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Industrial site of out-of-operation Baikalsk Pulp and Paper Mill as a potential source of pollution in Lake Baikal coastal zone

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ABSTRACT. The Baikalsk Pulp and Paper Mill (BPPM) was functioning on the shore of Lake Baikal for almost 50 years. These years of operation inevitably imposed specific adverse effects on the lake ecosystem. Finally, the BPPM was closed in 2013 but with no measures to mothball the plant. During 2013–2016, we observed a few atypical phenomena through our complex investigations of the coastal zone area next to the BPPM. Water temperature measurements, hydrochemical analysis, and FTIR spectroscopy showed these phenomena as unusual for Lake Baikal's pristine coastal areas. (1) We noted an anomalously high temperature of the interstitial water of the beach next to the BPPM. The water from the hole on the beach reached a temperature of 25 °C in June 2013. (2) The hydrochemical parameters of the water from the beach were analyzed, and the interstitial water there exhibited an appreciably lower concentration of oxygen and higher content of sulfur, sodium, and chlorine comparing the lake's water. (3) We discovered the specific black depositions to cover the stones of the target beach. The IR spectrum of those depositions was found to be identical to the BPPM sludge-lignin. All these observations and results of chemical analyses point out that the industrial site of the nonfunctioning plant is still a substantial source of pollution, which negatively influences the Southern Baikal coastal zone ecosystem.

Keywords: Baikalsk Pulp and Paper Mill, interstitial, Lake Baikal, pollution, sludge-lignin

1. Introduction

Lake Baikal, the oldest and deepest freshwater lake on the Earth, has always been the focal point for scientists and the world community. The uniqueness of the lake is a universally accepted fact, validated by incorporating of this natural object into the UNESCO List of the World's Heritage in 1996. Baikal – the colossal reservoir of freshwater, which contains a tremendous volume (over 23 000 km^3; Lake Baikal..., 1998). Facing the global deficit of drinking water, the problems of Lake Baikal protection and its ecosystem monitoring should become a matter of paramount importance (Timoshkin et al., 2019). Considering all these facts, the decision to construct on the lake's shores the pulp and paper mill seems quite paradoxical. Nevertheless, in 1966, one of the most significant sources of local pollution, the Baikalsk Pulp and Paper Mill (BPPM), started its production on the shores of Lake Baikal.

The pulp and paper industry is one of the leading branches of the forestry industrial complex in Russia: about 20 large pulp and paper mills operating in the country's territory (~150 mills in total; Russian Timber Industry, 2021). This industry is highly resource-intensive and requires much pure water (Fediaeva, 2007). That is why pulp and paper mills are located near big water bodies. For instance, in Russia, the biggest mills are operating on such water bodies as the Northern Dvina River and its tributaries (e.g., Vychegda River), Selenga River, Angara River, Volga River, Lake Onega, and Lake Vygozero (Moiseeva, 2005; Ermolina et al., 2011; Timakova et al., 2014).

The negative impact of the pulp and paper industry on the environment is rather various and complex. This industry is a significant source of pollution of atmospheric air, surface and ground waters, and soil (Fediaeva, 2007; Gavrilescu et al., 2012). A kraft pulping mill contaminates the air by specific sulfur compounds: hydrogen sulfide, methyl sulfate compounds (dimethyl sulfide and methyl mercaptan), and sulfate aerosols (sulfates and sodium sulfides; Obolkin et al., 2010). Mill energy units (e.g., thermal power stations or TPSs)

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emit sulfates, sulfur, and nitrogen dioxides. All such emissions could be involved in cloud formation that resulted in the acidification of precipitation (Obolkin et al., 2010; 2016).

In the sense of water management and conservation, the pulp and paper industry may be considered one of the most concerning. Pulp and paper manufacture is a large consumer of water and, even worse, it discharges high rates of wastewaters containing ~200 different chemicals (Fediaeva, 2007; Anikanova, 2009). Wastewater is high in chemical oxygen demand, total suspended solids, nitrogen, and phosphorus. In addition, it generates chlorinated organic compounds, including dioxins, furans, and other absorbable organic halides (Gavrilescu et al., 2012). Thorough studies of diverse freshwater ecosystems under the influence of pulp and paper manufacturing have demonstrated structural changes in their biota and water quality deterioration (Productivnost Baikala..., 1974; Moiseeva, 2005; Ermolina et al., 2011; Timakova et al., 2014).

Last but not least, pulp manufacture generates large quantities of solid wastes, which are landfilled (Fediaeva, 2007; Gavrilescu et al., 2012). The main subject of this paper is the impact of untreated wastes of BPPM stored in its landfills. Sludge-lignin, lime mud, fly ash, and solid municipal wastes have been accumulating at the plant's landfills for almost 50 years of its operation. Nowadays, ecology experts call the BPPM an "object of accumulated environmental damage" (Kolotov et al., 2021). World environmentalists include dangerous BPPM's legacy into the list of the immediate threats for the Lake Baikal ecosystem (Simonov et al., 2022).

Now the management of these accumulated wastes is the most acute problem facing ecologists since the BPPM production abandonment. The allocation of vast amounts of such wastes in a seismically active zone accompanied by a mudflow danger (Laperdin et al., 2016) might lead to a large-scale ecological disaster in the future (Suturin et al., 2021). A sudden discharge of all accumulated hazardous wastes of the plant into Lake Baikal will equal the volume of the BPPM effluents during 700 years of its operation (Kolotov et al., 2021). Moreover, the overfilled plant waste pits near the lake present another ecological risk – contamination of the lake's coastal zone. At first, we faced the threatening effects of the BPPM in 2013, when we observed warm dark-colored water flowing on the shore next to the mill's territory. Later, we detected the thermal and chemical contamination of the coastal zone there that is the focal point of this work.

2. Materials and methods

2.1. Study site

Lake Baikal is located on the border of Eastern Siberia and Central Asia (Fig. 1). Its maximum depth is 1 637 m and estimated age is over 25 million years (Lake Baikal…, 1998). Since the late 20th century, the opinion of experts on the state of Lake Baikal ecosystem was based on its high self-purification ability, sustainability, and a huge body of water regarded as the protection against the pervasive global changes even under the growing load of economic activities on its shores (Grachev, 2002). However, the first decades of the 21st century showed that, notwithstanding its enormous volume, the lake could not evade the imminent large-scale ecological crisis.

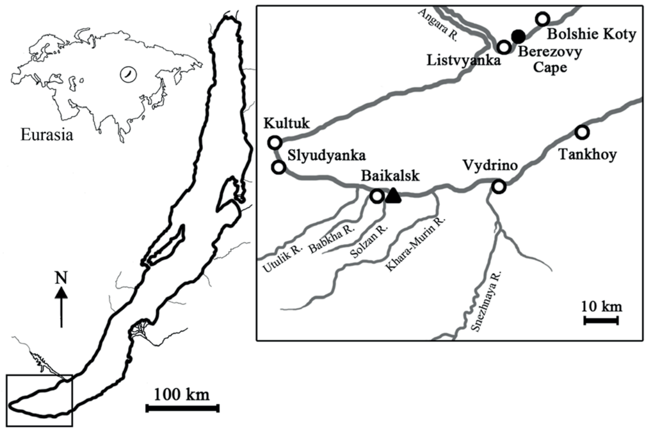

Fig.1. Schematic map of Lake Baikal (on the left) and the map of its Southern basin (on the right). Hollow circles mark large settlements. A black circle marks the location of reference site for the FTIR analyses of interstitial water. A black triangle marks the location of the BPPM.

According to (Kravtsova et al., 2014; Timoshkin et al., 2014), the earliest negative changes in the lake coastal zone became clear during 2010–2011. Currently, the lake-wide problems include changes in zonation and the species composition of the benthic algae and macrophytes, growth of their biomass (filamentous algal blooms), and the large coastal accumulations of the detritus formed by these rotting algae (up to 90 kg m^{-2}), active proliferation of the benthic cyanobacteria, including toxin-producing species, mass sickness and mortality of endemic Lubomirskiidae sponges (Timoshkin et al., 2019). One of the most evident reasons for these changes is nutrient pollution in groundwater (Timoshkin et al., 2018), but complex changes in climate (Potemkina et al., 2018), nutrient transport, lake hydrodynamics, and food web structure may also facilitate these emerging threats (Vadeboncoeur et al., 2021).

Above-mentioned ecological problems have been observed in almost the entire lake area, including certain remote localities, but the primary focus of this paper is the local source of pollution, the Baikalsk Pulp and Paper Mill (Fig. 2). The mill is situated in Baikalsk town, on the eastern shore of Southern Baikal (Fig. 1). The BPPM started production in 1966. Almost 50 years of the plant operation included the periodical slowdowns and even an attempt to transfer the mill to a closed cycle in 2008. After lengthy proceedings, the BPPM was finally closed in 2013, though it has stopped only the malodorous emissions and the direct discharge of industrial wastewaters into the lake.

The main reason for concern – no measures were taken to mothball the plant (Kolotov et al., 2021). The overfilled BPPM waste pits were just abandoned with no recycling procedures. At present, the wastes from sulfate pulp production (sludge-lignin, wastes from the chemicals' recovery process, ash from the TPS, and solid municipal wastes) occupy ~350 ha, including Babkhinsky and Solzansky landfills, and the BPPM industrial site (Fig. 3).

For the sampling and measurements, we chose the beach next to the BPPM industrial site (Fig. 2B, Fig. 3). The initial reason for choosing exactly this beach area was detecting a dark-colored warm water flow, which was seeping through the beach pebbles. Further, this water flow is referred to as a "warm brook" for the sake of convenience. We should note that the "warm brook" we found was an irregular phenomenon, and the intensity of its seeping was variable. Theoretically, it could depend on the amount of atmospheric precipitation that feeds groundwaters and also on the seismic activity. For instance, we observed a small but visible trickling brook in 2013, whereas there were only a few small puddles on the beach during 2014–2015.

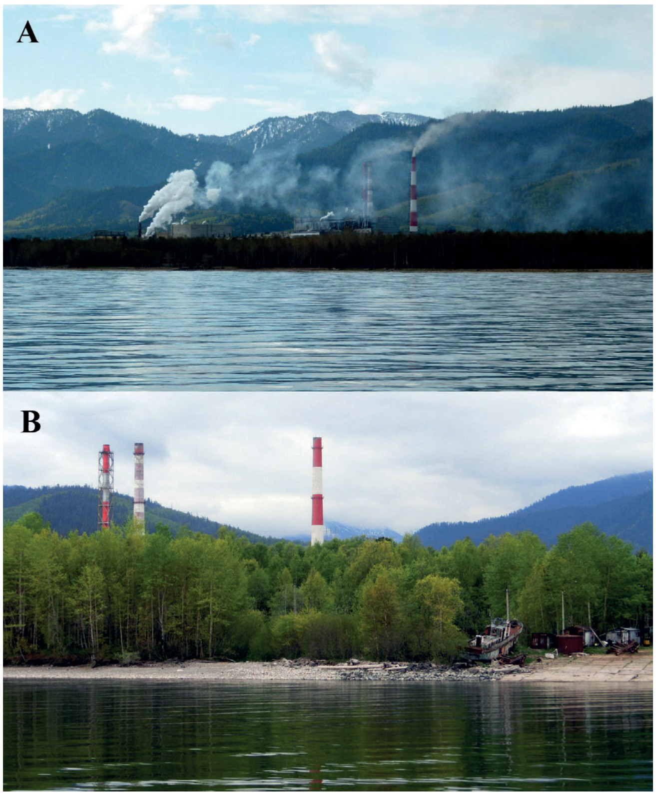

Fig.2. Industrial site of BPPM. (A) View from the water (May 31, 2013). (B) Coastal area near the target beach where the smoke-stacks of the mill's thermal power station are clearly visible (June 11, 2014).

2.2. Sampling and measurements

This study is based on the complex inter-disciplinary investigations of the Limnological Institute staff aboard the research vessel "V.A. Koptyug". The bulk of the data was collected during the expeditions in June and September 2013–2016.

2.2.1. The water temperature and hydrochemical characteristics

The temperature was measured for the water's edge and interstitial water using the Checktemp thermometer. We considered water's edge samples to be water from a depth of 10–20 cm, and interstitial samples to be water from holes on the beach. On the target beach, we usually dug holes 0.5–1 m above the water's edge. These holes were about 20–50 cm deep and about 50–60 cm wide. As soon as water filled the dug holes, we started sampling and measuring, so that the interstitial water parameters did not change under the influence of various factors (e.g., solar activity, precipitation, and atmospheric oxygen). During 2013–2014, the measurements were taken according to the following schemes (Fig. 4, Fig. 5). As the central point, we chose one where the outlet of the "warm brook" was visible. Other temperature measurements were taken both eastward and westward from the central hole with an interval of ~5 m along the water's edge.

During 2013–2015, the hydrochemical characteristics were estimated for the following samples: (1) water of "warm brook", (2) the interstitial water from the hole, (3) the edge's water, (4) pelagic lake's water. The SCUBA divers collected the last type of samples at the depth of ~7 m. Before the analysis, we used the acetate cellulose membrane filters (0.45 μm pore size) to remove the suspended particulate matter from the samples. The dissolved oxygen (DO), electrical conductivity (EC_{25}), pH, and the chemical oxygen demand (COD) were measured in the unfiltered water.

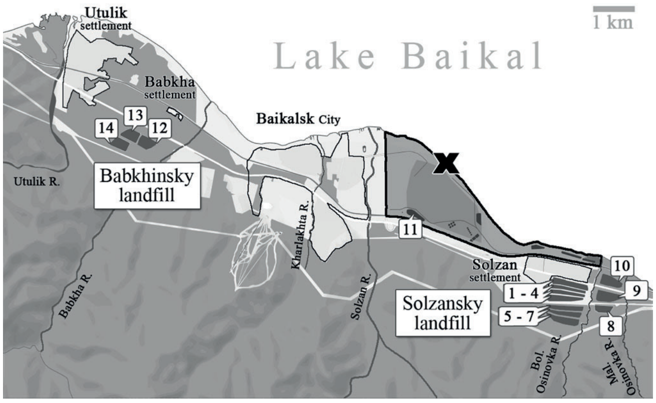

Fig.3. Location of the BPPM industrial site and its two landfills (Babkhinsky and Solzansky), storing different types of industrial and municipal wastes. The BPPM's area is shaded, and 14 plant's waste pits are marked with their numbers. A black cross sign marks the target beach.

Concentration of sodium (Na^+) was determined by the flame emission method on an Atomic Absorption Spectrophotometer 30 (AAS-30; Carl Zeiss Jena, Germany) with 5–7% error (Fomin, 2000). Anion concentrations (chloride (Cl^-), sulfate (SO_4^{2-}), nitrate (NO_3^-)) were measured by high-performance liquid chromatography on a chromatograph Milichrom A-02 (EcoNova, Russia) with 7–10% error (Khodzher et al., 2016). Dissolved oxygen concentration was measured *in situ* by the Winkler test with 3% error. Nutrient analyses (phosphate (PO_4^{3-}), ammonium (NH_4^+), nitrite

(NO_2^-)) were performed with a spectrophotometer KFK-3 (Zagorsky Optical-Mechanical Plant, Russia) with 10–20% error. Mineral phosphorus concentrations (soluble reactive phosphate) were estimated with ammonium molybdate, ammonium nitrogen concentrations were measured with indofenol, and nitrite concentrations were estimated with the Griess reagent (Boeva, 2009).

We measured the electrical conductivity at the sampling sites with the portable conductometer (Horiba, Japan) and the pH with the use of the pH meter (Hanna, Germany). Chemical oxygen demand was quantified by permanganate index (estimation error up to 40%).

2.2.2 Fourier-transform infrared (FTIR) spectroscopy

We analyzed two types of samples from the target beach with FTIR spectroscopy: (1) interstitial water samples and (2) the samples of solid black depositions from the stones. These measurements were performed with an Infralum FT-801 spectrometer (SIMEX, Russia) using KBr pellets. In the case of the interstitial water samples, we chose the beach of Berezovy Cape as a reference site (Fig. 1).

To analyze the solid black depositions from the beach stones, the samples were extracted with 0.1 M NaOH, precipitated with 2 M H_2SO_4 at pH 3, centrifuged, washed with water, and dried in a vacuum. Mechanical scraping may lead to contamination by the inorganic components of a stone, so extraction was indispensable. The procedure of extraction we used was described in (Tiranov, 1997).

2.2.3 Earthworms sampling

In June 2013–2016, we quantified the number of earthworms (Annelida, Oligochaeta, Lumbricidae) in the splash zone of the target beach (Table 1). The earthworms were sampled from squares with a side of 1 m. The results were performed as a mean number of individuals with a standard error of mean per square meter of the beach (ind. m^{-2}). For comparison, we used data obtained in the splash zone of Bolshie Koty Bay (Fig. 1). In general, the ecology of the splash zone as a part of the coastal zone of Lake Baikal has been little known (Timoshkin et al., 2012). Concerning earthworms as inhabitants of the lake splash zone, to date, there are only sparse observations on their number got by our research group for Bolshie Koty Bay in 2010–2013 (Timoshkin et al., 2012; Zvereva et al., 2012). Environmental conditions of the target beach and the beach of Bolshie Koty Bay differ, but the absence of data makes us to compare these sites for Lumbricidae abundances.

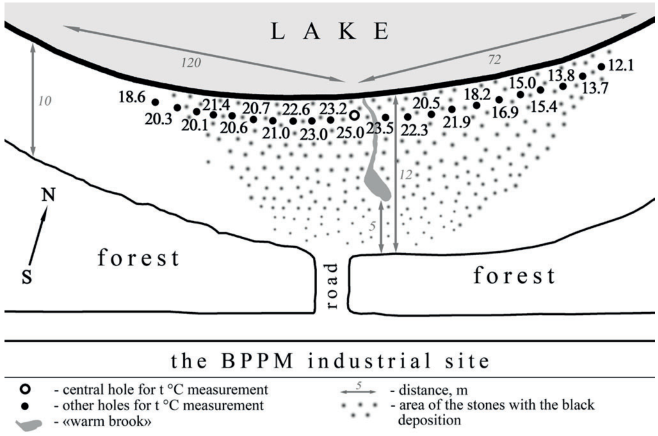

Fig.4. The temperature of the interstitial water of the beach next to the BPPM industrial site (May 31, 2013).

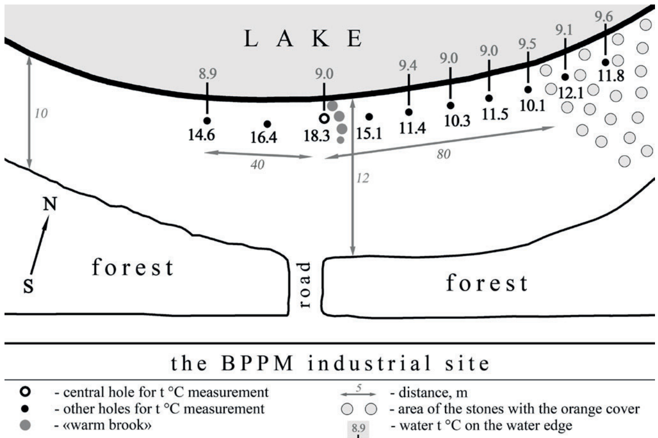

Fig.5. The temperature of the interstitial water of the beach next to the BPPM industrial site (June 11, 2014).

3. Results and discussion

3.1. Temperature deviations of the interstitial water

During routine sampling on the beach and in shallow parts, the limnologists registered a few phenomena unusual for the lake's pristine coastal areas. A thermal anomaly was one of the atypical cases observed. We registered a temperature of 25 °C in a hole dug above the water's edge on the shore. The temperature measurement with 5 m intervals in both directions from the central hole showed a downward gradient (Fig. 4). The interstitial water temperature gradually decreased from 25 °C to 18.6 °C westward and up to 12.1 °C eastward. Concurrently, the lake water temperature did not rise above 6.5–7.5 °C at the water's edge.

