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REPRODUCTIVE BIOLOGY AND THE LIFE CYCLE OF THE DIATOM *NITZSCHIA* CF. *THERMALOIDES* INHABITING MUD VOLCANOES OF CRIMEA

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In biology, it is important to study the ability of organisms to exist under extreme conditions, to which the phase of the life cycle, related to sexual reproduction and the possibility to leave the next generation, is especially sensitive. *Nitzschia* cf. *thermaloides* Hustedt was found in high abundance in samples from puddles and lakes formed in the areas of action of mud volcanoes of the Bulganak mud volcano field (Kerch Peninsula, Crimea). Individual clones were isolated from the samples by the micropipette technique and introduced into a culture; their crossing made it possible to initiate heterothallic sexual reproduction. The aim of this work was to study reproductive biology and the life cycle of the alga from a biotope with extremely high levels of irradiance, temperature, and salinity. For the first time for this species, a description of the sexual process corresponding to IB2a type according to Geitler classification is given. The cardinal points have been defined, which represent critical cell sizes that determine the transition from one phase of the life cycle to another. The full range of cell sizes is given, from the initial ones that appeared as a result of sexual reproduction to the smallest ones observed when kept in the culture. The main postulates of the life-cycle theory are considered allowing to analyze the natural population of the alga. The population of *N.* cf. *thermaloides* was found to exist in the sampling site for a long time and to be represented by cells in all phases of the life cycle.

Keywords: diatoms, *Nitzschia* cf. *thermaloides*, mud volcanoes, sexual reproduction, life cycle

Diatoms are known not only for their great species diversity, but also for their significant evolutionary plasticity, which allowed them to adapt to existence on the bottom and in water column of marine and freshwater basins, in soils, and on wet surfaces. Some diatom species are tolerant to conditions of extreme temperature [Nikulina, 2010], salinity [Nevrova, Shadrin, 2005; Senicheva et al., 2008], and high irradiance, including ultraviolet light [Peletier et al., 1996]; in the absence of light, they can grow mixotrophically [Abdullin, Bagmet, 2016]. In samples of water flowing out of mud volcanoes of the Bulganak mud volcano field (Kerch Peninsula, Crimea), 15 microalgal species were registered; out of them, 6 represented the phylum Bacillariophyta, including *Nitzschia* cf. *thermaloides* Hustedt, 1955 [Ryabushko, Bondarenko, 2020]. Due to this finding, a number of questions arose concerning the fundamental aspects of the biology of these species living under unique conditions of temperature, salinity, and irradiance. First of all, we were interested in reproductive biology of diatoms and their response to extreme conditions. What is the lifespan of populations found in the mud volcanoes? What are the rates of division of diatoms in these biotopes? Are the species capable of reproducing in generations under these conditions, passing through all phases of the life cycle, *inter alia* the process of sexual reproduction? These questions determined the aim of our work.

MATERIAL AND METHODS

The objects of the study were isolated from samples taken on 15.10.2020 in the Bulganak mud volcano field at the points with the coordinates given in Table 1.

Table 1. Sampling points in the Bulganak mud volcano field (Kerch Peninsula, Crimea), 15.10.2020; water salinity in the samples

Mud volcano	GPS coordinates		Water salinity, ‰
	N	E	
Andrusov	45°25′35.9″	36°28′38.9″	30, 35, 68
Pavlov	45°25′33.0″	36°28′44.1″	18, 35, 56
Tishchenko	45°25′33.3″	36°28′25.4″	20
Central Lake	45°25′23.7″	36°28′38.4″	19, 26

Four mud volcanoes were explored: Andrusov (Fig. 1A), Pavlov (Fig. 1B), Tishchenko (Fig. 1C), and Central Lake (Fig. 1D). The Bulganak mud volcano field belongs to mud volcanic formations with dispersed centers of eruptions (Fig. 2); hills and gryphons (local centers of mud volcanic activity) are located in a relatively small area. There are hills slightly rising above the surrounding landscape, from which gas and liquid silt are released, spreading in a thin layer and forming shallow puddles, and mud lakes of a smaller or larger area (for example, Central Lake). The second morphotype of volcanoes is cone-shaped hills, excreting rather viscous mud flowing down the slopes (Tishchenko mud volcano).

Brines of the mud volcanoes are of the hydrocarbonate-sodium type (hydrocarbonate-chloride sodium or chloride-hydrocarbonate sodium); those are characterized by an increased content of bromine (85–210 mg·L^{-1}) and iodine (23–45 mg·L^{-1}) [Matyunina, 2019]. The chemical composition of the brines of the mud volcanoes is variable: it can change significantly even within a few hours. The mineralization of erupted brines does not exceed 20–23 g·L^{-1} [Kayukova, 2018; Kurishko et al., 1968]. However, under conditions of high irradiance and intense winds, due to water evaporation in effluent brines, a noticeable increase in the concentration and precipitation of salts is recorded locally. Mineralization can reach 110 g·L^{-1} [Kurishko et al., 1968].

