estimation of the given fluorescence relationships of the phytoplankton sample. This approach was used to compare *M. aeruginosa* planktonic forms with the algal-bacterial complex of the reservoir-cooler during the vegetative season in 2005 (Fig. 5). In this figure, the ED range is represented as a horizontal corridor distinguishing *M. aeruginosa* from *Aph. flos-aqua* and *A. flos-aqua* during mass development from mid-June to September. In the area located above the horizontal corridor, the algal-bacterial complex can be considered as poor (containing less cyanobacteria), and in the area below the horizontal corridor, as rich of *M. aeruginosa*.



**Fig. 5.** Euclidean distance between the point, which characterizes the *M. aeruginosa* position in coordinate space X = F410/F510 and Y = F540/F510, and points, which are found on the base of the given fluorescence relationships of phytoplankton samples from the reservoir-cooler of Beryozovskaya SDPS No. 1 in 2005.

### Discussion

Our investigation into the fluorescence qualities of *M. aeruginosa* colonies revealed peculiarities in the fluorescence spectra of this species with respect to green algae and diatoms as well as the cyanobacteria *Aph. flos-aqua* and *A. flos-aqua*. Minimal values of ED between the benthic and planktonic forms of *M. aeruginosa* were in agreement with the conclusions of Raps *et al.* (1985). They established the adaptation of *M. aeruginosa* to light; the quantity of photosynthetic units containing phycocyanin and chlorophyll *a* per cell changes, whereas the structure and molecular composition of phycobilisomes remain constant. This can be combined with the conclusion made by Moezelaar and Stal (1994), who found that settled benthic colonies of *M. aeruginosa* switch from a photoautotrophic to fermentative metabolism. Fermentation of endogenous stocks of carbohydrates may thus begin with the onset of anaerobic conditions. Further, *M. aeruginosa* is capable of fermenting exogenous glucose, and the energy provided by this fermentation is sufficient to sustain cellular growth (Moezelaar and Stal, 1997). Thus, colonies can stay in a quiescent state for up to 14 years (Rinta-Kanto *et al.*, 2009) and maintain reduced enzymatic activity even at a depth of 40
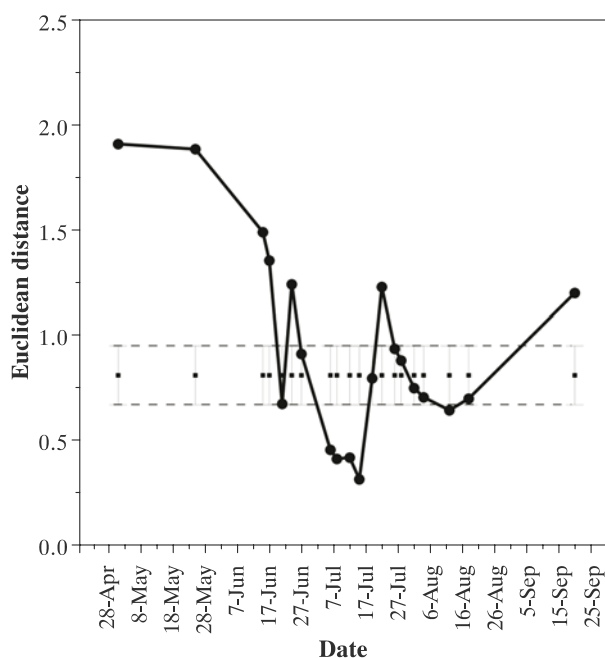
m (Zwart *et al*., 1998). Settled colonies form a pool for the restoration and reoccurrence of blooms.

This property of *M. aeruginosa* is extremely important in fluorescence analysis. Fixed relationships of *M. aeruginosa* fluorescence signals in coordinate space, $X = F_{410}/F_{510}$ and $Y = F_{540}/F_{510}$, chosen as a reference point (Fig. 4), allow estimation of algal-bacteria community changes based on cyanobacteria appearance, degree of mass development, and transition to the most dangerous conditions, under which *M. aeruginosa* develops in high quantities and threatens the reservoir.

Genetically, *M. aeruginosa* is rather diverse, even within a small body of water, which confirms the conclusions made by other authors (Kurmayer *et al*., 2003; Rinta-Kanto and Wilhelm, 2006; Hotto, 2007). Use of the phycocyanin intergenic spacer and flanking regions has allowed demonstration of a bulk of genotypes in a single body of water in Brazil (Bittencourt-Oliviera *et al*., 2001). Our results show that even one planktonic ecological form, forming a single fluorescence spectrum, includes a variety of strains. Different genotypes provide this species with good ecological adaptability. However, different genotypes isolated from one reservoir do not form a single cluster and thus mix with other genotypes of other geographical origin. *Microcystis* genotypes seem to be distributed worldwide, as was also previously reported for bacterioplankton species (Zwart *et al*., 1998). Zwart *et al*. (1998) stated that this can be explained by the fact that these organisms possess unique functional capabilities, thus enabling them to compete successfully in a wide range of freshwater environments.

In conclusion, *M. aeruginosa* was found to be not only a dominant cyanobacteria species in the artificial reservoir but also a species that exists in different ecological forms with different distinct fluorescence spectra. This feature can be exploited for the prediction of harmful blooms in reservoirs. Genetically, the population of *M. aeruginosa* is very heterogeneous, which is an important property that promotes its ecological success in water body.

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