Long-term observations of Baikal near-shore water temperature dynamics, with Bolshie Koty Bay selected as the reference lakeshore site (Fig. 1), revealed that the interstitial water temperature in the holes varied within 9–13 °C from late May to early June and was only a couple of degrees warmer than the

Table 1. Earthworms (Annelida, Oligochaeta, Lumbricidae) sampling data for the splash zone of the beach next to the BPPM site (2013–2016).

Sampling date	May 31, 2013	June 11, 2014	June 13, 2015	June 12, 2016
N samples	4	4	16	8

temperature near the water's edge (Timoshkin et al., 2017). According to the same data, water temperature in the holes may reach 15–17 °C only on the hottest summer days. Consequently, the temperature of the interstitial water of the target beach was 2–3 times higher compared to the normal situation, and this phenomenon was registered at ~200 m along the shoreline in 2013 (Fig. 4).

The measurements of the interstitial water temperature were made likewise at the same beach in early June 2014 (Fig. 5). In that case, we did not register such noticeable upsurge in the temperature. Only the central hole showed a relatively higher temperature for this period (18.3 °C). The temperature gradually reached typical values with distance at different sides from the central hole: 14.6 °C westward and 11.8 °C eastward (Fig. 5).

The mill with two big landfills and 14 waste pits occupied a vast territory (Fig. 3). The groundwater layer is found between 4–5 m to 20–25 m depths on the territory of the BPPM. At the shoreline of Lake Baikal, the groundwater level is located close to the Earth's surface (Anikanova, 2009). Since 2000, the source of wastewater contamination at the BPPM's site has been localized by a protective groundwater dam. However, a special plant's service had registered an increase in the chemical concentration in the groundwater in 2007 (Anikanova, 2009). This fact reasonably indicates the depreciation of the protective constructions, the state of which is now not monitored. According (Kolotov et al., 2021) waste pits No. 1 and 8 of Solzansky landfill showed a high level of groundwater pollution.

Due to the above circumstances, we tend to associate increased temperatures of interstitial water in 2013–2014 with polluted groundwater infiltration. Several publications (Anikanova, 2009; O probleme…, 2009; Suturin et al., 2015; 2021) clearly indicate that there is an industrially polluted groundwater ridge under the BPPM's site. Anikanova (2009) reported that the intensity of the underground flow is irregular and could be related to the seismic events that induced the discharge of the industrial groundwater. The case of the record-breaking high temperatures in 2013 could point out not only the mixing of industrial and natural groundwater but to an emergency release of warmed-up technical waters into the coastal zone.

The water temperature is one of the universal ecological factors for the dwellers of the shallow and interstitial zones as well as for the rest of the poikilothermic hydrobionts. It is well-known that the temperature of the environment is an important driver that affects the rate of biochemical reactions (Konstantinov, 1986). Water is a relatively stable medium, and its temperature changes gradually owing to its thermal properties (Bezmaternykh, 2009). Such stability allows the hydrobionts to adapt to a specific temperature range. For instance, the Baikal amphipods common for the lake coastal zone, *Eulimnogammarus verrucosus* (Gerstfeldt, 1858) and *E. vittatus* (Dybowsky, 1874), prefer a water temperature of 5–6 °C (Timofeyev and Shatilina, 2007). Therefore, a dramatic temperature rise can negatively affect the Baikal hydrobionts. Moreover, the majority of Baikal organisms are oxyphilous, and a temperature increase can exacerbate their breathing conditions.

3.2. Hydrochemical parameters of the interstitial water

Hydrochemical parameters of different water samples were analyzed: (1) water collected from the "warm brook", (2) interstitial water from a hole on the target beach, (3) the water from the lake's edge, (4) the lake's pelagic water (Fig. 6). In our case, the

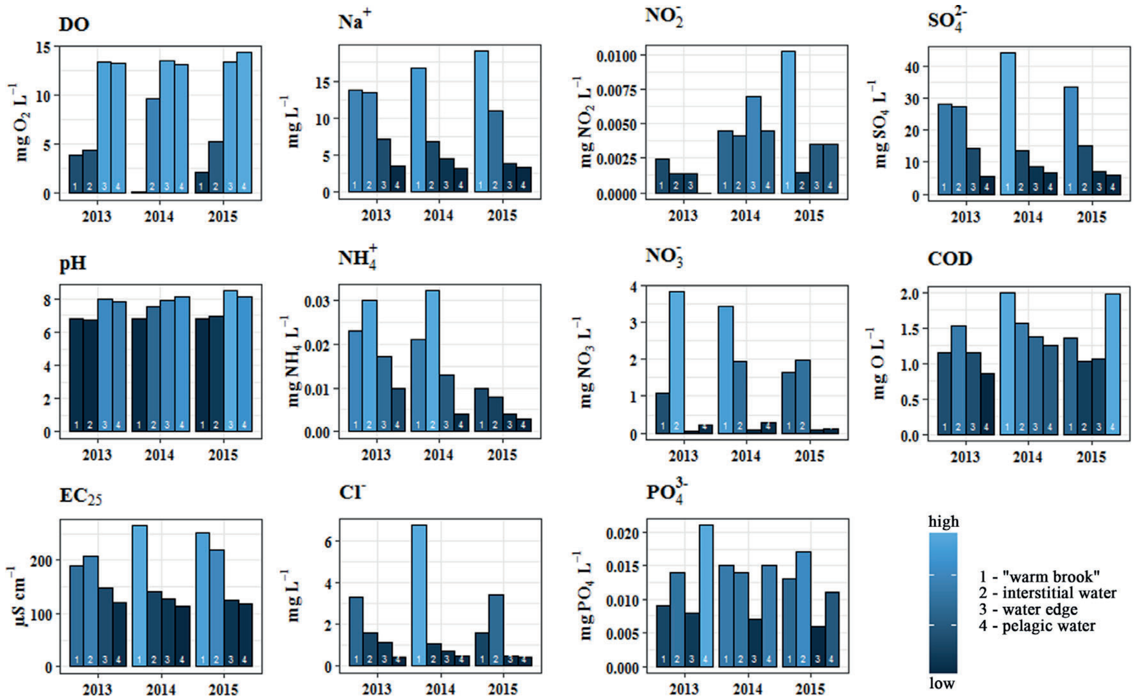

Fig.6. Results of hydrochemical analyses for (1) water of the "warm brook", (2) the interstitial water from the hole, (3) the edge's water, (4) pelagic water of Lake Baikal. Samples were collected in the coastal area adjacent to the BPPM site during 2013–2015. The less intensive color of a bar depicts a higher value of a parameter.

oxygen concentration in the water was one of the most indicative characteristics. It is quite clear that the oxygen content was significantly lower in the hole and "warm brook" comparing the lake's water. We noted the oxygen concentration was close to zero in the water from the "warm brook" in 2014. As for the pH, its measurements displayed stable low alkalinity in the water edge's and pelagic samples (~8), whereas the "warm brook" and the hole values were closer to neutral. The electrical conductivity in the "warm brook" during 2014–2015 testified to a moderately increased water mineralization there (up to 250 μS cm^{-1}).

In the context of our study, the sulfate content could be the most useful hydrochemical indicator for monitoring. Air emissions and wastewaters produced during sulfate pulping include large amounts of various sulfur compounds that entail contamination of rather spacious areas in the BPPM vicinity. While the mill was functioning, sulfates and other sulfur compounds were registered in the atmosphere (Obolkin et al., 2010; 2016) as well as in the water (Anikanova, 2009). The "warm brook" water showed the highest concentrations of SO_4^{-2} (28–44 mg L^{-1}), which could signify the influence of the BPPM site on the chemical composition of the groundwater.

FTIR analysis also showed the presence of sulfur compounds in interstitial water samples from the target beach (Fig. 7). The spectra of solid residues obtained after the evaporation of water from the reference site (Berezovy Cape) presented the following absorption bands associated with organic matter: 870 cm^{-1}, 1410–1530 cm^{-1}, and 1645 cm^{-1} (Fig. 7A). The band at 1130 cm^{-1} is associated both with fluctuations of an ether group (C-O-C) and inorganic compounds containing oxygen (Si-O-Si, Al-O-Si, Al-O-Al groups). Examination of the interstitial water samples from the beach next to the BPPM exhibited characteristic IR spectral deviations (Fig. 7B). The bands at 640 cm^{-1} and 1140 cm^{-1} allowed us to suggest the presence of sulfonate compounds.

Sodium and chlorine could be the other possible pollution tracers, as it was used in pulp production in the BPPM. Concentration of Na^+ showed a quite clear pattern (Fig. 6): there were high concentrations in the "brook" and interstitial, but low ones in the waters of Lake Baikal. The Cl^- concentration followed this pattern. Drawing a conclusion about high concentrations of these tracers, we keep in mind that normal mean concentration for the water of Southern Baikal is ~3.3 for sodium and ~0.4 for chlorine (Khodzher et al., 2017). Other chemicals as nitrogen compounds, phosphates and organic matter concentration did not demonstrate any obvious patterns in the distribution (Fig. 6).

In 2008, according to the government document (O probleme…, 2009), monitoring of the groundwater at Solzansky landfill revealed a stable high concentration of toxic elements exceeding maximum permissible concentrations (MPC) accepted for fishery water bodies of the premium category: iron (up to 4 MPC), manganese (up to 13 MPC), copper (up to 22 MPC), zinc and aluminum (up to 3 MPC), methanol (4 MPC), and formaldehyde (30 MPC). Higher concentrations of

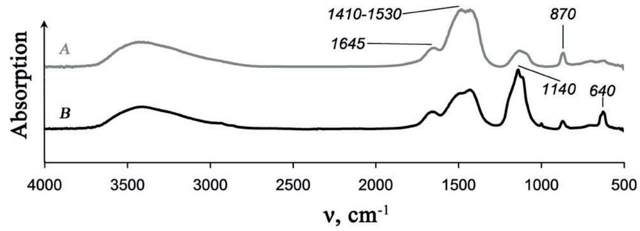

Fig.7. FTIR spectroscopy measurements of solid residues after evaporation of interstitial water. (A) The beach of the Berezovy Cape (reference site). (B) The beach next to the BPPM site.

petroleum products and lignin were constantly reported in the groundwater, and higher amounts of organic matter were observed periodically. Chromaticity and concentrations of sulfate ions were also high. The study (Kolotov et al., 2021) revealed an increase in the content of heavy metals in the splash zone of this area.

Data obtained in our work also demonstrate that the groundwater flow contains high concentrations of pollutants (Fig. 6) that were going on entering the lake. For instance, in 2013, the sulfates in the water at the shoreline next to the BPPM site were three times as high as the typical Baikal water values: 14.4 mg L^{-1} vs. ~5.5 mg L^{-1} (Khodzher et al., 2017). The influence of this runoff on the interstitial waters of the beach was quite remarkable: the hydrochemical parameters in the interstitial rarely differed from the "warm brook". Particularly, the changes in the oxygen concentration were obvious, while it is vital for oxyphilous Baikal dwellers. The crucial role of the dissolved oxygen concentration for abundant endemic species of the upper littoral zone of the lake has been already established by several studies (Zaitseva et al., 2008; Timofeyev and Shatilina, 2010).

Needless to say, the effects on the shallow-water hydrobionts are far from extreme as the water of the "warm brook" entering the littoral zone is strongly diluted by the lake waters. However, we have solid grounds for suspecting local negative effects on the beach interstitial. A low concentration of oxygen from the "warm brook" water accompanied by an increase in organic matter content can lead to a drop of the oxygen concentration to zero, as registered in 2014 (Fig. 6). Though some inhabitants of the splash zone may be tolerant to hypoxia (Zvereva et al., 2015), the near-complete absence of dissolved oxygen is highly stressful for its biota (Medvezhonkova et al., 2018).

3.3. Depositions on the stones forming the target beach

Besides the other phenomena that are abnormal for the pristine shores of Lake Baikal, we discovered specific depositions covering the beach boulders and pebbles. In 2013, we localized a relatively large zone of the beach (Fig. 4), where each stone had a black slimy ring-like stripe (Fig. 8). This substance from the stones was collected to be analyzed by FTIR spectroscopy. We compared the obtained results to the spectra of the pulp production wastes as untreated sludge-lignin and a dry solid residue of the plant wastewaters (Fig. 9). It was revealed that the IR spectrum of the black depositions and the spectra of the BPPM sludge-lignin were identical (Fig. 9), which enabled us to ascertain the origin of the substance from the beach stones. Finding lignin on the beach suggests the contamination of the lake, but we can only speculate about the source of the pollution. Theoretically, the surface runoff formed by the atmospheric precipitations may carry out solid residues from the BPPM's waste pits to the shore.

Lignin is a high-molecular aromatic compound, the decomposition of which produces toxic low-molecular substances: phenols, methanol, and carbon acids. Lignin produced through a sulfate pulping (sulfate lignin) harms hydrobionts when its concentration in water is higher than 100 mg L^{-1} (Kalinkina, 1993). Moreover, lignin is highly resistant to degradation, which leads to its accumulation in water in a concentration that becomes hazardous to aquatic organisms (Kalinkina, 1993). For instance, the structural changes in the lake's macrozoobenthic communities were most evident on the sediments with a layer of black pulp flakes and slime residues (Productivnost Baikala..., 1974). Long-term studies of Chironomidae taxocenosis in the Utulik-Murino region revealed a biodiversity decline on such highly polluted sediments and the appearance of eutrophication indicating species (Erbaeva and Safronov, 2010).

Concerning the lignin input, the authors of this paper came across an interesting phenomenon in this coastal area – a high population density of earthworms (Annelida, Oligochaeta, Lumbricidae) in the splash zone of the target beach (Table 2). Earthworms are typical inhabitants of soil and forest litter, with an extraordinary contribution to soil formation and functioning. It is noteworthy that the number of these oligochaetes is rarely above 1 ind. m^{-2} in the splash zone of Bolshie Koty Bay (Zvereva et al., 2012), whereas we observed up to 15 ind. m^{-2} in the splash zone of the beach next to the BPPM in 2013 (Table 2).

Lignin contamination of this area appears to be one of the possible reasons for the observed abundance of earthworms on this beach. The earthworms alone are not able to decompose either lignin or pulp (Marhan and Scheu, 2006). Fungi and bacteria are the primary lignin destructors (Buswell et al., 1987). There is evidence that soil communities of microorganisms change their structure under the lignin contamination: yeast and micromycetes begin to dominate (Zyrianova, 2003). Apparently, lignin affects the number of Lumbricidae indirectly: changes in the community composition of microorganisms and fungi may attract earthworms there.

Fig.8. Boulders and pebbles with abnormal black depositions. The beach next to the BPPM site (May 31, 2013).

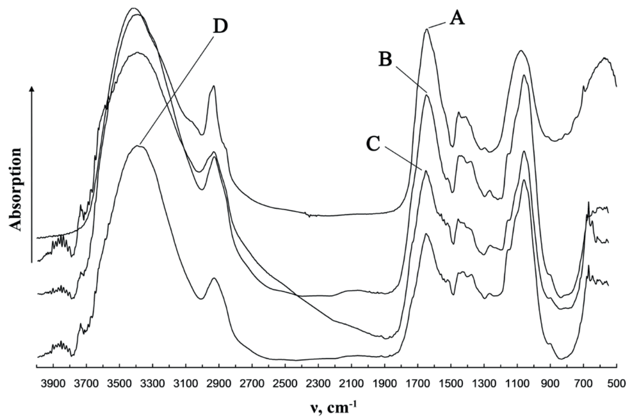

Fig.9. FTIR spectroscopy analysis of the depositions on the stones from the beach next to the BPPM site compared to the data on pulp production wastes. (A) Acid-precipitated alkaline washout from a stone. (B), (D) Untreated sludge-lignin. (C) Dry residue of the BPPM wastewaters.

4. Conclusions

The results of our research during 2013–2016 revealed a few facts that allowed us to conclude that even after its closure, the BPPM was threatening Lake Baikal. We hypothesized this influence includes the pollution of the groundwater via drainage flows and surface runoff with precipitation. As a result, we observed the rise of the interstitial water temperature, oxygen deficit, high concentration of pollution tracers compounds (sulfur, sodium, chlorine), and the presence of lignin on the beach adjacent to the plant – all these factors can cause alterations in the thermal and chemical regime of the coastal zone. These drastic fluctuations of

Table 2. The number of earthworms (Annelida, Oligochaeta, Lumbricidae) in the splash zone of the beach next to the BPPM site (2013–2016).

Sampling date	May 31, 2013	June 11, 2014	June 13, 2015	June 12, 2016
Number, ind. m^{-2}	15 ± 2	6 ± 2	4 ± 1	2 ± 1

environmental factors could affect the biota inhabiting the beach and shallow-water parts of the lake. The present investigation is one more argument in favor of the urgent need for adequate management and recycling of the BPPM wastes, the intrusion of which into the lake might lead to a massive ecological disaster in the future. The authors believe the findings will serve not only as a baseline for the risk assessment but an impetus for the regional and federal government of the Russian Federation to determine emergency actions for the BPPM wastes elimination and its site active remediation.

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Conflict of interest

The authors declare no conflict of interest.

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Comparative analysis and reconstruction of phylogenetic position of sunbleak *Leucaspius delineatus* (Heckel, 1843) from the Irkutsk Reservoir

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ABSTRACT. This work was aimed to a comparative analysis and reconstruction of the phylogenetic position of sunbleak *Leucaspius delineatus* (Heckel, 1843) from the Irkutsk Reservoir. Determination of a species specific fragment of the *Cox1* gene allowed to reveal a unique common haplotype present in all specimens analyzed. Comparative analysis and phylogenetic reconstruction based on nucleotide sequences available in genetic databases showed that the studied species is represented by three phylogenetic lineages. Intraspecific differences of nucleotide sequences of fragments of the *Cox1* gene amounted to 5.5%, and within phylogenetic lineages - < 3%. The studied fishes were related to the genetic lineage widely distributed in Europe and northwestern Russia. It is shown that all specimens with 100% genetic similarity belong to the same genotype, which is due to recent introduction of sunbleak into Pre-Baikal and to the “founder effect”.

Keywords: *Leucaspius delineatus*, invasive species, *Cox1* gene, Irkutsk Reservoir, Baikal Region

1. Introduction

Nowadays, the popularity of studies on invasive species of hydrobionts has increased considerably worldwide (Novoa et al., 2020; Kovalenko et al., 2021). For this reason, molecular genetic studies are particularly important in developing standards for monitoring and in assessing of long-term trends in ecosystem status, including the proxies describing expansion and impacts of invasive species, and in predicting genetic trends and potential consequences of their invasion (Darling et al., 2017; Pérez-Portela et al., 2018; Belle et al., 2021; Simberloff, 2021).

Sunbleak *Leucaspius delineatus* (Heckel, 1843) is a small-sized freshwater species of family Leuciscidae (Fig. 1). The natural habitat of sunbleak is the rivers of Eastern Europe. In Russia, it occurs in all Baltic rivers east of the Neva River; in some lakes of the Onega and Northern Dvina basins; in the Caspian Sea basin (the Volga River, from the upper reaches to the mouth, rivers of the Republic of Dagestan to the Kuma River); in the basins of the Black and Azov Seas – the Don and Kuban’ Rivers as well as in rivers on the shore of the Black Sea (Atlas …, 2003). Currently, sunbleak is spreading far beyond its natural areal (Reshetnikov et al., 2017) due to unintended introduction and subsequent self-propagation. Sunbleak penetrated into Pre-Baikal water bodies in 1973 together with fish breeding material of carp *Cyprinus carpio* Linnaeus, 1758, brought into a Kudareya pond (the basins of the Kuda and Angara Rivers) from the fish farm “Zerkal’ny” in Novosibirsk Region (Demin, 1997; Demin and Abramenok, 1998). It is now known that sunbleak inhabits the mainstream of the Angara and Irkut Rivers, as well as in the downstream of the Kaya and Olkha Rivers (Matveev and Samusenok, 2009; Ponkratov, 2014; Yuriev et al., 2021).