In puddles and mud lakes, diatoms form a biofilm of a characteristic brown-green color (Fig. 1A). The biofilm is located both on the surface of the liquid phase and on the bottom in the spots where the water column is shallow and the suspension is mostly settled. Depending on the location, the area of continuous biofilm can be of several square decimeters or more.

Single cells of *N.* cf. *thermaloides* were isolated from samples delivered to the laboratory by micropipette technique using inverted microscopes Nib-100 (China) and Altami INVERT 3 (Russia) (Fig. 3); those gave rise to 15 clonal cultures. The cultures were kept in 100-mL glass Erlenmeyer flasks in a modified ESAW medium [Polyakova et al., 2018] and inoculated into fresh medium every 10–14 days. Conditions of maintaining and the method of clones naming are described in more detail in [Davidovich et al., 2017]. The clones were kept and crossed in glass Petri dishes (40–50 mm in diameter) in a medium with the same salinity as they were in the samples. Initial salinity of the modified ESAW medium (36‰) was adjusted to the required level either by diluting it with distilled water or by adding sodium chloride. Salinity was measured with an RHS-10ATC refractometer (China). In the samples, salinity differed significantly depending on a sampling point (see Table 1). Specifically, in three samples of mud flowing out of the Andrusov mud volcano, which were taken on an area of < 1 m^2, salinity was 30, 35, and 68‰. Apparently, the reason for such noticeable differences are salts occurring in the samples which were deposited on the underlying surface because of water evaporation. In a sample taken directly from the vent (Tishchenko mud volcano), salinity was 20‰.

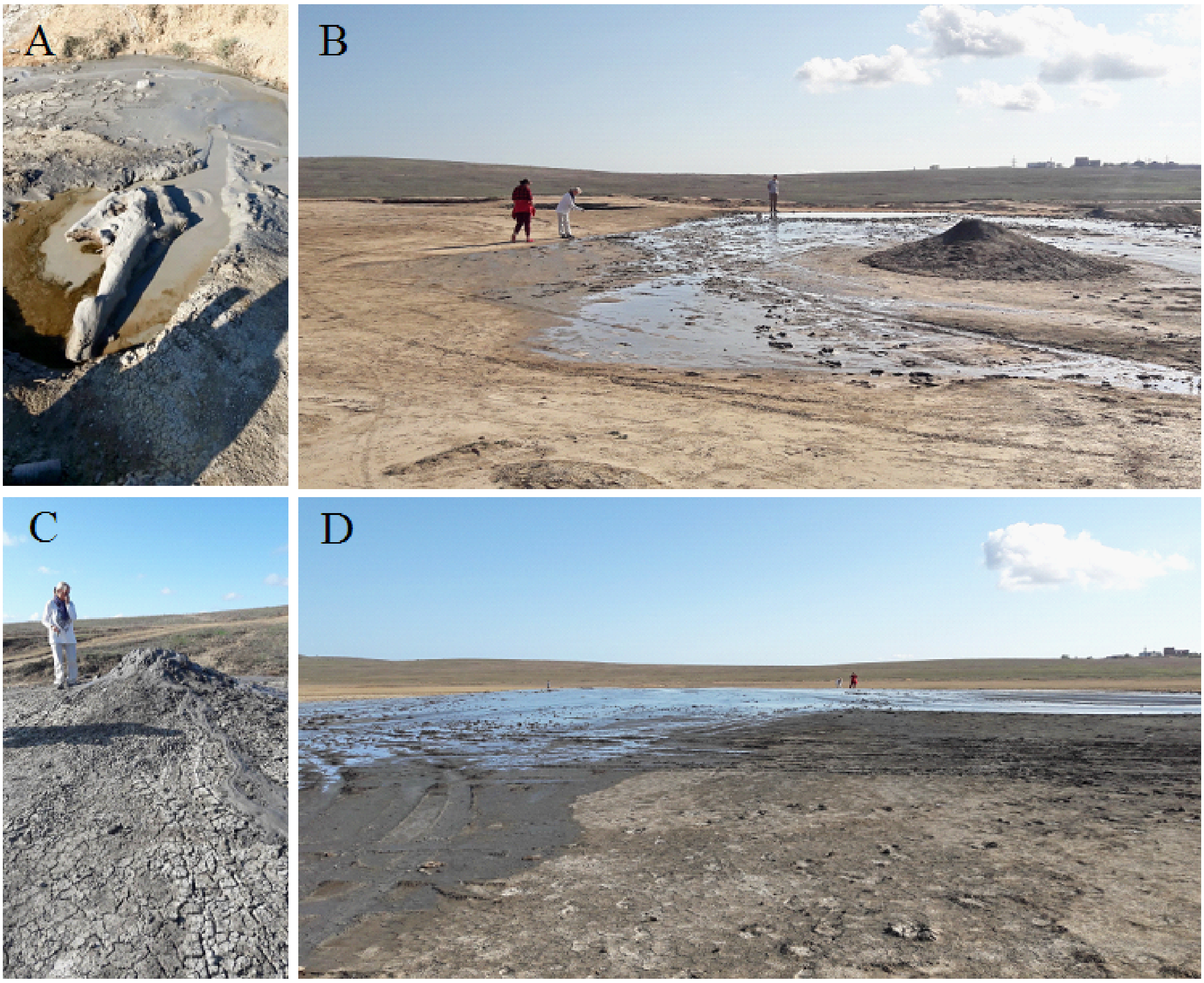

Fig. 1. The mud volcanoes – Andrusov (A), Pavlov (B), Tishchenko (C), and Central Lake (D) – of the Bulganak mud volcano field during the sampling period (Kerch Peninsula, Crimea)

For electron microscopy, frustules were boiled in hydrogen peroxide (35%) and washed (7–8 times) with distilled water; then, cleansed valves were placed on an object stub and coated with gold. Microphotography was carried out under a Hitachi SU3500 scanning electron microscope (Japan). Cell length and width were measured using a calibrated eyepiece ruler by direct observation under a microscope or a Moticam 1080 digital camera (China) with the supplied software. The obtained data were statistically processed in Microsoft Excel. Mean values (*M*) are given with a standard error (*SE*) and sample size (*N*): *M* ± *SE*.

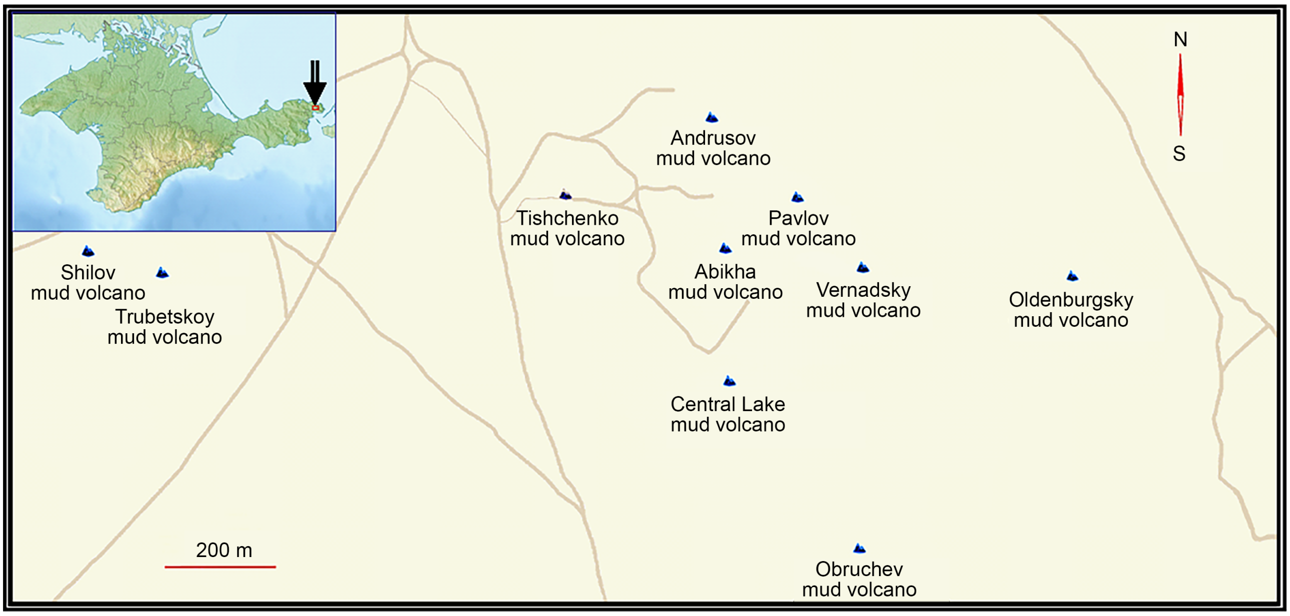

Fig. 2. Location map of the mud volcanoes of the Malo-Tarkhan (left cluster) and the Bulganak (right cluster) mud volcano fields (Kerch Peninsula, Crimea)