Fig.1. Sunbleak *Leucaspius delineatus* (Heckel, 1843).

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The appearing of sunbleak in the Irkutsk Reservoir was noted earlier (Yuriev et al., 2021), it results probably as well from an unintended introduction. Due to this fact, it is important to study genetic peculiarities of sunbleak in local ichthyocenoses. The aim of the study is to perform a comparative analysis and phylogenetic reconstruction of sunbleak from the Irkutsk Reservoir.

2. Materials and methods

The catching site (52°12’37’’N, 104°25’28’’E) is located in the Irkutsk Reservoir at the Angara River (Fig. 2). The fishes were caught with hooks at the depth of 2-3 m in July-August, 2019.

Euthanasia of fish was done with an overdose of anesthetic (GOST 33219-2014, 2016) using a 2% lidocaine solution (Lidocaine Bufus, Renewal, Russia). The samples wGOST 33219-2014ere handled in ice in the laboratory and stored at the temperature of -20°C until analysis. The mass and standard lengths of the studied fish (average ± SE) were 2.6 ± 0.2 g and 5.8 ± 1.4 cm, respectively.

Molecular genetic analysis was performed on 20 mature specimens. For DNA extraction, 25 mg of muscle tissue was collected from each specimen. Each sample was used for DNA extraction with the DNA-sorb-AM extraction kit (AmpliSens, Russia) according to the manufacturer’s instructions. The primers COIintF_MiSeq: 5´tcgtcggcagcgtcagatgtgtataagagacagg-gwacwggwtgaacwgtwtayccycc, dgHCO2198_MiSeq: 5´gtctcgtgggctcggagatgtgtataagagacagtaaacttcagg-gtgaccaaaraayca3´ (Leray et al., 2013) were used to amplify the *Cox1* gene fragment. The sequence is registered in the GenBank database under the number MZ818000.

The sequence obtained was aligned with all sequences of the *Cox1* gene of sunbleak presented in the GenBank database using the Clustal W program (Larkin et al., 2007).

Evolutionary model selection (Nei and Kumar, 2000) and visualization of phylogenetic relationships were performed using MEGA7 software (Kumar et al., 2016). Evolutionary history was inferred by a maximum likelihood method based on the Kimura’s two-parameter model (Kimura, 1980), using the discrete gamma distribution to model differences in evolutionary rate among sites (K2 + G).

3. Results and discussion

Determination of the species specific fragment of the *Cox1* gene allowed identification of a single haplotype found in all specimens. Comparative analysis and phylogenetic reconstruction based on the nucleotide sequences accessible in the genetic databases showed that the studied species is represented by three phylogenetic lineages (Fig. 3). The nucleotide differences of the fragments of the *Cox1* gene in sunbleak in the comparative data analysis from the GenBank database were 5.5%, and within the phylogenetic lineages the nucleotide sequences differed by < 3%.

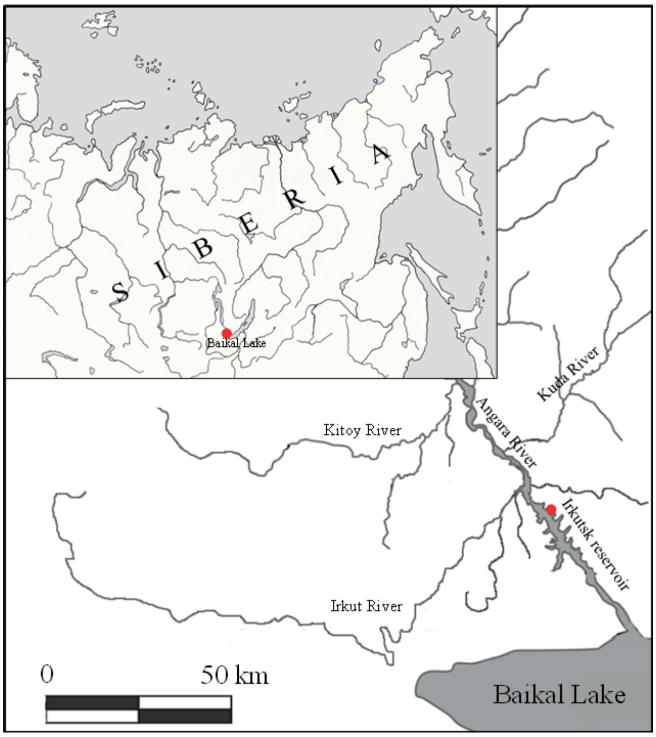

Fig.2. Sampling site, the Irkutsk Reservoir (the Angara River).

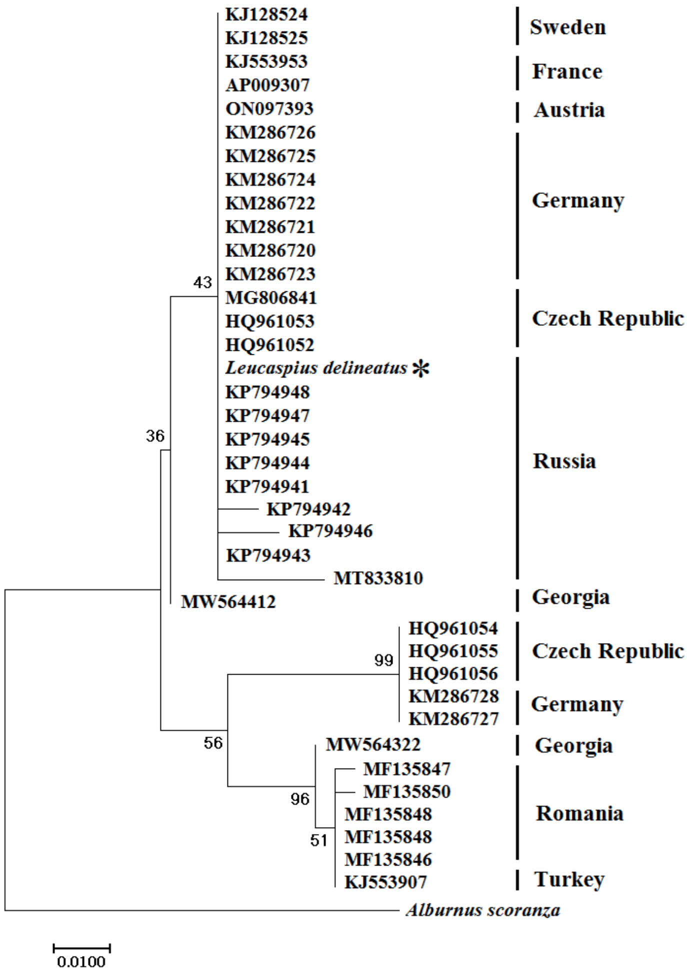

Fig.3. Phylogenetic reconstruction based on *Cox1* gene fragment. *L. delineatus* from the Irkutsk Reservoir (the Angara River) is marked with *.

The previously obtained data of morphological and molecular genetic analyses of sunbleak from the basin of the Pechora River indicate a common mechanism of the origin of the populations of this species on the territory of the Komi Republic and their long-term inhabiting the Pechora basin (Rafikov et al., 2015; Rafikov, 2018). Geographical variability of populations from water bodies of the basins of the Vychegda and Pechora Rivers results from natural species expansion and from its long-term living under the conditions of local landscape-geographical zones (Rafikov, 2018). No genetic differences were found between sunbleak from the Irkutsk Reservoir (the Angara River) and the Pechora River. The specimens we studied were found to be genetically close to fish inhabiting the Bröl River and other German creeks, the Rhone River in France, Lake Skvatsjon in Sweden, the Eger and Jordanka Rivers in the Czech Republic and the Vychegda River in the European part of Russia.

Two other phylogenetic lineages of sunbleak are reliably recognisable as separate clades in the phylogenetic tree. Their distribution is related to Romania – Turkey and the Czech Republic – Germany, respectively (Fig. 3). Two genetically different lineages of sunbleak inhabit the same water bodies: in the Czech Republic (the Eger River) and Germany (creeks).

4. Conclusions

We did not find any genetic differences between sunbleak from the Irkutsk Reservoir (the Angara River) and the Pechora River. This fact confirms the information on occasional introduction and expansion of the species from the European part of Russia into Siberian water bodies. Nowadays, the only one genetic variant in sunbleak, which adapted successfully to hydrological conditions of Siberian water bodies, is found. It is likely that they first inhabited bodies of water in Eastern Europe and from there expanded their range eastward. It is also probable that it was this genetic lineage that proved more successful in adaptation to the new habitat conditions. The lack of genetic diversity in the fragment studied is probably due to the “founder effect” – a decrease in genetic diversity when new areas are inhabited by a small number of representatives of this species, i.e. when a small number of fish are introduced. Sunbleak is an alien species in Siberian water bodies, and its adaptability and food concurrence as well as expansion just near Lake Baikal now require the greatest attention and careful study.

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Conflict of interest

The authors declare that they have no competing interests.

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Short communication

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Movies about diatoms and their movements

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ABSTRACT. Diatoms are unique unicellular photosynthetic organisms that have siliceous frustules around their cell membrane. Diatom cells are capable of independent movements in the aquatic medium, which is important for their life strategy. This article summarizes my main observations on the movements of diatoms, including some hypotheses. The material is presented in the form of six video films.

Keywords: diatoms, movement, gliding motility

The video films showing the movements of various diatom cells were created during the preparation of a series of works in this field. Files are available at http://limnolfwbiol.com/index.php/LFWB/article/view/994/684 under Creative Commons Attribution 4.0 International Public License. There are six videos:

1. "Bertrand1990 1 L'Equilibre.avi" (Bertrand, 1990; 1991a).

The characteristic shape of the diatom *Rhoicosphenia abbreviata* would seem to not allow apical and transapical movements. This study demonstrates with the help of analyses of graphical statics that the forces involved explain and confirm the reality of the observed movements.

2. "Bertrand1991 2. Synthase-Mouvements.avi" (Bertrand, 1991b; 1992).

This film shows all possible movements of raphid diatoms performed in the aquatic environment as well as a synthetic table showing the relationship with substrates. Apical, and transapical movements, horizontal, vertical, polar and conical rotations of 12 species are shown. The totality of all possibilities is executed by *Gomphonema acuminatum*.

3. «Bertrand1994 3 Cymbella triangulum.avi» (Bertrand, 1994a; Bertrand and Coste, 1994).

This video shows for the first time the diatom *Cymbella triangulum*, new to the European continent. Its movements, dimensions, morphology and ecological preferences are described.

4. "Bertrand1994 4 Cocconeis pediculus.avi" (Bertrand, 1994b; 1995a).

This video shows how easily the diatom *Cocconeis pediculus* performs a transapical movement despite the cylindrical-concave shape in the apical direction of its hypovalve. The diatom generally uses this particular geometry to position itself tightly on supports such as green algae of the genus *Cladophora*.

Brief biography J.R. BERTRAND

J. BETRAND born on 15.03.1931 in St. Benoit sur Loire France
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Amateur microscopist from 1942 to 1986.
Profession: engineer in industrial mechanics from 1960 to 1985.
Independent researcher from 1986 to now (2022)
Member of the Association des Diatomistes de Langue Française (ADLaF)
Member of the International Society for Diatom Research until 2015

Specialist in diatom movements from 1990 to 2005
Specialist in pond ecology from 2007 to 2017.
Specialist in diatoms on lichens from 2015 to present.
Taught diatoms in a French public service Agence de l'Eau; DREAL (3 years)
Taught general microscopy at the Natural History Museum of Orléans (12 years)

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5. “Bertrand1995 Les Diatomees.avi” (Bertrand, 1995b).

This documentary introduces the microscopic world of diatoms, unsuspected by most people. How to find them, how to observe them, their delicate beauty, their way of life, their usefulness, and how they inspired artists.

6. “Bertrand1999 Les Diatomees.avi” (Bertrand, 1999).

This video traces the search for the causes of diatom movements to the absence of rigid supports, i.e., just using the surface tension of water. It shows the researcher’s background, calculations, thoughts and the means implemented to try to propose a credible hypothesis.

A summary of my theories of diatom motility is given in Bertrand (2021).

Conflict of interest

The authors declare that they have no competing interests.

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Review

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Copepod cryptic species as aquatic invaders

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ABSTRACT. The dispersal of aquatic organisms has especially increased since the 19th century, mainly due to the introduction of metal ships with ballast compartments or with birds. Along with easily visible aquatic organisms, there are invasions of less noticeable aquatic organisms, like copepods, which can mainly be recognized by experts. A special group is made up of cryptic species for which morphological identification is inaccessible or very difficult. This review is devoted to the identification, description and analysis of the dispersal routes of three copepod cryptic species complexes into waterbodies. Using molecular-genetic methods, *Eurytemora carolleeae* introduction were revealed in 2007. After describing this invader as a new species, its distribution was also studied using morphology. The invasions of *Acanthocyclops americanus* and *Eurytemora caspica* were mainly studied using morphological methods since the species have already been described; however, molecular-genetic methods were also used to confirm their distinctions from local forms. The real distribution of the former "cosmopolitan" species *Eucyclops serrulatus* was studied using a multidisciplinary approach that combined molecular-genetic, cross-hybridization and morphological methods. Judging by the distribution of local *E. serrulatus* sensu stricto populations in coastal waterbodies of most continents, this species can apparently serve as a good marker of the species dispersal processes with ships. The three possible scenarios resulting from cryptic species introductions and their interaction with local fauna were competitive displacement (*A. americanus*), competitive coexistence (*E. carrolleeae*) and independent development without competition (*E. serrulatus*). The role of cryptic species invasion in aquatic biodiversity modification is discussed.

Keywords: cryptic species dispersal, *A. americanus*, *E. carrolleeae*, *E. caspica*, *E. serrulatus*, scenario of introduction, competitive displacement and coexistence, biodiversity

1. Introduction

The study of biological invasions has become one of the leading areas of aquatic ecology since the second half of the 20th century. Biological invasions are also called biological pollution and most often they are the result of human activity. The main mechanism for the intercontinental dispersal of aquatic organisms is the transport of organisms by ballast water of large-tonnage vessels (Gollasch et al., 2002).

The construction of various hydrotechnical structures (e.g., shipping channels, reservoirs, etc.) also violate biogeographic barriers. The introduction of alien species can cause significant and sometimes catastrophic changes in the ecosystem; for example, the introduction of the ctenophore *Mnemiopsis leidyi* A. Agassiz, 1865 into the Black and Caspian Seas, the zooplankton crustacean *Cercopagis pengoi* (Ostroumov, 1891) into the Baltic Sea or the mollusk *Dreissena polymorpha* (Pallas, 1771) in the Great Lakes of the North American continent. At the same time, most invasions remain unnoticed and usually do not cause any catastrophic events in the invaded community. A special place among invasions is occupied by cryptic invasions or invasions by cryptic species. Cryptic species are practically indistinguishable by morphological characteristics, but have significant differences at the genetic level (Geller et al., 2010). According to Novak (2011) a cryptic invasion is 'the occurrence of a species or genotype that was not previously recognized as alien in origin or not distinguished from other aliens'.

The first mention of cryptic species bioinvasions was back in 1996 in an article by Carlton (1996) where he used the term "cryptogenic species" and defined them as completely different from what we are "the species that are neither clearly native nor exotic in a biocommunity".

Cryptic species can differ in many parameters including physiological ones, and those differences may cause harmful changes in ecosystem function or

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productivity. Population shifts may eventually have important consequences for biodiversity, biogeography, conservation or fisheries management and ecosystem productivity (Knowlton, 1993; Lee, 2000; Gelembiuk et al., 2006; Declerck et al., 2015; Papakostas et al., 2016). Such invasions might also have important implications for disease transmission; for example, copepods are known as median hosts of many parasites and diseases (Arnold and Yue, 1997; Colwell, 2004; Piasecki et al., 2004; Lee et al., 2007).

Cryptic speciation is especially developed in the aquatic environment due to the predominant development of chemosensory systems for recognizing one's own species there rather than morphological characteristics (Morais and Reichard, 2018). Copepoda is a good example of such organisms, and according to Jarić et al. (2019), they have additional preferences for cryptic invasions like small body size, less-accessible habitats (e.g., aquatic or belowground environments), endoparasitism, camouflage, systematically complex and/or poorly studied species group, interspecific morphological homogeneity and taxonomic instability. According to Panov and Caceres (2007), developed dormancy stages are also a facilitating factor for invasions.

There are few publications devoted to cryptic species invasions among Copepoda: one parasitic copepod (Goedknegt et al., 2018) and our publications on the above-described species (Alekseev and Souissi, 2011; Miracle et al., 2013; Sukhikh et al., 2013; Sukhikh and Alekseev, 2013; Sukhikh et al., 2019; Alekseev et al., 2020; Alekseev, 2021). There are additional publications on other Entomostraca, mainly Cladocera (Mergeay et al., 2005; Ishida and Taylor, 2007; Sharma and Kotov, 2015; Kotov et al., 2020; Taylor et al., 2020) and two articles on *Malacostraca* (Roman, 2006; Grabowski et al., 2012). Many other organisms from different taxa are listed as cryptic invaders in reviews such as plants, algae, Annelida, Hydrozoa, Mollusca, Bryozoa, parasitic Platyhelminthes, Pisces, etc. (Miura, 2007; Morais and Reichard, 2018).

Probably, until a sufficient amount of data has been accumulated and methods for studying such cryptic invasions (mainly methods of molecular genetics according to Jarić et al. (2019)) will become more affordable, small organisms like copepods will be studied less in the sense of cryptic invasions. Here, we reviewed four Copepoda cryptic invasions. Each has its own introduction and naturalization scenario, which might be common for other Copepoda invasions.

The aim of our review is to describe three dispersal scenarios of cryptic copepod species into Eurasian water bodies and the related consequences for local ecosystems.

2. Biological invasion of *Acanthocyclops americanus* (Marsh, 1893) from North America to Eurasia

The history of bioinvasion of the North American cyclopoid *Acanthocyclops americanus* (Marsh, 1892), which is a cryptic species of two European species, *Acanthocyclops vernalis* (Fischer, 1853) and *Acanthocyclops robustus* (Sars G.O., 1863), is in some respects opposite to the case of *Eurytemora carolleeae* described below. *Acanthocyclops americanus* was described in the late 19th century as a distinct taxon from the Great Lakes vicinity of North America. At the beginning of the 20th century, it was found in England, which had the closest maritime ties with the United States at the time, and where the most modern ships equipped with ballast compartments had cruised on a permanent basis since 1880. The species was correctly identified and defined as invasive from North America by Lowndes (1926; 1928).

In the middle of the last century it was found on the mainland in water bodies along the Atlantic coast of Europe in France and Spain in a mass form (Dussart, 1967; 1971), then as a dominant species of summer zooplankton in large reservoirs built on the Dnieper and Dniester (Monchenko, 1961), and then in the reservoirs of the Volga cascade (Vijushkova and Kuznetsova, 1974; Alekseev and Kossova, 1976) and the fresh part of the Caspian Sea (Chuykov, 1986). Further study of its distribution in Europe was temporarily interrupted, and the species passed into the category of cryptic due to a taxonomic mistake of the most prominent expert in the field of copepod taxonomy, Prof. Kiefer. He drew attention to the great similarity between the invader and one of the two native Eurasian species, *A. robustus*, which was described much earlier than the American taxon, but the holotype and type material for it were absent. To address this issue, Kiefer visited Oslo, Norway and took a sample from the lake Sars was working on. Comparing the type population from Sars's lake with individuals of *Acanthocyclops* from other water bodies of Europe, where the invader was already dominant, Kiefer became convinced of their complete identity and synonymized the American species with the species described by Sars, thus making it an artificial cryptic species under the name "*A. robustus*".

In our opinion, Kiefer's taxonomic mistake was caused by the rapid dispersal of the American taxon and its aggressiveness as a more active predator than the native forms. Thus, by the time Kiefer visited Oslo, this species had not only been introduced, but had practically displaced the native species from the lake ecosystem. This can be seen from Kiefer's drawings and was subsequently confirmed by a detailed sampling of the lake and its catchment (Miracle et al., 2013; Alekseev et al., 2020). The native species described by Sars still existed in this lake, but only in the mouths of small river tributaries of the lake; it was displaced by the American invader in the entire pelagial and littoral zones of the lake (Alekseev, 2021). To confirm this hypothesis and the actual validity of both the American and European taxa (in fact, to split this artificial cryptic taxonomy of Kiefer), it was necessary to conduct a molecular genetic examination and compare the type populations of American *A. americanus* and the two European taxa described in the 19th century from Norway by Sars and from Russia by Fischer — *A. robustus* and *A. vernalis*.

Two descriptions were added to these undoubtedly valid species from the water bodies of

France and Norway (Mirabdullayev and Defaye, 2002; 2004), which appeared due to attempts to sort out this confusion and turned out to be young synonyms of controversial cryptic species (*Acanthocyclops trajani = A. americanus*; *Acanthocyclops einsle = A. robustus*) (Miracle et al., 2013).

The phylogenetic tree that we made based on the sequences from the database GenBank (Fig. 1) and the original ones from the habitat types of three these *Acanthocyclops* species show that *A. americanus* from the United States, Mexico, the Czech Republic and Spain are practically identical, while the European forms, *A. vernalis* from Russia and *A. robustus* from Norway, are clearly separated from them. It is interesting to note that the distribution of native *Acanthocyclops* species most likely covers the entire Palearctic, which is confirmed by the discovery of forms very close to *A. robustus* in the area of Lake Baikal (see Fig. 1), 5000 km away from Norway and separated by mountain ranges and catchments of several large rivers.

It should be noted that not all copepodologists agreed with Kiefer's revision. In the identification keys in Ukraine and Russia, and hence in the studies of biodiversity in the territory of the former USSR, the dispersal of the American species continued to be recorded (Monchenko, 1974; Alekseev, 1995; 1998; Kruppa, 1998; Alekseev et al., 2002).

It was shown that after its appearance in the Volga delta (Alekseev and Kosova, 1976) and the Northern Caspian Sea (Chuykov, 1986), the main route of seasonal migration of waterfowl nesting in the Arctic zone of Western and Central Siberia, this species was found as an abundant form of summer zooplankton in water bodies of northern Kazakhstan, Crimea, the Trans-Urals and in even reservoirs of the Yenisei River (such as the Bratsk reservoir) (Alekseev, 1998; Kruppa, 1998; Anufriieva et al., 2014; Alekseev et al., 2020).

Such a rapid settlement of the vast territory covering the divided catchments of the largest rivers of Europe and Asia was possible, in our opinion, due to the active transfer of the invader by birds. This was facilitated by a specific form of physiological protection (winter diapause), which is reactivated in the invader only after the water warms to above 16 °C (Alekseev, 2021). As a rule, migrating birds fly north to their nesting sites following the melting of ice in local water bodies when the water temperature even near the shores of lakes and rivers is only a few degrees above zero. While feeding on bottom remains of plants etc. in this zone of waterbodies, birds capture cysts with dormant stages of invaders and then transfer them over considerable distances (geese up to 1000 km per flight). Both local species of this genus, preferring cold water in the beginning of their life cycles, have already been reactivated by this time and lack the physiological and constitutional (cysts) protection to migrate with birds in this way. Examples of external views of similar cysts protecting dormant copepodites can be seen in Figure 2.

Other reasons, along with efficient transportation by birds and often highly toxic ballast waters of ships, which is facilitated by the protective role of diapause, are rooted in the peculiarities of the invader's biology, which determine its advantage in the competitive struggle with native species. Some of the reasons for the displacement of native species by the invader have already been described in our previous paper, which included a higher growth rate, higher fecundity in comparison with both local *Acanthocyclops* species and adaptive behavioural features of its juveniles that reduce the risk of cannibalism (Alekseev, 2021).

Furthermore, one additional feature of the biology of the invader that was not previously considered but is important is the behaviour of adults in the process of hunting and feeding. Many planktonic invertebrates, being active predators, show dominance of hunting instinct over self-feeding (the so-called wolf in a flock of sheep effect, in which a predator kills many more prey than it can eat or carry away) (Monakov, 2003). Our observations on the feeding behaviours of sexually mature females of the invader on the larvae of the fire shrimps hatching from resting eggs showed that one female is capable of killing dozens of larvae in a short time, which is many times greater than her daily food intake (Alekseev and Pugachev, 1978). This phenomenon, called overconsumption, is known in many, mainly pelagic, copepod species. Both native *Acanthocyclops* cryptic species from Eurasia live in the littoral zone or at the bottom of waterbodies and do not show such high hunting activity (Monakov, 2003). In our opinion, this overconsumption feeding behaviour of the invader that allows it to be dominant. (Alekseev et al., 2002; 2020). We observed this domination usually in shallow-pelagic ecosystems such as city ponds in Belgium (Alekseev et al., 2002).

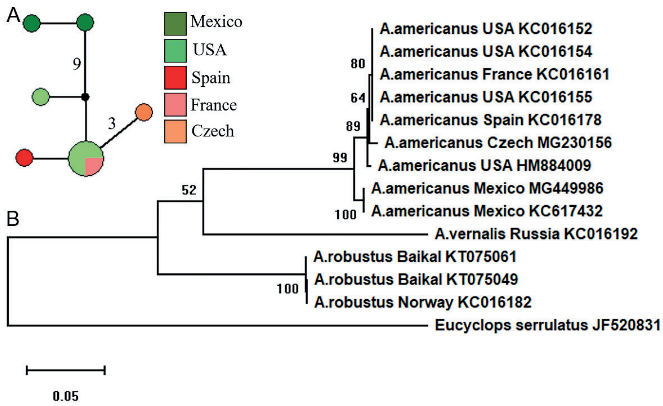

Fig.1. A) Median joining networks of the mitochondrial cytochrome oxidase subunit I (COI) of the mtDNA gene from *Acanthocyclops americanus* (Marsh, 1893) retrieved from GenBank. The black dot indicates the median vector. B) Maximum likelihood phylogenetic tree based on COI sequences of *A. americanus* using 13 sequences retrieved from GenBank. Bootstrap values for maximum likelihood (ML) (≥ 50%) are given for nodes. Accession numbers are given after the species name and geographical location. *Acanthocyclops vernalis* (Fischer, 1853), *Acanthocyclops robustus* (Sars G.O., 1863) and *Eucyclops serrulatus* (Fischer, 1851) are used as outgroups.

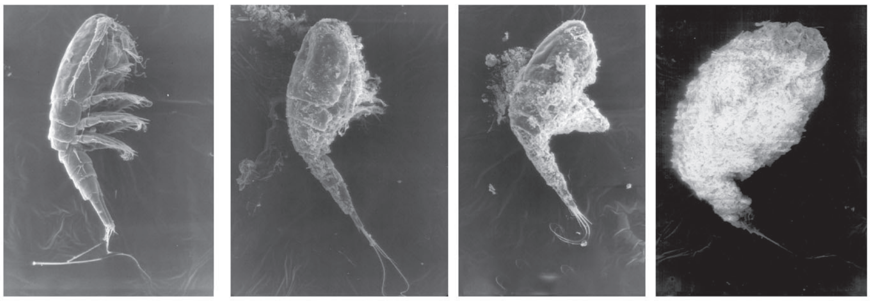

Fig.2. *Diacyclops thomasi* (S. A. Forbes 1882) cysts found by us in the lakes of Quebec at a latitude climatically close to Eurasia.

Such feeding behaviour, along with a high capacity for uncontrolled dispersal, represents, a particular danger of the invading species for Arctic waterbodies such as polygonal tundra lakes, in which the biodiversity of invertebrates is limited to several highly specialized species of crustaceans (Abramova et al., 2017). At present, the dispersal of the invader to the north is apparently limited by a short period of temperatures effective for its life cycle (above 16 °C). As a result, the range of this species does not reach the Polar Circle (64°N) and does not cover the tundra zone (Alekseev, 2021) (Fig. 3).

However, in the event of further climatic warming, the situation may change and the invader can reach arctic area in the nearest future. In that case the biodiversity and productivity of polygonal lakes of the tundra, which provide the main food for nesting waterfowl and juveniles of valuable fish species in the north, may radically change. So effective predator as *A.americanus* is capable of controlling even so large animals like phyllopods (Alekseev and Pugachev, 1978). The phyllopodsform the basis of nutrition for the juveniles of most waterfowl species in this region. . Apparently, it will not be possible to track further change the distribution of *A.americanus*, but it is necessary to follow the development of these species distribution to the north.

3. Settlement of the Palearctic species *Eucyclops serrulatus* (Fischer, 1851) in coastal waterbodies of other continents and zoogeographic zones

The second example, and also the second variant, of the bioinvasion of cryptic species is the dispersal of *E. serrulatus* along the main routes of navigation, apparently throughout the entire period of the sailing fleet. During this period, water was supplied and renewed in places where the ships sailed, usually the estuaries of rivers on the sea and ocean shores. The water was taken and transported after preliminarily emptying the remains and washing out the wooden barrels. The water in these barrels was not boiled, and the organisms contained in it, which were resistant to a decrease in oxygen content and a lack of food, as well as usually to an increase in temperature, were transported step by step, as local populations formed farther and farther along trade routes, geographical research and the colonial conquesting.

Cyclopids of the subfamily Eucyclopinae occupy a leading place among the relatively small number of species that meet the above requirements. *Eucyclops serrulatus* itself has long been considered cosmopolitan (i.e., living on all continents, against which the leading

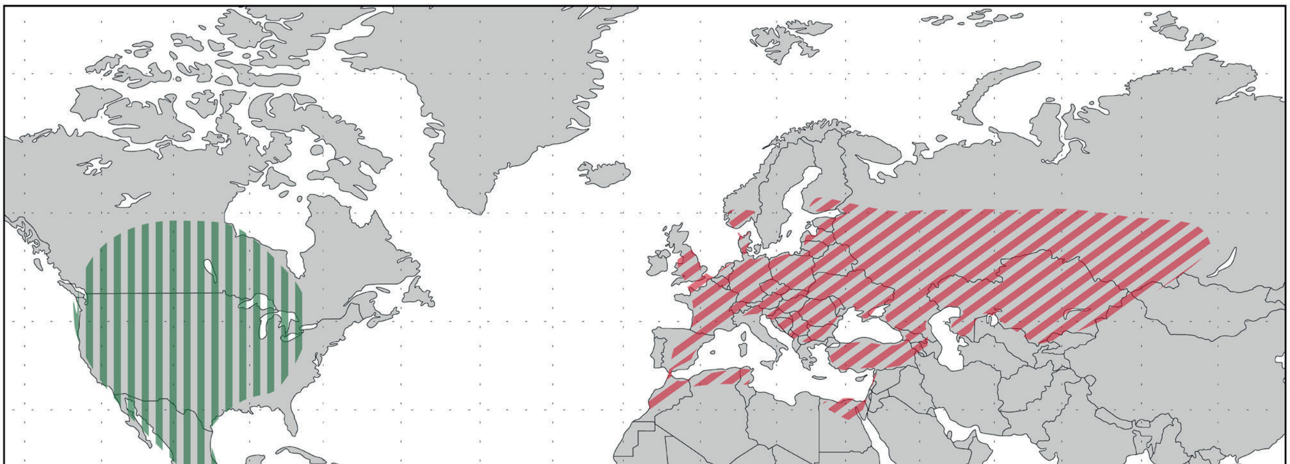

Fig.3. Confirmed native area (in green) of *Acanthocyclops americanus* (Marsh, 1893) and the extent of its invasion in the Palearctic (in red).

taxonomists objected back at the beginning of the last century. Thus, Kiefer explained a phenomenon of cosmopolitanism as a result of insufficient knowledge of species morphology among distant populations.

Indeed, the redescription of a single "cosmopolitanian" type species of *E. serrulatus* sensu stricto using several methods, including hybridization and molecular genetic diagnostics, led to the separation of about 30 species of this complex and, in turn, to the limitation of the natural range of *E. serrulatus* to part of the Palearctic (Alekseev et al., 2006; Alekseev and Defaye, 2011; Fig. 3). At the borders of this area, the species is represented by several subspecies, and outside it is replaced by other taxa, more or less corresponding to their niche properties (eurythermal, polygenerative, meiobenthic, substrate-bound, small collector-detritus eater) (Alekseev, 2019).

At the same time, the study of near-port regions including river deltas and adjacent waterbodies in areas that are very remote from the natural range often reveals the presence (never as dominants, but in a noticeable density comparable to native species) of representatives of the Palearctic species *E. serrulatus* sensu stricto. It has been reliably established (at least at the morphological level) that there are such populations of *E. serrulatus* sensu stricto cut off from the main area of North America, Australia, a number of countries in Southeast Asia (Singapore, Malaysia, Thailand), New Zealand and Tasmania (see Fig. 4).

All of these territories were included in the circle of possession or influence at the level of colonies of Great Britain, the largest maritime power for centuries. There is some evidence that these isolated colonies are the result of biological invasions of these species from Europe that occurred slowly over several hundred years, starting with the period of geographical discoveries. A reasonable settlement pattern of this kind (from one freshwater intake to another) is described at the beginning of this section. To validate this concept, it is necessary to conduct large-scale molecular genetic studies.

The nucleotide sequences of mitochondrial and nuclear DNA are already available in the database (Sukhikh and Alekseev, 2015; Kochanova et al., 2021) and make it possible to not only identify various haplotypes of the Palearctic species *E. serrulatus*, but also to use them to identify penetration vectors, including other invasive species, into various regions of the Earth (Fig. 5).

Such a dispersal of the species apparently occurs without significant rearrangements in local communities since almost everywhere *E. serrulatus* was found several other species, undoubtedly native, were also found in comparable numbers. It is known that this species coexists within its own range, and apparently diverges very effectively along microniches with several representatives of this genus. This complex in the Palearctic includes *Eucyclops macrurus* (Sars G.O., 1863), *Eucyclops macruroides* (Lilljeborg, 1901), *Eucyclops denticulatus* (Graeter, 1903), *Eucyclops speratus* (Lilljeborg, 1901) and *E. serrulatus*. Together with *E. serrulatus*, these species live in approximately equal numbers in the same biotope in the littoral part of lakes and rivers (Rylov, 1948; Monchenko, 1974). The apparent lack of competition seems to be compared with the well-known Hutchinson's paradox of plankton (Hutchinson, 1961), which states that species similar in ecological requirements and occupied niches coexist in the plankton of lakes without displacing each other. The ecological consequences of such a "cryptic" bioinvasion, an example of which is the resettlement of *E. serrulatus*, remain to be studied.

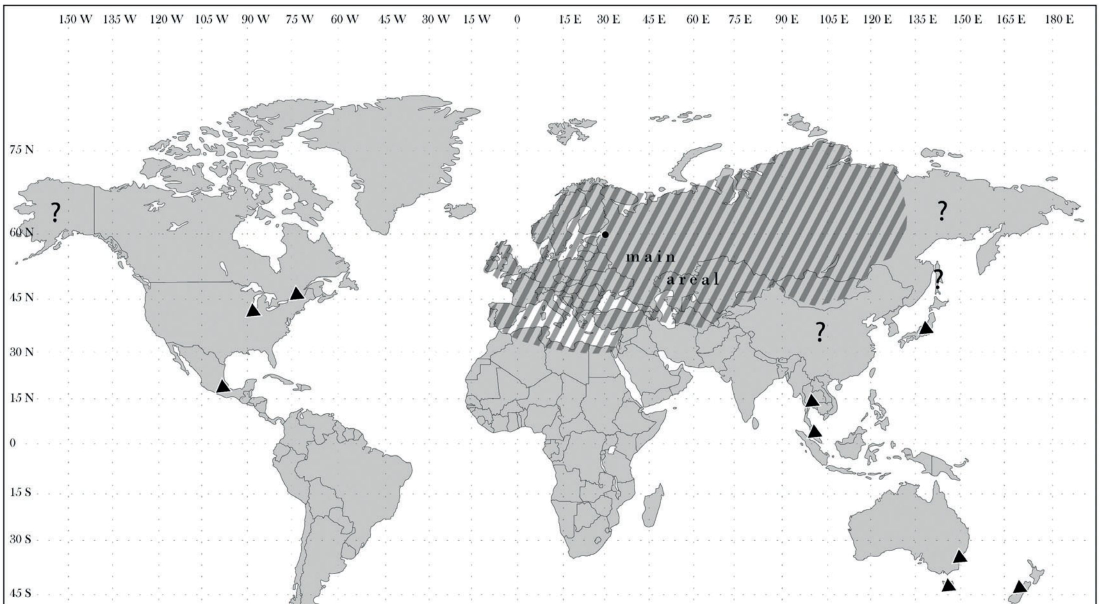

Fig.4. Main range of *Eucyclops serrulatus* sensu stricto (Fischer, 1851) (shaded) and its distant local populations (marked as triangles); the black point indicates the type locality and circles indicate localities of some confirmed findings (Alekseev and Defaye, 2011).

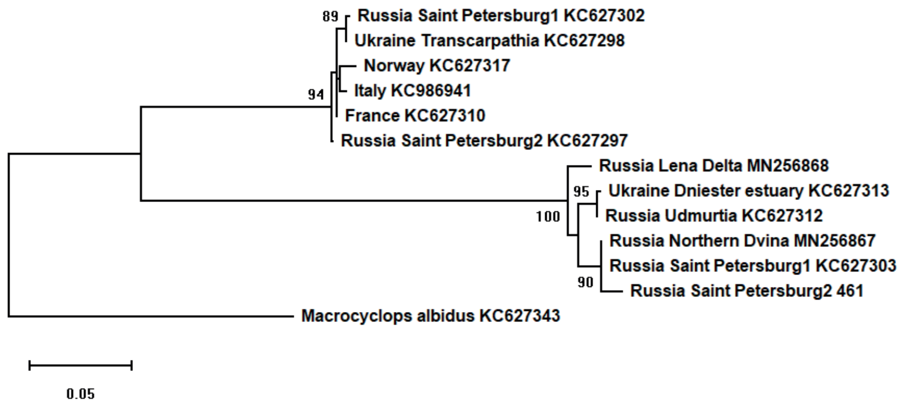

Fig.5. Maximum likelihood phylogenetic tree based on the 13 sequences retrieved from GenBank using the cytochrome oxidase I (COI) mtDNA gene from *Eucyclops serrulatus* (Fischer, 1851). Bootstrap values for maximum likelihood (ML) (≥ 50%) are given for nodes. Accession numbers are given after the species name and geographical location. *Macrocyclops albidus* (Jurine, 1820) was used as the outgroup.

The spread of *E. serrulatus* and similar species contributes to the emergence of the phenomenon of “neo-cosmopolitanism” which is not based on the historical reasons for the settlement of species across continents (the existence of Pangea, drift of continents, evolution of the Tethys Sea, etc.), but on human-mediated dispersals.