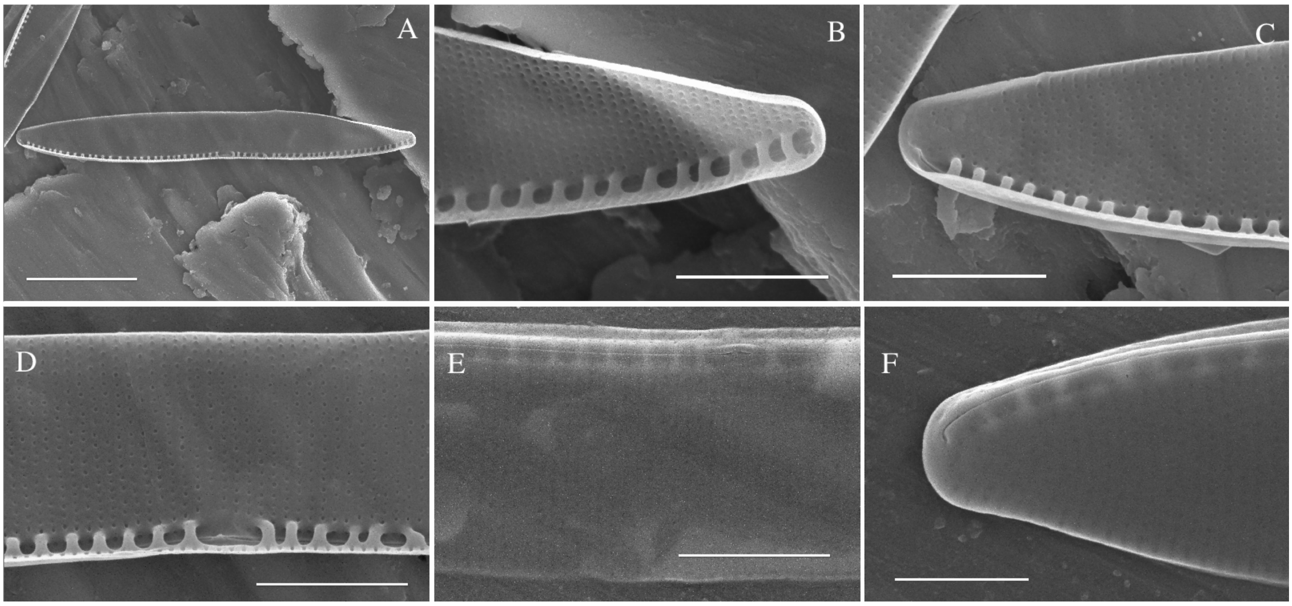

Fig. 3. *Nitzschia* cf. *thermaloides*, scanning electron microscopy. Internal (A–D) and external (E–F) valve view. Scale bar: A, 10 μm; B–E, 3 μm; F, 2 μm

RESULTS

For the first time, we observed *N.* cf. *thermaloides* sexual reproduction in samples with the nutrient medium added even before the isolation of individual clones. In total, 17 initial cells were found and measured, their mean apical size turned out to be (68.7 ± 0.5) μm (maximum was 71.0 μm). The length of the valves of the empty open frustules of parental cells, lying next to them, averaged (37.4 ± 0.7) μm ($N = 14$; maximum was 40 μm). Even these preliminary data allowed concluding as follows: in the process of sexual reproduction, there was a twofold increase in size, and the cell length of slightly more than 70 µm was obviously close to the maximum species-specific length. Subsequent measurements of cells (not only from the natural population, but also obtained by crossing the isolated clones) made it possible to identify the sizes that determine the phases of *N.* cf. *thermaloides* life cycle (Table 2).

Table 2. The apical size of *Nitzschia* cf. *thermaloides* vegetative, gametangial, and initial cells

Cells	Sample size (*N*)	Cell length, µm	
		min	max
Vegetative	1,190	15	84
Gametangial	103	33	43
Initial	202	56	84

Measurement of cells from the natural population (*N* = 502) showed that their length varied from 15 to 74 µm. The histogram of the distribution of cell sizes in terms of the frequency of occurrence in the natural population is shown in Fig. 4. By crossing the isolated clones, initial cells were obtained; their maximum size was 84 µm. The length of the largest gametangial cells was 43 µm (52% of the maximum size); this is the transition boundary from the pre-reproductive to the reproductive phase of the life cycle.