4. Biological invasions of *Eurytemora carolleeae* Alekseev and Souissi, 2011 and *Eurytemora caspica* Sukhikh et Alekseev, 2013

The *Eurytemora affinis* species complex is a group of species inhabiting the Holarctic (Lee, 1999). Since the first species description by Poppe in 1880, it was clear that the species has a very high level of morphological polymorphism, even in the Baltic Sea that is close to the type locality where few subspecies were described (Sukhikh et al., 2016). Genetic analysis of extensive population samples of *E. cf. affinis* using the mitochondrial genes 16S rRNA and cytochrome c oxidase 1 (CO1) revealed a significant genetic heterogeneity in the northern hemisphere (Lee, 1999; 2000). As a result, *E. affinis* was recognized as a cryptic species complex (Lee, 2000).

Due to the worldwide distribution of the species complex, its euryhalinity and central position in the food web, it has been well studied using morphological characteristics (Lee and Frost, 2002; Alekseev and Souissi, 2011; Sukhikh et al., 2013), genetic tools (Lee, 2000; Winkler et al., 2011; Sukhikh et al., 2013; 2016; 2019; 2020a; 2020b), hybridization methods (Prof. Sami Souissi, personal communication) and searches of physiological features (Knatz, 1978; Hirche, 1992; Devreker et al., 2008; 2010; Beyrend-Dur et al., 2009; Dur et al., 2009; Lloyd et al., 2013; Lajus et al., 2015).

The phenomenon of cryptic speciation was supported by hybridization experiments that showed reproductive isolation among some North American populations (Lee, 2000) and between North American and European populations (S. Souissi, unpublished data). Furthermore, significant ecophysiological differences between one North American and one European population were found (Beyrend-Dur et al., 2009). Nevertheless, no valid species were distinguished in this complex since morphological stasis was established for these species (Lee and Frost, 2002).

However, when a *Eurytemora* population of the Gulf of Finland was studied with molecular-genetic tools in the frame of the project on biological invasions in 2007, the estuarine North American *Eurytemora* was revealed (Alekseev et al., 2009; Sukhikh et al., 2013). These studies provided the basis for detailed morphological analyses. A new set of morphological signs allows us to overcome the limitation of the morphological stasis hypothesis. As a result, the North American *E. cf. affinis* (USA) was described as a new species, *E. carolleeae* Alekseev and Souissi, 2011. Later, the Asian *Eurytemora* population from the Caspian Sea was also described morphologically as a new species, *Eurytemora caspica* Sukhikh and Alekseev, 2013. Thus, the species complex is currently represented by three species: *E. affinis* with a Palearctic distribution; North American *E. carolleeae*; and Asian *E. caspica* Sukhikh and Alekseev, 2013.

It is interesting that, according to pictures and descriptions of *Eurytemora* species in English waters (Gurney, 1931), it seems as though the American invader *E. carolleeae* already inhabited this area of water at the beginning of the 20th century. Perhaps it was an invasion through ship ballast water, similar to the case of *Eurytemora americana* Williams, 1906, which was originally discovered in 1933 in the same area (Lowndes, 1931).

The presence of the invasive *E. carolleeae* species in European waters has only been reported in specific locations, namely the Gulf of Finland, the Gulf of Riga, Amsterdam channels (Sukhikh et al., 2013), the Oder River (Sługocki et al., 2021), Kiel Bight, Mecklenburg Bight, the Arkona Sea, the Bornholm Sea, the Eastern Gotland Sea (Wasmund et al., 2013) and perhaps in British waters (Gurney, 1931) (Fig. 6, Fig. 7).

Experimental studies comparing the fitness traits (development time, clutch size and longevity) of *E. affinis* (from the Seine estuary, France) and *E. carolleeae* (from St. Lawrence salt marshes, Canada,

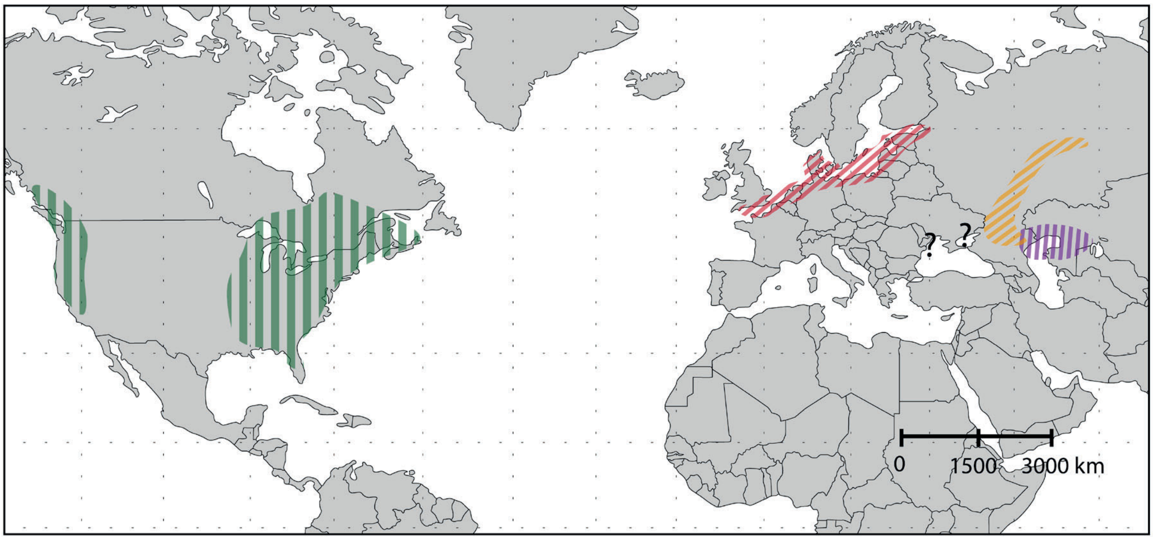

Fig.6. Confirmed native area of *E. carolleeae* Alekseev and Souissi, 2011 (in green) and *E. caspica* Sukhikh et Alekseev, 2013 (in violet) and the extent of the invasions in the Palearctic for *E. carolleeae* (in red) and *E. caspica* (in orange). Question marks are questionable areas for *E. caspica*.

and Chesapeake Bay, USA) have confirmed the higher fitness of the North American population (Beyrend-Dur et al., 2009; Devreker et al., 2012) compared to the European one (Dur et al., 2009; Devreker et al., 2012). In addition, field measurements have suggested that, in both populations, egg production decreases when temperatures rise above 18 ˚C (Lloyd et al., 2013; Pierson et al., 2016). This corroborates results from laboratory experiments (Devreker et al., 2012).

The population dynamics of both species coexisting in the Gulf of Finland are largely parallel and exhibited one or two summer population density peaks at the same time. Invasive *E. carolleeae* is usually second to *E. affinis* in terms of density (the invader accounts for about 30% of the total adult species density for the two species). It was observed that the invasive species has a larger body size and different reproductive traits that could facilitate displacing native *E. affinis* species by alien *Eurytemora* (Sukhikh et al., 2019). Moreover sometimes only *E. carolleeae* was observed in samples; this suggests a major shift in zooplankton populations, featuring a replacement of *E. affinis* by invasive *E. carolleeae*. However, the shift in zooplankton was temporary since samples devoid of *E. affinis* were recorded only two times in the summers of 2010 and 2015. These seasons featured unusual temperatures — hot in 2010 and cold in 2015 (https://en.wikipedia.org; https://en.wikipedia.org/wiki/2010_Northern_Hemisphere_summer_heat_waves). Summer in 2021 was even hotter than in 2010 and water in the Russian part of the Gulf of Finland at the end of July reached 25 ˚C (http://weatherarchive.ru/Pogoda/Lomonosov/July), whereas the yearly mean water temperature in the eastern part of the Gulf of Finland usually varies between 0 (winter) and 18–20 ˚C (summer) (http://weatherarchive.ru/Sea/Ust-luga/July). We did not find total loss of the native species in this year, but density of the alien *E. carolleeae* was six times higher than *E. affinis*, which is abnormal for the Gulf of Finland community (unpublished data). These uncommon temperature conditions probably negatively affected native *E. affinis* populations without reducing population densities of invasive *E. carolleeae*. The temperature tolerance of the invasive copepod species is possibly wider as water temperatures in its native Chesapeake Bay range between 5 ˚C and 25 ˚C (Kimmel et al., 2006). *Eurytemora carolleeae* is also characterized by high egg productivity in the same food conditions (Pierson et al., 2016; Sukhikh et al., 2019), which could favour its rapid spread in the area. Invasive species may be more successful than native ones in fast-changing

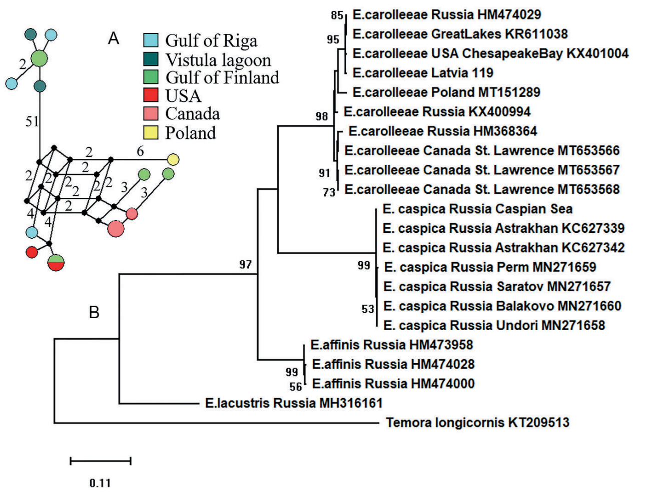

Fig.7. A) Median joining networks of the mitochondrial cytochrome oxidase subunit I (COI) mtDNA gene from *Eurytemora affinis* (Poppe, 1880) (upper) and *Eurytemora carolleeae* Alekseev et Souissi, 2011 (lower) retrieved from GenBank. Black dots indicate median vectors. B) Maximum likelihood phylogenetic tree based on COI sequences of *E. carolleeae, E. affinis* and *Eurytemora caspica* Sukhikh et Alekseev, 2013, constructed using the 22 sequences retrieved from GenBank. Bootstrap values for maximum likelihood (ML) (≥ 50%) are given for the nodes. Accession numbers are given after the species name and geographical location. *Eurytemora lacustris* (Poppe, 1887) and *Temora longicornis* (Müller O.F., 1785) were used as outgroups.

environmental and temperature conditions. Another parameter confirming more favourable environmental conditions for the invader in the Baltic is fluctuating asymmetry (FA). Lajus et al. (2015; 2020) compared levels of FA for populations of *E. carolleeae* from their native Chesapeake Bay and for the native and invasive species in the Gulf of Finland. FA is often used to monitor stress of different origins (Zakharov, 1989; Graham et al., 2010; Beasley et al., 2013). FA was larger for native *E. carolleeae* (Chesapeake Bay) compared to invasive *E. carolleeae* (Gulf of Finland). Interestingly, *E. affinis* from the Gulf of Finland has almost the same FA as the invasive *E. carolleeae* species. This may be the result of generally less stressful environmental conditions in the Gulf of Finland compared to the Chesapeake Bay. The Gulf features different temperature conditions and fewer salinity changes due to the absence of tides. FA of *E. caspica* from the Caspian Sea was minimal within all studied populations (Lajus et al., 2015).

Caspian *Eurytemora* is not as well studied in terms of physiology. According to Krupa (2020), its optimum temperature range is 21–22 °C, which is higher than the values given for *E. affinis* populations in European waterbodies. Initially, the species' area was rather restricted and was represented by northern Caspian Sea and lower Volga River. However, due to the construction of a chain of large water reservoirs along the Volga River, every year the species occupies more and more northern parts of the Volga River and its tributaries up to Perm (Fig. 6 and Fig. 7). Today *E. caspica* reaches the 58th latitude compared to its original range, which was around the 40th–50th latitude (Lazareva, 2018; 2020; Sukhikh et al., 2020b). Considering global warming and ship connections, we can expect further distribution of the species. *Eurytemora caspica* also occurs in central and eastern Kazakhstan (Krupa et al., 2016; Krupa, 2020) and in the Volga-Don channel built in the middle of last century (Lazareva, 2020; 2021). According to the published pictures the species under other names (*E. affinis* and *Eurytemora hirundoides* (Nordquist, 1888)), *E. caspica* is also found in the Black Sea and Sea of Azov (Samchishina, 2005), possibly due to previous invasions via the Volga-Don channel (Lazareva, 2021).

Concluding our review of the introduction of the American species into European waters, we should point out a relatively stable ratio of of its population compared to the native species, which is violated in favour of the invader in years with extreme temperatures. The simultaneous resettlement of the Caspian endemic, which has taken place recently, is due, in our opinion, to hydro-construction (reservoirs on the Volga River) and the creation of navigable canals between the basins of the Black, Caspian and Baltic Seas. The observed tendency of *E. caspica* to move further north allows us to expect to find this species in the Gulf of Finland of the Baltic Sea in the near future.

5. Conclusions

The described examples of biological invasions of cryptic species make it possible to distinguish at least three types of interactions with closely-related species in invaded waterbodies:

- Effective competition, leading to the rapid displacement of native species from their niches, which may be accompanied by taxonomic problems, among other things. Example: *Acanthocyclops americanus* as invader from North America to Eurasia.
- Division of the existing niche between two competitors of practically equal strength and maintenance of a constant relationship between them for a long time, accompanied by periodic changes in the position of one or the other due to fluctuations in environmental conditions (e.g., meteorological conditions). Example: the invasion of *Eurytemora carolleeae* into the Baltic Sea and the Atlantic coast of Europe.
- "Easy" joining of the invader to the community of closely-related species that are similar in ecological requirements and a new community is not formed as observed in the case of *Eucyclops serrulatus* invasion.

Biological invasions can increase biological diversity, leave it unchanged, cause the replacement of one species by another or reduce its population in the event of the penetration of active predators and effective competitors. The dispersal of invasive species of continental hydrofauna, initially based on anthropogenic transportation after crossing serious zoogeographic barriers (such as oceans), can subsequently develop rapidly using local biological routes of dispersal, for example waterfowl. The rate of dispersal in this case can be very high, especially when transportation occurs during the diapause stage of an organism with increased resistance to unfavourable environmental conditions. In our opinion, in recent years a new phenomenon of the distribution of species over distant continents has begun, which should be called "neo-cosmopolitanism". This neocosmopolitanism is based not on the historical reasons for the settlement of species across continents, but on human-mediated dispersals.

Despite the fact that to date, confirmed invasions of cryptic copepod species have been identified only among *Eurytemora* and *Acanthocyclops*, this rather reflects the level of Copepoda research from the point of cryptic bioinvasion, and in many genera of the order there are potential candidates for cryptic invaders (almost all taxa showing cryptic species). This project may be especially pertinent in the context of global climate warming and growing anthropogenic pressure on aquatic ecosystems.

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

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Substrates for cell cultivation based on thermosensitive imidazole copolymers

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ABSTRACT. Cell cultures are needed in various fields: the study of cell structure and function, models in drug screening, and other biomedical applications. Most tissue-derived cells can only grow on solid substrates. Thus, cell culturing involves three steps: cell adhesion on the surface, cell growth and division on the surface, and cell detachment from the surface for further use. Thermo- and pH-sensitive polymers are promising substances for cell culture coatings. Changes in temperature and/or pH can drastically change surface properties, resulting in gentle cell detachment. We synthesized copolymers with pendant imidazole and hydrophobic groups that exhibit the properties of weak polymeric bases capable of thermosensitivity due to hydrophobic interactions. Plastic surface can easily be coated with copolymers by pouring over the copolymer solution. The modified plastic surface is a good substrate for culturing adenocarcinomic human alveolar epithelial cells. The cells show strong adhesion to the copolymer film and high viability after detachment under the influence of temperature and/or pH changes.

Keywords: thermosensitive polymer, imidazole, polyelectrolyte, surface modification, cell culture, cell adhesion and detachment

1. Introduction

Adhesive cell culture on various substrates such as glass, polystyrene, natural and synthetic polymers has become a popular method for supporting cells *ex vivo* in laboratories. They are used to gain knowledge about biological processes inside cells, to study the toxicity and efficacy of drugs in a cell model before animal and human studies, to develop new biomaterials for medical applications, and to create 3D models of cell cultures closely resembling specific tissues and organs for a more accurate physiological response to the research object. (Verma et al., 2020). Although some cells grow in suspension and do not depend on attachment to the substrate (e.g., blood cell lines), most tissue-derived cells die from anoikis without proper cell adhesion or aggregation (Paoli et al., 2013).

One of the main challenges of modern 3D cell culture is that most methods result in cell aggregation into spheroids 20-500 μm in diameter, since such aggregation is limited due to the lack of a vascular system for oxygen and nutrient delivery (Langhans, 2018). However, detaching a cell sheet from the surface without breaking intercellular interactions during cell detachment with only cell-substrate connections broken can create sheets with adjustable widths by adding layers on top of each other, and the sheet area will be limited only by the selected culture dishes. Cell sheet production has a future in transplant medicine by creating cell sheets from different cell types (Yamato et al., 2001; Sekine et al., 2013). Moreover, in ordinary cell culture there is a need to maintain a certain number of cells in a Petri dish by manually detaching the cells with chemical agents (Na_2EDTA and trypsin) after the cells have grown a monolayer on the substrate. In the monolayer the cells cannot continue their reproduction and release a huge number of metabolites that can trigger the processes of cell aging and programmed cell death - apoptosis. In cell biology, smart materials with properties of cell detachment under certain stimuli are needed to simplify manipulations, improve cell detachment and create new models for drug screening and transplantation medicine.

Natural cell adhesion happens through the interaction of cell membrane receptors (integrins) with extracellular matrix proteins, such as collagen,

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fibronectin, laminin, which have special amino acid sequences with adhesive properties (RGD: Ruoslahti, 1996; GFOGER: Knight et al., 2000; PHRSN: Dillow et al., 2001). Nevertheless, the easiest way to achieve cell adhesion in a daily cell culture is to use plasma-treated polystyrene laboratory tissue culture dishes, where the surface charge of the dishes will depend on the gases used to treat the plasma (Lerman et al., 2018). Mostly, it is a net negative charge or both negative and positive charges (Barker and LaRocca, 1994). Interestingly, the cell membrane has a negative charge (Fairhurst et al., 2007; Gallardo et al., 2017) and for passive adhesion to matrix positive charge on the surface of biomaterial is essential. After passive adhesion, the cell begins to spread over the surface and establish strong focal adhesions using integrins and the actin cytoskeleton, which require an energy source, adenosine triphosphate (ATP), and are called active adhesion. (Okano et al., 1995). Studies of functional groups similar to those in amino acids (-COOH, $-CH_2OH$, $-CONH_2$ and $-CH_2NH_2$) have shown that polar and positively charged surfaces have better conditions for cell growth, adhesion and proliferation than negatively charged surfaces (Lee et al., 1994). Cell detachment techniques such as trypsin, Na_2EDTA, and scraping reduce cell viability, disrupt the integrity of membrane receptors, disrupt intercellular connections, and the ECM molecules secreted by cells, which reduces the quality of experiments and makes it impossible to collect cell sheets for regenerative medicine.

Stimulus-sensitive polymers have become a valuable tool in cell biology due to their unique properties of harmless cell detachment with increased cell viability and preservation of cell membrane receptors (Chen et al., 2015; Kurashina et al., 2017), cell sheet recovering by keeping intact cell-cell junctions and extracellular matrix (ECM) (Akiyama and Okano, 2015) in comparison with more conventional detachment methods: chelating agents (Na_2EDTA) and enzymes (trypsin). Smart polymers are sometimes used in cell sorting methods (Matsuda et al., 2007; Zhao et al., 2016). Poly-(N-isopropylacrylamide) (PNIPAM) is the gold standard and widely investigated polymer in this field because the hydrophobic character of the polymer changes to a highly hydrophilic one when the temperature changes from 37˚C to 20˚C, which impairs cell adhesion to the polymer substrate, but electron beam (EB) irradiation, which is used to graft PNIPAM onto laboratory dishes, is an expensive procedure. Although some modified PNIPAM coatings can overcome this disadvantage, they still face another problem: making a surface with sufficient polymer grafting density. (Akiyama and Okano, 2015).

Although temperature is the most abundant stimulus in cell culture, there are pH (Chen et al., 2012), mechanical stress (Akiyama et al., 2018) and electricity (Gao et al., 2016). Moreover, we believe that only one stimulus cannot provide sufficient quality for cell sorting, and that a larger number of modifiable parameters improves the procedure. In addition, the combination of stimuli allows for finer regulation of cell detachment. That is why new polymers with temperature and pH stimulus were synthesized to enhance cell adhesion and detachment parameters, cell sheet collection, and cell sorting quality. One of the main features of these polymers would be a simple modification of most substrates connected with cell culture (cover glass, polystyrene, PDMS) that have a great potential in creation of smart materials in cell culture like microfluidics.

The work is aimed at synthesizing thermo- and pH-sensitive polymers, coating plastic surfaces, and studying cell growth on the modified surfaces. The pH sensitivity of the polymers was achieved by introducing imidazole groups, which are not strongly protonated at neutral pH and therefore should be less toxic than amine groups.

2. Materials and methods

2.1. Chemical reagents and cell culture

Acryloyl chloride, 3-(1H-imidazol-1-yl)propan-1-amine, dipropylamine, triethylamine (Sigma-Aldrich, Acros Organics, USA) were purified by distillation before use. Dibenzylfluorescein was used without purification (Sigma-Aldrich, USA). DMF was dried with $CuSO_4$ (30 minutes) and distilled at 5 mm Hg. Asobis(isobutyronitrile) (Sigma-Aldrich, USA) was recrystallized from ethanol. NaOH was purified from carbonate impurities by filtering its 50% solutions. Ethanol were purified according to (Keil et al., 1966) Dichloromethane and 3-methylbutan-1-ol were purified by distillation. Solutions were prepared using deionized water (resistance 18.2 MΩ·cm, Millipore Simplicity UV, USA). A cellophane membrane (3.5 K) was used for dialysis.

CalceinAM (Invitrogen), Propidium Iodide (95%, J&K Scientific), Fetal Bovine Serum (FBS, Biowest), NaOH (98%, Ekspit, Russia), Dulbecco's modified eagle medium (DMEM), gentamicin (10 mg/mL), phosphate-buffered saline (PBS) (pH = 7.4) were obtained from Biolot, Russia. Sterile 24-well microbiological polystyrene plates were used (non-pyrogenic, non-cytotoxic, DNase/RNase/DNA – free, SPL Life Science Co., Ltd). A549 adenocarcinomic human alveolar epithelial cell line (from American Type Culture Collection ATCC) were obtained by Biolot, Russia.

2.3. Modification of plastic microbiological assay plates

24-well microbiological polystyrene plates were modified with a polymer solution in $EtOH:CH_2Cl_2$ 85:15 v/v mixture. Poly(vinyl butyral) (PVB) or 3-methylbutan-1-ol were added to the polymer solution with the aim to obtain non-fragile films (Table 1). Solutions were evaporated under a closed cap for 48 h on an orbital shaker (90 rpm). The plates were stored at room temperature for 24 h and heated for 2 h at 80°C. The fluorescent dye dibenzylfluorescein was added to the polymer solutions in order to visualize the coatings and check their stability. The coatings were stable for 7 days at 37°C in phosphate buffer (pH 7.4, 50 mM).

Table 1. Compositions for the plates modification

Coating	Polymer	Polymer conc., %	Additive	Additive conc., %	Solution volume, µL	*Coating thickness, µm
PV5-1-A	ZS-682-5	0.1	PVB	0.01	300	1.307
PV5-2-A	ZS-682-5	0.1	3-methylbutan-1-ol	0.005	300	1.307
PV6-1-A	ZS-682-6	0.1	PVB	0.01	300	1.307
PV5-1-B	ZS-682-5	0.02	PVB	0.01	300	0.261
PV5-2-B	ZS-682-5	0.02	3-methylbutan-1-ol	0.005	300	0.261
PV6-1-B	ZS-682-6	0.02	PVB	0.01	300	0.261

* Theoretically, taking the polymer density as 1.

2.3. Study of the cell growth on the modified surfaces

24-well assay plates modified with different polymers were sterilized under UV light (250 nm) for 30 minutes. A549 cells were seeded at concentration 50,000 cells *per* well and incubated in DMEM medium supplemented with 10% fetal bovine serum, gentamicin (50 µg/ml) at 37 ºC, 5% CO_2 in a humidified atmosphere for 72 h until a monolayer cell formation for most samples. Then cells were imaged on an inverted Microscope Leica DMi8 (cells unaffected by CalceinAM chelating properties), washed with 1x phosphate-buffered saline (PBS), stained by CalceinAM for 30 minutes, washed with PBS again, stained by propidium iodide (1 µg/ml) for 10 minutes, and washed with PBS. Fluorescent images were obtained via Microscope Leica DMi8 in a Rhodamine and FITC filters. All images were edited via LAS X software.

2.3. Instrumentation

^{1}H NMR spectra were recorded using a Bruker DPX 400 (Bruker Biospin Corporation, Billerica, MA, USA) at 400.13 MHz, in DMSO-d_6 for ZS-682-5 and in D_2O for ZS-682-6. IR spectra were recorded on an Infralum FT-801 instrument (SIMEX company, Novosibirsk) using KBr pellets.

The molecular weight (MW) of ZS-682-5 in chloroform was determined by the light scattering method (Photocor, Russia) using plots of inverse scattering intensity versus concentration. MW was calculated from the segment cut off by a straight line on the y-axis, according to the Debye equation.

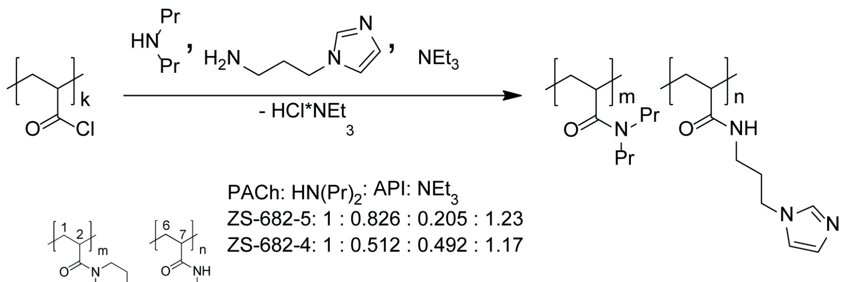
Potentiometric titration was carried out on a Multitest ionometer (NPP Semiko, Novosibirsk, Russia) in water with the addition of 0.1 M NaCl, the polymer concentration was 7.5 mM. Before measurement, HCl was added to the solution to pH 2.5 and the solution was titrated with 0.1M NaOH.

Fluorescence microscopy was carried out using a MOTIC AE-31T and Leica DMi8 inverted microscopes. Ultraviolet analytical cabinet "UFK-HD" (LLC "PETROLASER", St. Petersburg) was applied to detect the stability of coatings in an aqueous environment using dibenzylfluorescein.

3. Results and discussion

3.1. Synthesis and study of copolymers

The copolymers were obtained by reacting poly(acryloyl chloride) with 1-(3-aminoprolyl) imidazole (API) and dipropylamine in the presence of triethylamine (Scheme 1)., similar to (Danilovtseva et al., 2017). Structure of the copolymers was confirmed by ^{1}H NMR (Fig. 1) and their composition was analyzed



Scheme 1. Synthesis of copolymers.

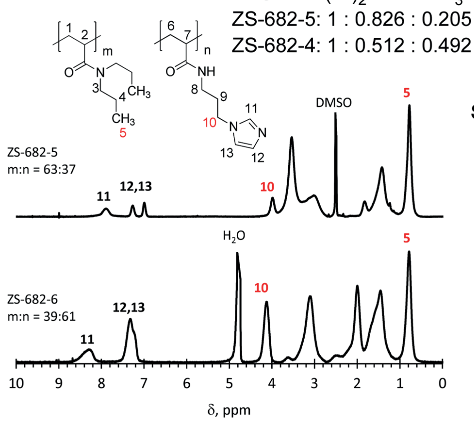


Fig.1. ^{1}H NMR of copolymer ZS-682-5 in DMSO-d_6 and ZS-682-6 in D_2O.

by signal intensity ratio at 0.5-1.1 ppm (CH_3 groups) and 3.9-4.4 ppm (CH_2 group attached to the imidazole cycle).

ZS-682-5 copolymer is insoluble in water, but soluble in organic solvents (ethanol, chloroform, dichloromethane). It exists in mono-macromolecular form in chloroform (hydrodynamic radius of 4.3 nm), and its molecular weight has been measured as 29.8 kDa. Comparing this value with the degree of polymerization of the original PACh (220 (Zakharova et al., 2018), we can conclude that there is no noticeable change in macromolecule length during the modification reaction. ZS-682-6 copolymer contains more hydrophilic groups and it is soluble in water and ethanol but insoluble in chloroform. Increasing the ionic strength (0.15 M NaCl, pH 4 and 7 buffers) prevents solubility in aqueous medium. This copolymer gives associates in aqueous solutions (hydrodynamic radius 56 nm), which do not allow a correct measurement of the molecular weight. Estimation of the molecular weight by minor monomolecular peak (3.3-3.6 nm) gives 30 kDa which agree with ZS-682-5 data.

The ZS-682-6 copolymer precipitates out of water at pH greater than 6.5 at 20°C. Light scattering (90°) and transmittance studies (Fig. 2) show the formation of turbidity at 46°C. The data of dynamic light scattering (Table 2) show the presence of macromolecules in aggregates of two sizes: 30 and 200-400 nm in radius. Large aggregates decrease in size and contribution to the scattering intensity when heated and disappear at 40°C. This behavior explains decrease in scattering from 10 to 30°C in Fig. 2. Heating ZS-682-6 should enhance the compactization of macromolecules and their aggregates due to hydrophobic interactions, resulting in redistribution between large and small aggregates. Polymer precipitation at about 50°C does not show large particles in DLS due to the effect of multiple scattering (Danilovtseva et al., 2015).

ZS-682-6 was studied by potentiometric titration at pH below 6.5, in the region of solubility (Fig. 3). pK of conjugated acid ionization of the imidazole units increases with increasing degree of ionization (α), because a decrease in the positive charge on the polymer chain hinders further elimination of protons from it. Increasing the temperature reduces the basicity of the imidazole units due to compactization of the macromolecules, which increases the electrostatic effect on the acid-base properties.

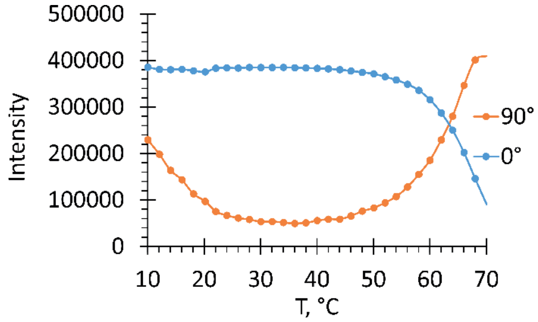


Fig.2. Light scattering (90°) and transmittance data for ZS-682-6 copolymer at pH 6 in sodium acetate buffer (20 mM). The polymer concentration was 7.5 mM (in polymer units) and the heating rate was 0.6 deg/min.

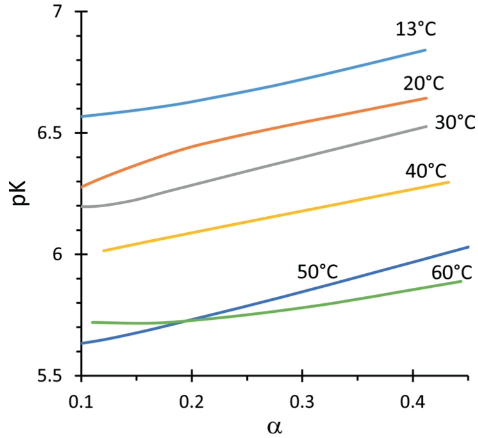


Fig.3. Dependence of conjugated acid pK vs. α for ZS-682-6 copolymer.

Table 2. Particle size in aqueous solution of ZS-682-6 at pH 6

T, °C	R_1, nm	I_1, %	R_2, nm	I_2, %
10	30	60	281	40
20	30	71	396	30
30	28	76	190	24
40	39	100	-	-
50	42	100	-	-
60	53	100	-	-

3.2. Observation of cell adhesion, growth, and proliferation on polymer coatings of different thicknesses

Using the polystyrene surface for control and synthesized polymers, cells were cultured on different substrates. Endothelial cells A549 were used as a well-characterized cell line with medium adhesive properties to substrates. In addition, A549 cells are a popular model for 3D cell culture due to their epithelial origin of lung adenocarcinoma and strong cell-cell interactions. Being too hydrophobic, untreated polystyrene showed a lack of cell attachment to its surface. Treatment with PV6-1-B polymer significantly improved cell adhesion to the substrate, although some cells remained rounded, i.e., poorly attached to the surface (Fig. 4). Meanwhile, a thicker version of this PV6-1-A polymer coating had no cell attachment and only irregularities in the polymer coating were visible. The cell morphology on PV5-1-B and PV5-2-B surfaces corresponds to a typical monolayer epithelial cell line with close cell-cell interaction. PV5-1-B and PV5-2-B are completely suitable for conventional cell culture. On thicker polymer layers PV5-1-A and PV5-2-A cell attachment was reduced to a small number that showed weak proliferation rate due to dependency on cell adhesion quality. Here, we recognized the importance of thickness in developing stimuli responsive polymer coatings. Thus, further experiments are needed to find the most appropriate polymer thickness to regulate cell adhesion.

3.3. Cell detachment under temperature and pH stimuli from different polymers

Response to the stimuli was examined by changing temperature by adding DPBS (DPBS temperature was similar to room temperature and corresponded to 25°C), or by simultaneously changing temperature by adding DPBS and increasing pH to 8.2 by adding 0.1 M NaOH solution. The PV6-1-B surface responded sufficiently to pH and temperature stimuli, and cells easily detached from the surface (Fig. 5). Moreover, using both stimuli, a change in pH and temperature, compared to a change in temperature, showed a significantly higher level of cell detachment, as can be seen by morphological changes from slight to complete rounding of the cells. The cells were then successfully detached from the surface. Although the PV5-1-B and PV5-2-Bsurfaces also showed some delamination in response to pH and temperature stimuli, it was limited to the center of the polymer coating, which may be due to the uneven thickness of the polymer layer, and cells are sensitive to the thickness of the cell layer.

3.4. Life/Dead cell staining after detachment

Cells recovered after detachment from various polymers were stained with two dyes: CalceinAM (green) for live cells and Propidium iodide (red) for dead cells (Fig. 6). It is worth noting that staining and imaging procedures take time and occur at room temperature, which led to rounding of cells on stimulus-sensitive polymers. Cells that were separated from the PV6-1-B polymer and then seeded onto the same new polymer layer showed a high concentration of separated cells. Moreover, the detachment procedure and prolonged cell culture on the PV6-1-B layer showed no significant signs of cytotoxicity even when compared to the mild chemical detachment method using Na_2EDTA. PV5-1-B and PV5-2-B layers showed poor cell retrieval after detachment even when both stimuli were used. Nevertheless, cells showed good viability in these layers.

Conclusions

New copolymers containing weakly basic imidazole groups and hydrophobic fragments were obtained. Water-soluble sample show termo- and pH-sensitivity. The plastic surface can easily be coated with copolymers by pouring over the copolymer solution. The modified plastic surface is a good substrate for culturing A549 cells. The

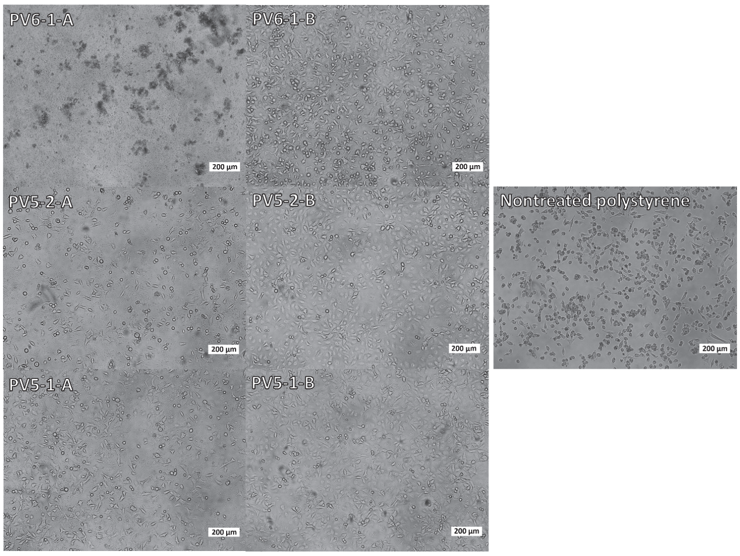


Fig.4. Adhesion of A549 cells to various substrates. Nontreated polystyrene showed poor adhesion, most cells are rounded. PV6-1-A didn't show any cell adhesion at all while cells on a thinner PV6-1-B layer have a moderate adhesion and only some cells are unattached. Although cells attached and proliferated restrictively on the PV5-1-A and PV5-2-A thick polymer coatings, the PV5-1-B and PV5-2-B polymer substrates showed perfect cell adhesion and resulted in a cell monolayer, proving the suitability of their composition for cell culture.

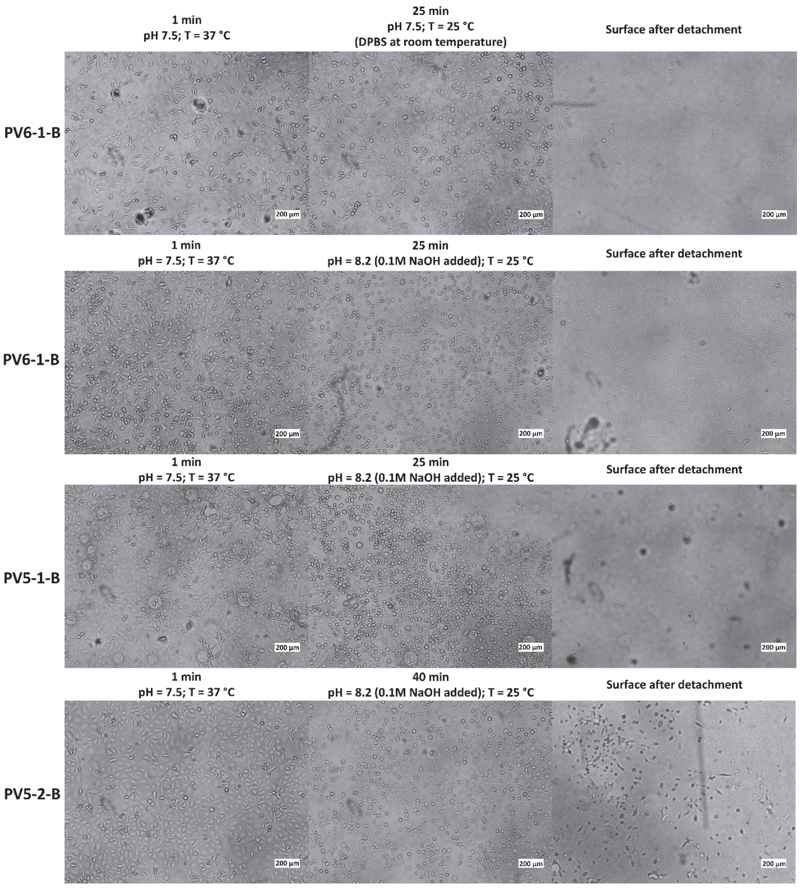


Fig.5. Characteristics of A549 cell detachment in response to changes in temperature and pH from polymer substrates. PV6-1-B demonstrated significant cell detachment in response to changes in temperature and pH across the surface. PV5-1-B and PV5-2-B delamination was limited to the center of the well surface and was more sensitive to changes in pH than to changes in temperature (not shown).