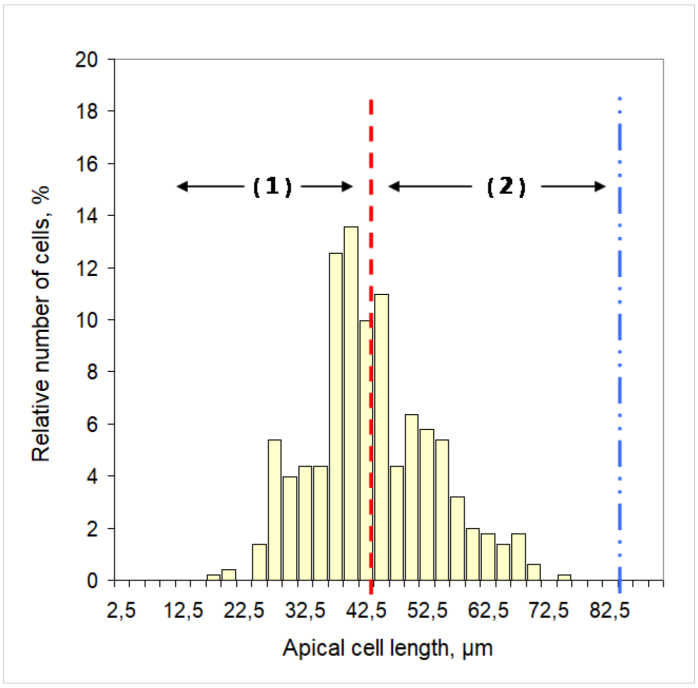

Fig. 4. Distribution of *Nitzschia* cf. *thermaloides* cell length (*N* = 502) by frequency of occurrence in the natural population of the mud volcanoes of the Bulganak mud volcano field. The dash-dotted line defines the maximum species-specific size. Cells smaller than the critical limit (a dashed line) are in the reproductive phase (1) and represent the effective fraction of the population. Larger cells, the size of which is between a dashed and a dash-dotted line, are in the pre-reproductive phase of the life cycle (2)

There was a direct correlation between length of parental and daughter cells (Fig. 5). After isolation, the clones were tested for crossability/non-crossability in pairwise combinations, and 15 clones randomly selected from the samples turned out to be sexually compatible strictly in accordance with their mating types. For a sample of 10 clones, a table of cross-mating is given (Table 3). The clones were unevenly distributed by mating types, in a ratio of 4 : 11.

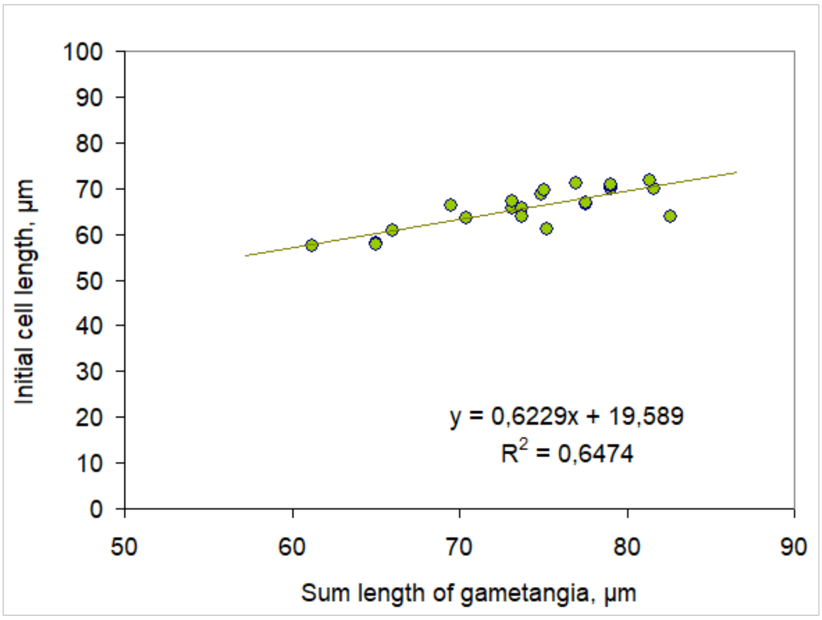

Fig. 5. Dependence of the initial cell length on the gametangial cell length in *Nitzschia* cf. *thermaloides* (N = 18)

Table 3. Cross-mating table (shows reproductive compatibility of *Nitzschia* cf. *thermaloides* clones isolated from the mud volcanoes)

Mud volcano	Clone		0.1026-YB	0.1026-YD	0.1020-OA	0.1020-OC	0.1029-YE	0.1029-YF	0.1029-YG	0.1025-YA	0.1020-OE	0.1020-OF
		mt	mt1	mt2	mt1	mt2	mt2	mt2	mt2	mt1	mt2	mt2
Andrusov	0.1026-YB	mt1										
Andrusov	0.1026-YD	mt2	**3**									
Pavlov	0.1020-OA	mt1	**0**	**2**								
Pavlov	0.1020-OC	mt2	**2**	**0**	**3**							
Pavlov	0.1029-YE	mt2	**3**	**0**	**3**	**0**						
Pavlov	0.1029-YF	mt2	**2**	**0**	**–**	**0**	**0**					
Pavlov	0.1029-YG	mt2	**3**	**0**	**3**	**0**	**0**	**0**				
Central Lake	0.1025-YA	mt1	**0**	**2**	**0**	**2**	**3**	**3**	**3**			
Central Lake	0.1020-OE	mt2	**3**	**3**	**3**	**0**	**0**	**0**	**0**	**3**		
Central Lake	0.1020-OF	mt2	**2**	**3**	**3**	**0**	**0**	**0**	**0**	**2**	**0**	

Note: mt, mating type; mating types (mt1 and mt2) are distinguished conventionally and marked in blue and red, respectively, for ease of perception. The table shows the maximum scores for the intensity of auxospore formation, obtained as a result of 3–5 times repeated experiments: 0, no auxospores were observed; 1, single; 2, non-abundant; 3, abundant. Dash indicates lack of data.