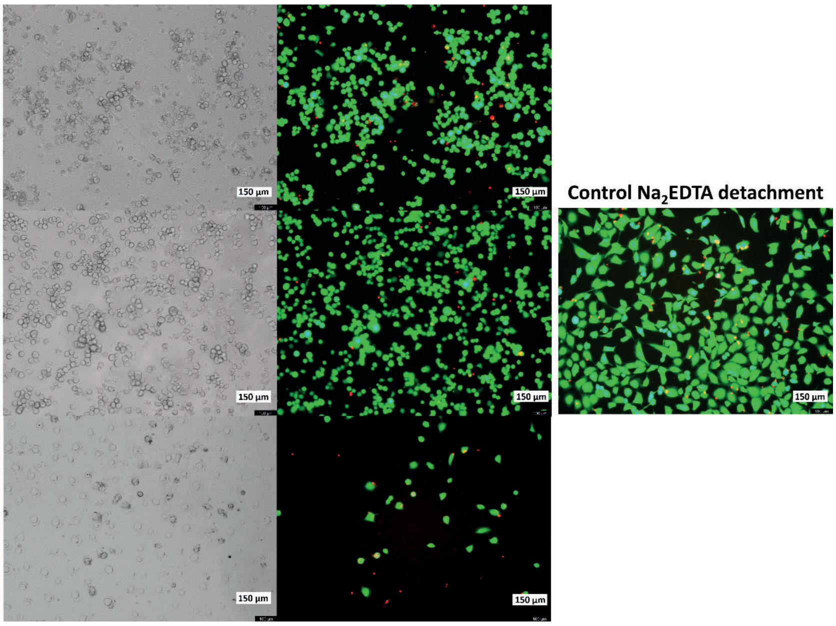


Fig.6. Life/Dead cell analysis after detachment and comparison with the control detachment via Na_2EDTA. A549 were detached at high concentrations from the PV6-1-B polymer under temperature and pH change. Moreover, cell viability after detachment from the PV6-1-B polymer is high and similar to the Na_2EDTA detachment. The number of cells detached from the PV5-1-B and PV5-2-B surfaces via stimuli were low although cells remained with metabolic activity (PV5-2-B has no significant difference from PV5-1-B).

cells show strong adhesion to the copolymer film and high viability after detachment under the influence of temperature and/or pH changes.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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Review

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Silica scaled Protista and Stomatocysts in East Siberia

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ABSTRACT. The review examines the work on biodiversity in the reservoirs of Eastern Siberia of a wide, but poorly studied group of organisms forming siliceous scales and bristles – these are scaled chrysophytes, chrysophycean stomatocysts and heterotrophic protists: rotosphaerids, colorless free-living thaumatomonad flagellates, centrohelid heliozoans. The difficulty in studying these objects is the need to use electron microscopy for their species identification. High biodiversity of silica scaled Protozoa has been revealed in the reservoirs of Eastern Siberia and the relationship of their species composition with the parameters of the aquatic environment, including in areas of local anthropogenic impact, has been shown. The recorded enrichment of the mouths of Arctic rivers with boreal species is important for predicting changes in aquatic ecosystems in the context of GCC. The proposed scenario of the settlement of siliceous chrysophytes at the beginning of the Holocene may be valid for other small planktonic organisms. The high degree of preservation of siliceous stomatocysts in sediments allows them to be used as an additional signal of changes in lake ecosystems in the past, this is based in particular on the reconstruction of the ecosystem and changes in the level of Lake Vorota (Yakutia) in the Holocene-Upper Pleistocene.

Keywords: silicon utilizing protists, scaled chrysophytes, heterotrophic protists, stomatocysts, Eastern Siberia

In addition to diatoms, whose role in the global Si cycle is well known (Nelson et al., 1995; Ragueneau et al., 2000; Tréguer and De La Rocha, 2013), other silicon utilizing microeucaryotes live in aquatic ecosystems – these are scaled chrysophytes, heterotrophic protists rotosphaerids, colorless free-living thaumatomonad flagellates, centrohelid heliozoans. These organisms transform silicic acid dissolved in water into elements of shells of a species-specific structure – siliceous scales and bristles (Fig. 1) or resting stomatocysts (Fig. 2).

The scaled chrysophytes include representatives of the Chrysophyceae class Pascher, from the families of Paraphysomonadaceae Preisig, Hibberd, Mallomonadaceae Diesing, Synuraceae Lemmermann. In total, about 350 taxa of silica-scaled chrysophytes are described (Guiry and Guiry, 2022). Scaled chrysophytes are a widespread group, they form a significant part of the plankton biomass of many freshwater reservoirs, there fore they play an important role in the structural and functional organization of freshwater ecosystems. Thaumatomonads are silica-scaled colorless free-living flagellates. Rotosphaerids are silica scaled organisms with filopodia that facilitate phagotrophic nutrition and a slowly rolling or creeping form of motility (Nicholls, 2012) Centrohelid heliozoans are predatory amoeboid flagellate protists, uniting more than 100 species (Zlatogursky, 2012; Shishkin et al., 2018). Representatives of these groups of heterotrophic protists play an important role in the food webs of aquatic microbenthos ecosystems, acting as passive predators. During periods of their maximum abundance, heterotrophic protists rise into the plankton for the purpose of settling (Ostroumov, 1917; Mikrjukov, 2002). Silicon utilizing protists and stomatocysts of

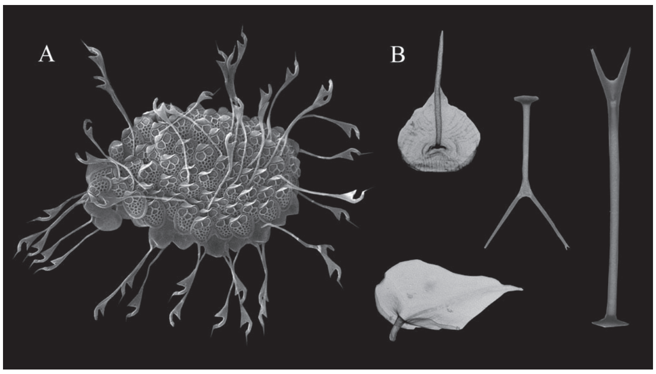

Fig.1. Scales of different silicon utilizing protists. A – a decayed cell of scaled chrysophytes; B – siliceous scales of centrochelid heliozoans.

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chrysophytes have micron sizes and species-specific ornamentation, which is detected using transmission and scanning electron microscopy methods.

In aquatic ecosystems, certain types of silicon utilizing protists are indicators of environmental factors such as pH, temperature, electrical conductivity, total phosphorus concentration, salinity, etc. (Hahn et al., 1996; Mikrjukov, 2002; Gavrilova et al., 2005; Leonov and Mylnikov, 2012; Siver, 2015; Prokina and Mylnikov, 2019; Prokina and Philippov, 2019; Lengyel et al., 2022). In addition, with an increase in the concentration of CO^2 in the atmosphere (Schindler, 2001; Rühland et al., 2008) in some boreal and Arctic lakes, an increase in populations of scaled chrysophytes is increasingly observed (Wolfe and Siver, 2013; Mushet et al., 2017). One of the characteristic features of scaled chrysophytes is the ability to form siliceous stomatocysts when changing chemical or physical parameters, In some temperate zones a low percentage of chrysophycean cysts often shows a more eutrophied stage, since these algae are most often less competitive in water with a high nutrient content (Smol, 1985). In the polar regions, this ratio has been proposed to be used to assess the ice cover (Smol, 1983; 1988). Later, the ratio of cysts to diatoms was used (Cumming et al., 1993), Later, the ratio of cysts to diatoms was used to illustrate the potential of cysts as indicators of lake water salinity in the past, and this ratio was also used to study the climatic trends of the Holocene history of Lake Losiny (Minnesota State) (Zeeb and Smol, 1993). The seasonality of their formation was determined by the layers of cysts in layered sediments (Battarbee, 1981; Peglar et al., 1984; Grönlund et al., 1986). Heterotrophic protists and centrohelid heliozoans live in a wide range of environmental factors and are considered eurybionts (Stoupin et al., 2012). However, in most publications there is no data on the parameters of the habitat during the sampling period, identifying the boundaries of such parameters at which a particular species was detected in natural samples would allow determining the autecology of species (Finlay et al., 1998) and to clarify the degree of their eurybiont. Thus, the study of silicon utilizing protists may have an application value for use in environmental monitoring.

The presence of relevant species and morphotypes of stomatocysts in samples can be detected not only from modern reservoirs, but also in sediments of different ages, therefore, silicon utilizing protists, like stomatocysts, are relevant objects not only in monitoring modern plankton, but also in paliolimnology, paleogeography, in terms of evolution and paleoreconstructions. For example, scaled chrysophytes belonging to the genus *Synura* Ehrenberg was very likely formed before the Cambrian period of the Paleozoic era (330 mya) (Boo et al., 2010). And already formed scales, having a different and complex structure, of heterotrophic protists of the genus *Rabdiophrys* Raine and scaled chrysophytes genera *Mallomonas* Perty and *Synura*, were found in deposits of the Eocene Giraffe Pipe sediments and in the Paleocene Wombat sediments in the area of the Lac de Gras kimberlite field in the Northwest Territories of Canada (64°44′ N, 109°45′ W; paleolatitude

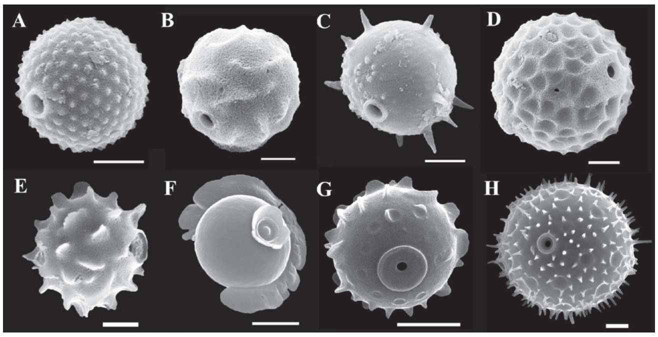

Fig.2. The variety of morphotypes of chrysophycean stomatocysts from Vorota Lake (Yakutia) (1-5) and Boguchany reservoir (6-8). A – Stomatocyst 031, Duff & Smol; B – Stomatocyst 498, Firsova & Bessudova; C – Stomatocyst 314, Firsova; D – Stomatocyst 506, Firsova & Bessudova; E – Stomatocyst 501, Firsova & Bessudova; F – Stomatocyst 489, Firsova & Bessudova; G – Stomatocyst 487; Firsova & Bessudova; H – Stomatocyst 076 Duff & Smol. Scale – 2 μm.

62°-64° N) (Siver and Wolfe, 2005a; 2005b; 2009; Siver and Lott, 2012; Siver et al., 2013; 2015; Siver and Skogstad, 2022). Chrysophycean stomatocysts in paleolimnological studies were assigned to one group and considered relative to the total number of diatoms calculated on the same microscopic preparations (for example, Smol, 1983; Stoermer et al., 1985; Grönlund et al., 1986; Harwood, 1986). D. Smol suggested (Smol, 1985) using the ratio of diatoms to cysts (D/C) as a coefficient of eutrophication of reservoirs. The proposed methods, with their relative simplicity, provide useful information about the environmental conditions in paleovodoems (Zeeb and Smol, 2001). Due to the complexity of determining the species of stomatocysts, the international group for the study of statospores International Statospore Working Group (ISWG) (Cronberg and Sandgren, 1986), digital designations of morphotypes of stomatocysts have been adopted. To date, more than 800 morphotypes of stomatocysts have been described in the world, of which more than 200 are known for Russia. On the territory of Russia, studies of modern and fossil stomatocysts were carried out in the lake. Baikal in the sediments of the Lena River and Lake "TS-9" on the Taimyr Peninsula, the Chukotka Peninsula, in Lake Khubsugul, Lake Teletskoye and in the North of Russia (Vorobyova et al., 1996; Wilkinson et al., 2001; Firsova and Likhoshway, 2006; Firsova et al., 2008; 2012; Bazhenova et al., 2012; Voloshko, 2016).

The first studies of scaled chrysophytes of Eastern Siberia using electron microscopy were carried out in the Khanty reservoir, the Khanty River, in small rivers flowing into the reservoir, and in the Big Khanty Lake (Balonov and Kuzmina, 1986); as a result, 29 species were identified. The use of new methods has made it possible to reliably identify 14 species of scaled chrysophytes in Lake Baikal (Vorobyova et al., 1992), and 5 species in the Irkutsk reservoir (Vorobyova et al., 1996). Later, foreign scientists investigated the species composition of scaled chrysophytes on the Taimyr Peninsula (Duff, 1996; Kristiansen et al., 1997). The study area included small unnamed lakes (Duff, 1996), Lake Taimyr, Lake Engelhardt, a lake in the north of the village of Khatanga, a lake in the Talnakh district (the city of Norilsk) and small temporary reservoirs (Kristiansen et al., 1997). A total of 23 species have been found on the Taimyr Peninsula. Recent studies on the scaled chrysophytes of Eastern Siberia have been conducted in the lake. Frolikha (Transbaikalia) (Gusev and Kulikovsky, 2013; Gusev, 2016). As a result, 10 species were identified.

The first study of chrysophycean stomatocysts carried out using electron microscopy on the basis of the cell ultrastructure department was published in 1996 by S.S. Vorobyova and co-authors. The article contained descriptions and illustrations of 7 morphotypes, 5 of which were described for the first time. As a result of studies of the continuous Baikal chronicle of sedimentary deposits of siliceous microfossils, mainly diatoms, the presence of siliceous cysts in sediments of Lake Baikal of different ages was noted (Bradbury et al., 1994; Likhoshway, 1999) and in modern bottom sediments (Stoermer et al., 1995; Likhoshway et al., 2005), but their structure has not been described.

The method of electron microscopy has significantly expanded the possibilities for studying these organisms. The species composition of scaled chrysophytes was studied in the zone of mixing of the waters of the Yenisei River and the Kara Sea, as well as in thermokarst lakes of the Lower Yenisei basin (Bessudova et al., 2015; Bessudova et al., 2016; Firsova et al., 2017; Bessudova et al., 2018a). In total, 40 species of scaled chrysophytes were found in the studied areas. Based on the original data obtained on the species diversity, the distribution of scaled chrysophytes relatively changing hydrochemical parameters of the environment in the river-sea water mixing zone, summary data taking into account the literature on the occurrence of these organisms and their autecology, a monograph has been compiled (Bessudova et al., 2016).

The study of scaled chrysophytes inevitably led to a more detailed consideration of heterotrophic protists due to the similarity in size, timing of development and structure of siliceous elements. Since earlier studies of the ecology of diversity and seasonal dynamics of silicon utilizing protists from reservoirs in Eastern Siberia have not been conducted, electron microscopy methods have opened up new prospects.

For the first time in Eastern Siberia, the species composition of heterotrophic protists in a system connected by watercourses, from Lake Baikal to the Kara Sea, has been studied. A total of 29 species of heterotrophic protists were found: 21 species of centrohelid heliozoans, 6 species belonging to rotospherids and one flagellate protist (Bessudova et al., 2021a). It is shown that the most diverse silicon utilizing heterotrophic protists are represented in the river-sea and river-lake ecotonic zones. It was revealed that even a small increase in salinity sharply limits the diversity of these organisms. However, two species with a wide ecological valence have been identified, occurring from the mouth of the Yenisei River to the northernmost section of the northeastern part of the Kara Sea (Bessudova et al., 2021a). The distribution of chrysophycean stomatocysts during mixing of fresh and salty waters was also studied (Firsova and Tomberg, 2012). 12 morphotypes of stomatocysts were found in plankton and sediments of mineralized meromictic Lake Shira (Khakassia, Russia) (Firsova, 2014).

The species composition and ecology of scaled chrysophytes and stomatocysts in the Baikal region have been studied in the most detail. In 2006, an atlas of chrysophycean stomatocysts was created, which included a detailed description of 93 morphotypes of fossil cysts from Holocene and Upper Pleistocene bottom sediments and 33 morphotypes from Baikal plankton (Firsova and Likhoshway, 2006). In the future, the seasonal dynamics of stomatocysts from phytoplankton of Southern Baikal was studied. It was revealed that siliceous stomatocysts of golden algae make a significant contribution to phytoplankton, reaching the highest values (46.8×10^3 cysts per l) in August-October with a minimum concentration of biogens. The D/C coefficient (the ratio of diatom and cyst cells) varies throughout the year, reflecting the seasonal succession of phytoplankton and changes in the concentration of biogenic elements in the photic layer. 50 morphotypes of cysts have been identified, which are divided into 25 groups according to morphological features. The selected groups of cyst morphotypes have different seasonal dynamics. Group 5 cysts (with spikes of various lengths on the equatorial and posterior parts of the cysts) dominate over the other morphotypes and reach the highest concentration (13.6×10^3 cysts per l) in August (Firsova et al., 2008). The geography of research gradually expanded. Siliceous microfossils were studied in the Upper Miocene deposits of Transbaikalia (in the Jilindin formation of the eastern part of the Amalat paleodoline of the Amalat plateau of the Vitim Plateau). It was noted that stomatocysts of chrysophycean algae were found almost throughout the section. A total of 26 morphotypes of cysts were found, among which 6 new ones were identified. The dominant (up to 57%) were smooth, without collar and ornamentation (*Mallomonas*, *Paraphysomonas* (Stokes) DeSaedeleer, *Chrysosphaerella* Lauterborn). The total number of cysts varied from 7 thousand to 82 million copies/g. The largest number of cysts (65-82 $\times 10^6$ cysts per g) was observed in the depth range from 176-172 m. The values of the D/C coefficient (2.7-6.1) proposed earlier to characterize the trophic capacity of the reservoir (Smol, 1985) at this horizon were minimal, which indicates a possible decrease in the trophic content of the water reservoir during this period (Usoltseva et al., 2008). In the Pleistocene-Holocene deposits of Lake Elgygytgyn (Chukotka), 8 morphotypes of cysts were found, of which 3 were new to science. It was shown that smooth unornamented cysts without collars and complex ornamentation (more than 40%) most often prevailed among the morphotypes. The distribution pattern of various morphotypes of golden algae cysts has been studied (Firsova, 2013). In the Miocene deposits of the Vitim plateau in the core of sle. 7236 in the depth range of 126-249 m, 60 morphotypes of cysts were found, of which 9 are new to science. To study the nature of the distribution of various morphotypes, they were divided into 28 groups (GV) according to the shape, ornamentation and structure of the collar. It was revealed that the distribution of morphotype groups in the core had a different character. In the middle part of the core (depth interval 199-168 m), the greatest variety of stomatocysts was noted. During this period, the conditions in the reservoir were most favorable for both the development of cysts and planktonic diatoms (Firsova et al., 2010).

In 2012, the analysis of microfossils from the core of Upper Pleistocene and Holocene sediments of the southern part of Lake Khubsugul revealed 36 different morphotypes of stomatocysts. Some of them were assigned to the genera *Mallomonas*, *Chrysosphaerella*, *Paraphysomonas* and *Dinobryon* Ehrenberg 20 morphotypes were described as new to science. The abundance and diversity of morphotypes of stomatocysts in the core sample varied depending on the age of the deposits. It is noted that the highest diversity of morphotypes is observed in the layers corresponding to the periods of development of the cold-water diatom *Cyclotella bodanica* Eulenstein ex Grunow. Smooth (unornamented) morphotypes are the most common in sediments. It has been established that the morphotypes H12, H19 and H22 are typical for Lake Khubsugul, which occur throughout the core depth and, at certain intervals, account for up to 40% of the total number of stomatocysts. The intervals when the dried-up southern part of the lake was again filled with water were characterized by the highest values of D/C, which means that the trophic level of the lake temporarily increased during these periods (Firsova et al., 2012).