In *N.* cf. *thermaloides*, the sexual process is preceded by pairing of parental cells (Fig. 6A); the formation of pairs could be observed on the second day after mixed inoculations were made. Cells in pairs were arranged more or less parallel to each other and showed a stable relationship. At the same time, cells did not secrete any mucus and did not form mucous capsules. Gametogenesis in parental cells (gametangial cells) usually began on the third day (after the light–dark period completion) in the morning and proceeded in pairs almost simultaneously, but not synchronously. Initially, the cell protoplast contracted along the apical axis (Fig. 6B). Resulting from transapical division, two identical gametes were formed in each gametangial cell (Fig. 6C). The gametes slightly increased in size, which is more typical of the gametes that were formed first (Fig. 6D). At the end of the formation, the gametes fused in pairs without any order (Fig. 6E, F); this resulted in the formation of two zygotes. The zygotes began bipolar growth, extending parallel to the apical axis of the gametangial valves (Fig. 6G). From this point on, cells should be called auxospores (Fig. 6H). Growing auxospores were usually arranged side by side, more or less parallel to each other and to the valves of parental cells; occasionally, auxospores were lined up along one axis (Fig. 6I). There were gametangial pairs characterized by disordered orientation of auxospores and valves of parental cells, for example, due to deformation of the perizonium, which determines the bipolar direction of auxospore growth (Fig. 6J).