The revision of the scaly chrysophytes of Lake Baikal made it possible to supplement the species list with 13 species, and amounted to 25 species (Bessudova et al., 2017). The seasonal dynamics of these organisms is revealed, it is shown that the greatest variety of scaly chrysophytes is characteristic of the Southern and Middle regions of Lake Baikal. The maximum abundance and species diversity was noted in the autumn period. Also, the composition of chrysophytic stomatocysts was replenished with 8 morphotypes new to Baikal, of which 5 are described as new to science. For 3x, the presence of scales and their structure made it possible to establish the species (Firsova et al., 2017; 2018)

A study of the mouths of rivers flowing into Baikal, small bays and straits revealed a high diversity of scaled chrysophytes, which amounted to 66 species (Bessudova et al., 2018b, 2018c) and morphotypes of stomatocysts – 58, of which 25 were described as new (Firsova et al., 2018). It is shown that large rivers – Upper Angara, Barguzin and Selenga affect the lake. Baikal, increasing the diversity of scaled chrysophytes in its Southern, Middle and Northern basins, however, due to the difference in habitat conditions, their distribution is limited. The greatest diversity recorded in shallow, well-warmed waters rich in nutrients is significantly reduced (from 66 to 17) when entering a cold oligotrophic lake. It was found that out of 25 species registered in the lake, 8 species were not found in the tributaries. Rare species have been found in the mouths of these large rivers, modified scales of the species *Mallomonas striata* Asmund have been observed. The change in the morphology of the scales could be triggered by a high phosphorus content (Bessudova et al., 2018b). In July 2018 in the floodplains of the Selenga and Barguzin rivers, a continuous period of low water was interrupted by high water. A study of the waters during this period showed that the species composition of the scaled chrysophytes on the one hand significantly decreased (to 23 species), on the other hand, it was enriched with new species for the Baikal region, amounting to 79 species (Bessudova et al., 2020). After analyzing two years of different water content, we came to the conclusion that the high diversity of scaled chrysophytes in the mouths of the main tributaries of Lake Baikal, Selenga, Upper Angara and Barguzin in low water conditions may be caused by previous floods. Flooding of floodplains led to the unification of small streams and lakes, which enriched their flora due to the spread of a wide range of species. The retreat of water stimulated the flowering of phytoplankton, in particular scaled chrysophytes, in warm and shallow reservoirs with a high level of biological productivity, which contributed to the diversity of scaled chrysophytes in the Baikal region. The alternation of floods and low water levels created different environmental conditions and stimulated the dynamics of the ecosystem, which allowed the formation of a “hotspot” for the diversity of scaled chrysophytes (Bessudova et al., 2020).

The waters of the largest hydroelectric power plant in Russia during the first years of operation at full capacity – the Boguchany reservoir have been studied. The influence of the source of the Angara River – Lake Baikal can be traced in the species composition of the scaled chrysophytes of the Boguchany reservoir. The species composition is not high and consists of only 23 species (Bessudova and Likhoshway, 2017). At the same time, 35 morphotypes of stomatocysts were found, 10 of them were registered for the first time in Russia and nine morphotypes new to science were described (Fig. 2) (Firsova et al., 2019). As a result of these studies, the list of chrysophycean stomatocysts of the Baikal region was expanded to 203. The data obtained not only expand the information about the diversity of stomatocysts, but also will allow further assessment of changes in the state of the reservoir.

After analyzing the scattered literature data on the distribution of scaled chrysophytes in northern reservoirs, above the 60th parallel north after the last glaciation of the Pleistocene, we identified hydrochemical parameters that significantly affect the distribution of these organisms (Bessudova et al., 2021b). Territories above the 60th parallel north were affected by glaciation at the end of the Pleistocene, and the lakes located here and their microflora were formed mainly at the beginning of the Holocene. We analyzed the distribution of scaled chrysophytes in 193 northern reservoirs. The formation of flora and species richness of scaled chrysophytes is most influenced by the location of the reservoir, temperature and water conductivity. Reservoirs similar in species composition can be significantly removed in the latitudinal direction. Eighteen species and one variety out of 165 discovered taxa found here have a clear similarity with ancient related groups; they are found in all studied regions and account for 6 to 54% of the total number of scaled chrysophytes. The settlement of scaled chrysophytes in northern reservoirs could occur at the end of the Pleistocene – the beginning of the Holocene along the circumpolar freshwater network of glacial-underground lakes, and the final composition of the flora was determined by the parameters of the habitat of each individual reservoir and the region in which the reservoir is located.

We continued our research on the biodiversity and ecology of scaled chrysophytes in collaboration with colleagues from the Institute of Cryolithozone Problems SB RAS in the remote and unexplored northern and Arctic regions of the Asian part of Russia, the Republic of Sakha (Yakutia). The biodiversity of silicon utilizing protists of reservoirs of Kotelny Island, Arctic, latitude 75°53 N was studied (Bessudova et al., 2022a (in press)). The research area is part of the Novosibirsk Islands archipelago. 17 species of silica scaled and 8 heterotrophic protists were found in the studied small reservoirs. On an Arctic island remote from the mainland, widespread species and cosmopolitans predominate, however, there are also some species specific to the area.

In large lakes located at the Cold Pole of the Northern Hemisphere – Labynkyr and Vorota, 23 species of scaled chrysophytes were identified (Bessudova et al., 2019). Rare species have been discovered, seasonal dynamics of chrysophytes has been investigated. 76 morphotypes of stomatocysts were also found in the waters of lakes, 51 of which are widespread, 24 of them were noted in Russia earlier and 25 were described as new (Firsova et al., 2020).

A high species diversity of scaled chrysophytes was revealed in the mouths of the Arctic rivers of Yakutia, numbering 82 species (Bessudova et al., 2021c), as well as in the Arctic waters of the Tiksi region, numbering 65 species (Bessudova et al., 2022b). New and rare Arctic species of scaled chrysophytes have been discovered (Bessudova et al., 2022b; 2022c). The hydrochemical parameters of the waters are analyzed and the main factors affecting their high diversity are identified (Bessudova et al., 2022b). The high diversity of these organisms is formed mainly from polyzonal and widespread species, but since 2008-2010 there has been an increase in the relative content of boreal species compared to the data obtained over the previous 30 years of studying northern reservoirs. The observed trends of climate warming may contribute to the Northward movement of representatives of the boreal flora. For a number of species, the research area is the northernmost habitat to date. Physico-chemical factors affecting the species composition and species richness of scaled chrysophytes in the study area have been identified.

The studied reservoirs of the Baikal region, the mouth of the Olenek River, as well as the Tiksi area can be considered “hot spots” of the scaled chrysophytes biodiversity, along with 3 previously marked points of the world. Also, in the mouths of the Arctic rivers of Yakutia and in small reservoirs of the Tiksi region, a high species diversity of silicon utilizing heterotrophic protists numbering 50 species was revealed (Bessudova et al., 2022d (in press)).

The study of the biodiversity of silicon utilizing protists using electron microscopy methods in combination with hydrochemical parameters has made a significant contribution to our knowledge of these organisms as indicators of changes in environmental conditions, which can serve as a criterion in assessing the state of modern reservoirs and an additional basis in the construction of paleoreconstructions in Eastern Siberia.

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Conflict of interest

The authors declare no conflict of interest.

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The problem of species delimitation within the endemic Lake Baikal sponges Lubomirskiidae

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ABSTRACT. The delimitation of species within the endemic sponge family Lubomirskiidae is difficult. Along with specimens falling within the range of morphological variability of previously described specimens, we found specimens with intermediate morphological characteristics. The largest number of such specimens had intermediate characteristics between representatives of the genera *Lubomirskia* and *Baikalospongia*. For some species of these genera, a common feature is the structure of megasclera in the form of oxeas of a similar size. At the same time, the genera *Swartschewskia* and *Rezinkovia* have less variable morphological characteristics, so the identification of species belonging to these genera based on morphology is not difficult. The results of our study indicate the need for delimitation of Lubomirskiidae species based on molecular data.

Keywords: Porifera, Baikal, Lubomirskiidae, biodiversity, species delimitation

1. Introduction

Sponges (Porifera) are important component of marine and freshwater ecosystems due to their species richness and widespread abundance (Diaz and Rützler, 2001; Bell, 2008; Van Soest et al., 2012). Baikal sponges make up the bulk of the benthic biomass in Lake Baikal (Timoshkin, 2001) and play an important ecological role as biofilters. Endemic sponge family Lubomirskiidae includes today 18 accepted species (Rezvoi, 1936; Efremova, 2004; Itskovich et al., 2015; Bukshuk and Maikova, 2020).

For sponges, the insufficiency of morphological data alone was noted for the development of taxonomy and the study of phylogeny (Van Soest et al., 2012). Molecular studies provide additional data to address these issues in the case of marine sponges (Morrow et al., 2012; Boury-Esnault et al., 2013). Molecular studies have also helped to determine the phylogenetic position of a number of cosmopolitan freshwater sponges Spongillidae (Addis and Peterson, 2005; Itskovich et al., 2008; Carballo et al., 2018; Erpenbeck et al., 2019; Sokolova et al., 2021). The use of molecular markers with different evolutionary rates also made it possible to identify founder species of endemic families in ancient lakes (Meixner et al., 2007; Erpenbeck et al., 2011; Itskovich et al., 2013a; Sokolova et al., 2020).

Species identification of Lubomirskiidae is difficult both on the basis of morphological and molecular methods. To date all molecular analyzes based on the protein coding (COXI, silicatein) and intron (ITS region rDNA and intergenic spacer region mtDNA) sequences have not supported the current morphology-based taxonomy of Lubomirskiidae (Meixner et al., 2007; Itskovich et al., 2008; 2013b).

Taxonomy and the definition of species criteria are the fundamental basis for theoretical and applied biological research. Recently, there has been an increase in the number of publications devoted to the issue of species boundaries in marine sponges, most of which indicate an underestimation of the existing biodiversity (Cruz-Barraza et al., 2012; Boury-Esnault et al., 2013; DeBiasse and Hellberg, 2015). Sponges present a goodmodel for testing hypotheses of species delimitation by study of molecular evolution and morphological variability.

The problem of species differentiation is especially relevant in Lake Baikal, which is a natural laboratory for the study of evolutionary processes. In Baikal along with other ancient lakes there is a process of accelerated evolution driven by the extreme and unique environmental conditions of the lake. Rapid radiation processes operate at all levels from cell to species and affect both genetic and anatomical characteristics. The high intraspecific morphological variability of Lubomirskiidae is probably the result of their morphofunctional and anatomical adaptations.

In this work, we analyzed the variability of morphological characteristics in representatives of 4 Liubomirskiidae genera.

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2. Materials and methods

Samples of sponges were collected by SCUBA diving at two collection points in Central Baikal: Maloe more strait, Kurma village (53°11’496”N 106.59’752”E), Maloe more strait, Zama village (53°28’275”N 107°32’022”E), Cape Izhimei on Olkhon Island (53°13’444”N 107°44’052”E) during expeditions performed in 2022. During the collection of samples, ecological parameters such as temperature, water quality and mineral composition, and substrate type were recorded, which will reveal the links between taxonomy and ecology, which is important for species delimitation. Underwater photo and video surveys were carried out and for all samples in vivo images were captured (Fig. 1, Fig. 2). Spicule and skeleton preparation were performed as previously described (Efremova, 2004) and examined using an Olympus CX22 microscope. The shape and consistency of sponges, osculum structure, skeletal characteristics, the form and size of spicules, the presence and shape of spicule spines, the nature of their distribution along the spicule and their variability in each sample were analyzed. Spicule sizes were determined by the results of measurements of 50 spicules within each sample.

3. Results and discussion

We collected and performed morphological analysis of 208 Lubpomirskiidae specimens. Most of them were identified as *Lubomirskia baikalensis, L. fusifera, L. abietina, L. incrustans, Baikalospongia intermedia, B. bacillifera, B. recta, B. martinsony, Rezinkovia echinata, Swartschewskia papyracea* (Fig. 1). About 20 samples had difficult species identification (Fig. 2). Some of them had spicule sizes beyond the previously described species. Some samples had combinations of features of different species.

The largest number of such specimens had intermediate characteristics between representatives of the genera *Lubomirskia* and *Baikalospongia*, but at the same time, we did not encounter any difficulties in identifying such a species as *Lubomirskia baikalensis* (in the case of adult branched specimens). For some species of these genera (for example, *Lubomirskia baikalensis, Lubomirskia incrustans, Baikalospongia intermedia*), the structure of megasclera in the form of oxeas of similar size is common. Important characteristics for the identification of these species are also the structure of the skeleton, the shape and size of the skeletal bundles, and the nature of their branching, however, these characters vary significantly. Oxeas are also present in Spongillidae, which lose gemmules when living in constant conditions in Baikal, and this sometimes makes it difficult to identify Baikal sponges even to the family level (Itskovich et al., 2015).

At the same time, the genera *Swartschewskia* and *Rezinkovia* have less variable morphological characteristics within the genus, so the identification of species belonging to these genera based on morphology is not difficult. *Swartschewskia papyracea* also differs in ecological features. This species lives on negative slopes, in places with low amount of light, that also affects the composition of its symbiotic community (Kaluzhnaya and Itskovich, 2016).

Our data are consistent with the data of other researchers, who also noted the blurring of morphological features and overlapping of species boundaries in Lubomirskiidae (Efremova, 2001; Masuda, 2009; Maikova et al., 2017). Some genetic markers also indicated the proximity of the genera *Lubomirskia* and *Baikalospongia* (Itskovich et al., 2013b; Maikova et al., 2017), however, the lack of separation of other Lubomirskiidae species using intergenic mtDNA regions indicates the need for additional studies. Rare deep-water species (*R. arbuscula, B. abyssalis*) and three

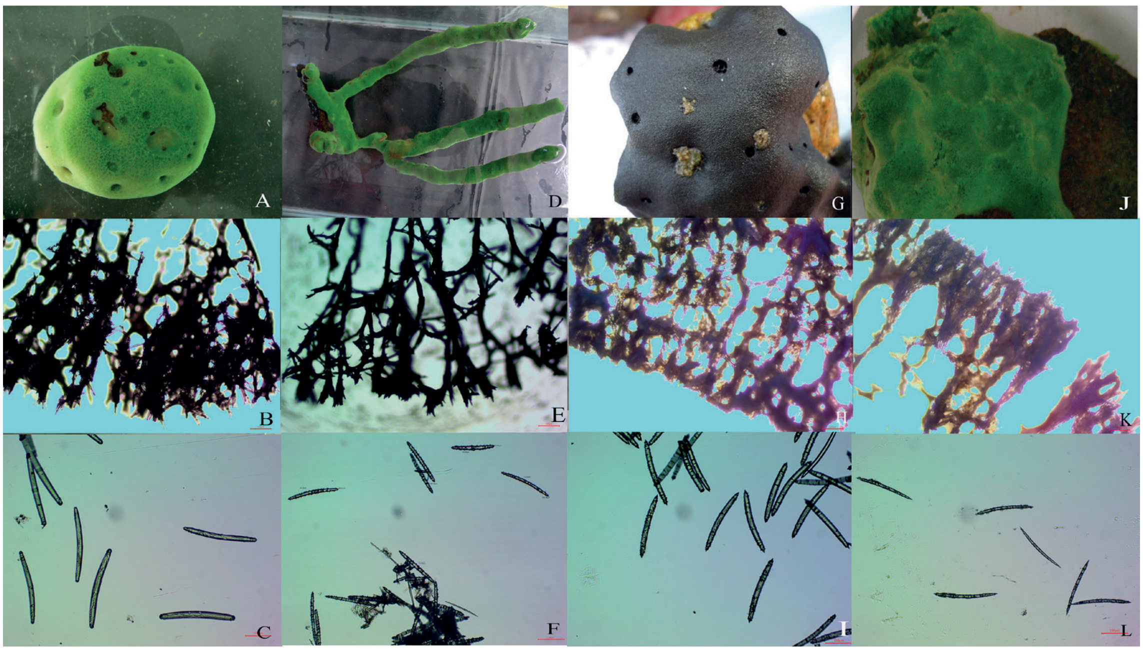

Fig.1. Sponge body, the skeleton and microscopy of megascleres, respectively, of (A–C) *Baikalospongia bacillifera*; (D–F) *Lubomirskia baikalensis*; (G–I) *B. intermedia*; and (J–L) *L. abietina*.

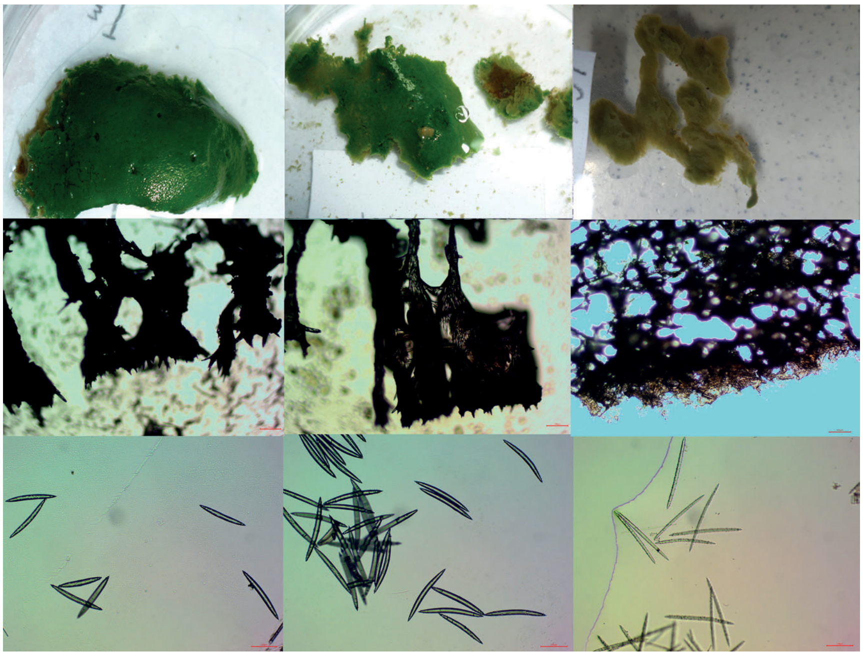

Fig.2. Sponge body, the skeleton and microscopy of megascleres of unidentified samples of Lubomirskiidae.

closely related species of the genus Swartschewskia should also become the object of close attention for species delimitation.

Marine sponges also have a phenotypic plasticity that can lead to misidentification of specimens and a problem to determine their low level relationships. For example, morphological studies of sponges of the genus *Callyspongia* have shown that some species are actually growth forms (Zea et al., 2014). However, molecular analysis revealed evolutionarily distinct lineages that were not concordant with current species designations in *Callyspongia* (DeBiasse and Hellberg, 2015).

The morphologically divergent specimens identified by us in this work may be either new species of Lubomirskiidae or indicate that the real number of sponge species in Baikal is overestimated. Molecular studies based on genomic data will help resolve this issue. A large-scale ddRAD analysis of the Lubomirskiidae specimens collected and analyzed by us is currently in progress.

4. Conclusions

The results of morphological analysis made it possible to identify samples with intermediate characteristics between representatives of the genera *Lubomirskia* and *Baikalospongia*. The delimitation of Lubomirskiidae species including these specimens, as well as a number of closely related and deepwater species, should be carried out using morphological, ecological and genomic data.

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Conflict of interest

The authors declare no conflict of interest.

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