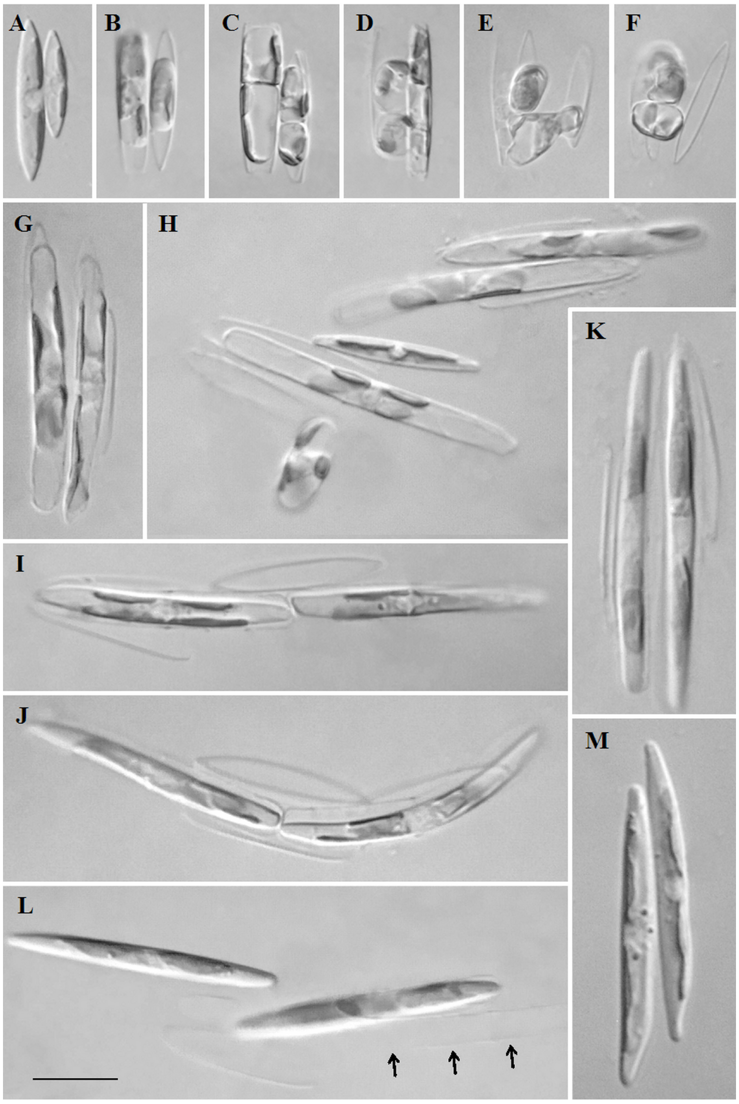

Fig. 6. The process of *Nitzschia* cf. *thermaloides* sexual reproduction: A, formation of pairs of gametangial cells; B, the beginning of gamete formation, the protoplast contracts along the apical axis; C, as a result of transapical division, two identical gametes are formed; D, gametes slightly increase in diameter; E, F, gametes fuse in pairs without any order; G, bipolar-growing auxospores; H, usual arrangement of auxospores – side by side and parallel to each other and to the valves of gametangia; I, occasionally, auxospores are located along one axis; J, developmental disorders result in disordered orientation of auxospores and valves of parental cells; K, inside fully formed auxospores, which have reached a size close to the maximum species-specific, the valves of initial cells begin to deposit (first epitheca; then hypotheca); L, initial cells, that recently left the perizonium (indicated by arrows); M, initial cells return to normal mitotic divisions, giving rise to a new generation of clones. Scale bar: 20 μm

With a sufficient duration of the light period (October–November), on the same day, auxospores that had grown to the maximum size could be detected; inside them, the valves of initial cells began to be deposited (Fig. 6K) – first epitheca, and then, after acytokinetic meiosis, hypotheca. The process of auxospore formation continued on the fourth day. Since initial cells proceeded to mitotic division, in the mixed cultures one could detect both initial cells that had recently left the perizonium (Fig. 6L) and the first post-initial cells morphologically slightly different from them (Fig. 6M).

Interestingly, syngamy was not always successful. Sometimes, only one pair of gametes fused; therefore, in some gametangial pairs, only one zygote and, accordingly, one auxospore was formed. Aborting the development of one of auxospores led to the same result. Sometimes, not a single gametangial pair could reach syngamy, even in those rare cases when gametangial cells had formed not pairs, but triplets. However, in general, the process of auxospore formation was intensive, and large cells of the new generation could be observed in mixed cultures in high abundance.

DISCUSSION

The theory of the life cycle of diatoms is quite well developed [Chepurnov et al., 2004; Drebes, 1977; Gastineau et al., 2014; Poulíčková, Mann, 2019; Roshchin, 1994; Round et al., 1990; *etc.*]. During the main part of their life cycle, these microalgae are in a diploid state, carrying out mitotic divisions; only gametes are haploid. Sexual reproduction, accompanied by meiosis, is an obligatory attribute of the life cycle in almost all diatoms, with rare exceptions. At the same time, one of the most remarkable features of these microalgae is that their life cycle is closely related to cell size. The following main key points regarding the vast majority of diatom species must be taken into consideration for performing an analysis of the natural population studied by us:

- Passing through all phases of the life cycle depends on the change in cell sizes.
- Due to the peculiarities of the structure and the way of frustule formation, the apical size in pennates and cell diameter in centric diatoms decrease with each vegetative division. In clones, a decrease in the mean size and an increase in the range of variation in cell sizes occur.
- Restoration of initial (maximum) cell sizes in a population is carried out in the process of sexual reproduction due to rapidly growing zygotes called auxospores.
- In the life cycle, pre-reproductive, reproductive, and post-reproductive phases can be distinguished (the latter one is not observed in all species).
- Duration of the pre-reproductive phase of the life cycle ranges from several months to several years, depending on the species and the cell division rate.
- Transition from the pre-reproductive to the reproductive phase is related to overcoming the critical size (one of the cardinal points of the life cycle), which in most diatoms is close to half the maximum species-specific size.
- Transition to the reproductive phase does not mean an unconditional entry into the sexual process. Its implementation requires a favorable combination of environmental factors and presence of a sexual partner (in the case of heterothallic reproduction, which prevails in most pennate diatoms). Most cells that have reached the reproductive phase continue to divide vegetatively and decrease in size.

- Regardless of the size of parental cells, auxospores usually grow to the size close to the maximum species-specific one. However, in some species, the size of daughter (initial) cells depends on the size of parental cells. In such cases, the size of the arisen initial cells may be less than the critical limit, which allows them to enter the sexual process immediately. All this results in two-stage auxospore formation.

Thus, two important events occur in the process of sexual reproduction in a diatom population: restoration of initial cell sizes and appearance of a new generation of clones with new genetic "patiences." Worth noting that a small number of Bacillariophyta species (extremely insignificant compared to the total number of those studied) do not change cell sizes during vegetative division. Apparently, for this reason, the sexual process has never been observed in them. Those include species which are model in many aspects but sexually exceptional: *Phaeodactylum tricornutum* Bohlin, 1897 and *Thalassiosira pseudonana* Hasle & Heimdal, 1970. These are the first two diatoms with a complete genome sequenced [Armbrust et al., 2004; Montsant et al., 2005].

Considering the above principles, it is possible to analyze cell distribution in the natural population of *N.* cf. *thermaloides* (see Fig. 3) by the life cycle phases. At the time of sampling, slightly more than a half of cells in the population were in the pre-reproductive phase. The effective fraction of the population accounted for 46%. The process of sexual reproduction occurs in the population, as evidenced by the finding of cells whose size was close to the maximum species-specific one. Obviously, appearance of such cells could result only from sexual reproduction. The size spectrum covers all phases of the life cycle: cells in the pre-reproductive and reproductive phases are equally represented. This state of the population can be called balanced. Moreover, given that it usually takes several years for diatoms to go through the entire life cycle [Mann, 1988], we can conclude that *N.* cf. *thermaloides* population is not ephemeral and inhabits this spot for quite a long time.

The trend characteristic of most diatoms was confirmed once again: the size boundary (the cardinal point), passing which cells reach the reproductive phase, is close to half the maximum species-specific size (in most of the species studied, it ranges from 45 to 55%) [Davidovich, 2000]. A consequence of the discovered positive correlation between the length of parental and daughter cells (see Fig. 4) is unequal duration of the life cycle of individual clones.

Isogamous sexual reproduction, accompanied by the formation of two gametes in each gametangial cell, was recorded in at least 15 genera of diatoms, phylogenetically both close to the genus *Nitzschia* Hassall, 1845 (for example, *Achnanthes* Bory, 1822) and distant from it (for instance, *Dickieia* Berkeley ex Kützing, 1844 or *Berkeleya* Greville, 1827), considering the largest phylogenetic tree constructed from data for 11 genes in 1,151 diatom taxa [Nakov et al., 2018]. However, in addition to number of gametes and their behavior, an important role in the classification of types of the sexual process is played by the following details: presence or absence of copulatory mucus secreted by gametangial cells; division of the cell protoplast in the apical or transapical plane during the formation of gametes and presence or absence of rearrangement of the latter; direction of movement of gametes; and mutual orientation of axes of auxospores and valves of gametangial cells. Given all the details, the sexual process observed in *N.* cf. *thermaloides* can be categorized as IB2a according to Geitler classification [Geitler, 1973].

In the mating system of the species, heterothallism prevails. Apparently, it is the only way to reproduce, given that no cases of homothallism have been revealed in clonal cultures so far.

In the future, the response of *N.* cf. *thermaloides* to quite variable (extreme for many other diatoms) conditions of temperature and high irradiance is to be studied. Moreover, the limits of the halotolerance of this species living under conditions of relatively high salinity, typical for puddles and lakes of mud volcanoes, are to be established.

Conclusion. In water bodies formed by emissions from the mud volcanoes of the Kerch Peninsula, *Nitzschia* cf. *thermaloides* is represented as a stable population containing cells in all phases of the life cycle (pre-reproductive, reproductive, and post-reproductive). The analysis of the size distribution of cells in the population, which became possible due to the established cardinal points of the life cycle, shows that the population is not ephemeral and is capable of reproducing in generations, despite living under extreme conditions.

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БИОЛОГИЯ ВОСПРОИЗВЕДЕНИЯ И ЖИЗНЕННЫЙ ЦИКЛ ДИАТОМОВОЙ ВОДОРОСЛИ *NITZSCHIA* CF. *THERMALOIDES*, НАСЕЛЯЮЩЕЙ ГРЯЗЕВЫЕ ВУЛКАНЫ КРЫМА

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В биологии актуально изучение способности организмов к существованию в экстремальных условиях, к которым особенно чувствительна фаза жизненного цикла, связанная с половым размножением и возможностью оставить следующее поколение. В пробах, которые были взяты из луж и озёр, образующихся в местах действия грязевых вулканов Булганакского сопочного поля (Керченский полуостров, Крым), обнаружена в большом количестве *Nitzschia* cf. *thermaloides* Hustedt. Из проб микропипеточным способом выделены и введены в культуру отдельные клоны, скрещивание которых позволило инициировать гетероталлическое половое воспроизведение. Целью работы было изучить биологию воспроизведения и жизненный цикл водоросли из биотопа с экстремально высокими уровнями освещённости, температуры и солёности. Впервые для данного вида приведено описание полового процесса, соответствующего типу IB2a по классификации Гайтлера. Определены кардинальные пункты — критические размеры клеток, обусловливающие переход из одной фазы жизненного цикла в другую. Приведён полный диапазон размеров клеток — от инициальных, появившихся в результате полового воспроизведения, до самых маленьких, наблюдавшихся при содержании в культуре. Рассмотрены основные положения теории жизненного цикла диатомовых водорослей, позволяющие выполнить анализ их природной популяции. Установлено, что популяция *N.* cf. *thermaloides* существует в месте отбора проб длительное время и представлена клетками, находящимися во всех фазах жизненного цикла.

Ключевые слова: диатомовые, *Nitzschia* cf. *thermaloides*, грязевые вулканы, половое воспроизведение, жизненный цикл