SALINITY OPTIMA FOR VEGETATIVE GROWTH AND SEXUAL REPRODUCTION OF THE DIATOM TOXARIUM UNDULATUM

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Distribution of diatom algae is limited by their tolerance to environmental factors. Although a genus Toxarium has been evolving for more than 100 million years, it is represented by only two species. Toxarium undulatum is widely spread in tropical and subtropical seas, and it can be also found in the Black Sea, the salinity of which is twice lower than the oceanic one. Ecological and psychological characteristics research of this species is of great interest in terms of its relationship to salinity. T. undulatum clonal cultures were sampled in the Donuzlav Lake connected to the Black Sea (southwest of the Crimean Peninsula) and on Gran Canaria coast (Canary Islands archipelago). Experiments on the salinity tolerance limits showed, that the Black Sea clones were viable in a range of at least 30 ‰ (12 to 42 ‰). The same wide range of salinity tolerance with slightly higher values was observed among oceanic clones of this species. Optima of vegetative growth and sexual reproduction were determined. Optima of the Black Sea clones appeared to be 27.8 and 27.2 ‰, respectively, which was significantly higher than salinity observed in population habitat. Similar higher optima of vegetative growth and sexual reproduction, compared with those salinity values, at which natural population developed, were observed for a number of other Black Sea diatoms, which proved their oceanic (Mediterranean) origin. It was concluded that T. undulatum, along with other species, began to populate the Black Sea basin about seven thousand years ago after Mediterranean Sea water started to flow into the freshened Novoevksinsky Sea-Lake through the Bosporus Strait. However, the evolution rate did not allow bringing physiological and ecological characteristics of the species studied into full agreement with environmental conditions. Oceanic origin is evidently seen in its physiological reactions to salinity. Possibility of speciation due to settlement of the Black Sea with oceanic species is discussed.

Keywords: diatom, Toxarium undulatum, evolution, salinity, tolerance limits

One of the largest diatoms, Toxarium undulatum Bailey, 1854, can be found in the Black Sea. This species is common for tropical and subtropical seas [22] characterized by a higher salinity than the Black Sea. It has previously been shown that a number of diatom species found in the Black Sea have salinity optima for vegetative growth and sexual reproduction higher than in their populations habitats [2, 3]. The most reasonable explanation seems to be possibility of Black Sea basin being populated with originally oceanic species evolutionarily adapted to 35–36 ‰ salinity after Bosporus Strait formation, 7–8 thousand years ago, when the highly desalinated Novoevksinsky Sea-Lake began to fill up by waters coming from the Mediterranean Sea [5, 7]. Study of T. undulatum physiological reactions has provided us with new data on this cosmopolitan species, common in all coastal seas of subtropical zone, and its relation to lower (Black Sea) salinity.
MATERIAL AND METHODS

Periphyton samples were collected from a depth of about 40 cm near the eastern shore of the Donuzlav Lake (south-west of the Crimean Peninsula, point with coordinates 45°22′23″N, 33°05′40″E) and on the Gran Canaria Island (Canary Islands archipelago, 27°59′27″N, 15°22′06″W). Single cells given rise to clonal cultures (Fig. 1) were isolated from the samples using glass micropipettes and inverted microscopes Zeiss Primover (Carl Zeiss AG, Germany) and Nib-100 (China).

![Fig. 1. *Toxarium undulatum*, attached to substrate with the help of mucilage secreted by one of the apical ends of the cell, forms tuft colonies in the process of division. A – clone 8.0827-Y, the Black Sea population; B – clone 9.0620-C, the Canary population. Scale bar is 100 μm](image)

The cultures were maintained in 100-ml Erlenmeyer glass flasks in the modified ESAW medium [9], periodically (every 7–14 days) transferred in the fresh medium. Conditions of maintaining and the method of clones naming are described in [1]. The Black Sea clones were maintained at 20 ‰, The Canary ones – at 30 ‰. In the sampling points, salinity was 20 ‰ and 36 ‰, respectively. Salinity was measured with RHS-10ATC refractometer (China).

After we had succeeded in stimulating sexual reproduction in mixtures of clones from the Black Sea population, two pairs of clones, (8.0820-E + 8.0830-K) and (8.0827-Y + 8.0830-K), were chosen for further experiments. As a result of the last pair crossbreeding, initial cells were obtained. They were characterized by sizes close to a maximum value for the species. Several postinitial cells (average size of 1270 μm) were chosen for the culture and used later in experiments as cells being at the beginning of their life cycle. In addition, clones 8.0822-D, 8.0920-E, and 8.1227-A from the Black Sea population with average cell sizes of 642, 518, and 963 μm, respectively, were used in the experiments. In clones 9.0618-A, 9.0618-E, 9.0620-B, and 9.0621-DD from the Gran Canaria population, the average cells sizes at the time of the experiments were 680, 212, 284, and 334 μm, respectively.

Salinity factor effect on algae was estimated by mortality of cells while transferring them from the medium in which they had been maintained in the collection to the new medium with salinity gradation: 8, 12, 18, 24, 30, 36, 42, 48, 54 ‰. The required salinity was obtained by either diluting the modified ESAW medium (36 ‰) with distilled water, or adding sodium chloride to it. The cells were put into 50-mm diameter glass Petri dishes (bottom area of 494 mm²). Mortality was determined as a ratio between a number
of dead cells and a total number of cells. The cell was regarded as dead based on visual estimation of the inner content state, mainly chloroplasts (their color, location, or complete absence). The number of cells was counted in ten Nib-100 microscope fields of view (field of view of 0.88 mm²) at 20× lens and 10× eye-piece magnification within five days: on the first, second and fifth days of the experiment – for the Black Sea clones, on the first, second, fourth and fifth days – for the Canary clones. The average death rate for each clone was calculated for all days of the experiment.

To determine the optima, we used a second-order equation approximating the data obtained. Coefficients of the equation were calculated by the least squares method [6]. The value of the argument of the first derivative of this equation, equaled to zero, determined the position of the optimum.

RESULTS

Both the Black Sea and oceanic *T. undulatum* populations demonstrated wide salinity tolerance, with tolerance range of at least 30 ‰ (Fig. 2).

![Fig. 2. Relative number of dead *Toxarium undulatum* cells when transferred from medium with salinity of 20 ‰ (A, the Black Sea population) and 30 ‰ (B, the Canary population) to the new medium with different salinity levels (on average for four clones for five days of growth in the new medium).](image-url)
The Black Sea clones were viable in a range 12 to 42 ‰, possibly slightly wider, taking into account 6 ‰ gradation step set in the experiments. The Canary clones tolerated salinity 18 to 48 ‰. A shift towards higher salinity, which was observed in the oceanic clones compared with the Black Sea ones, could be explained by the fact that before experiments, they had been grown at different salinity levels (30 and 20 ‰, respectively). However, some authors believe that preliminary adaptation of diatoms to salinity levels extreme for these species cannot result in a noticeable increase of tolerance limits [8].

For the Black Sea clones, a relationship was found between the cells size and the number of dead cells, when transferred into the new medium, on average for five days of growth at nine experimental salinity levels (Fig. 3). The largest cells, arose as a result of the reproductive process and being at the beginning of the life cycle, turned out to be the most sensitive to changes in growth conditions. This dependence was not observed among the Canary clones. It should be noted, however, that their sexual reproduction has not been studied, and the largest cells at the beginning of the life cycle have not been obtained.

**Fig. 3.** Mortality of *Toxarium undulatum* cells depending on their apical length (when transferred to a new culture medium, on average for nine salinity levels); □ are the Canary clones; ● are the Black Sea clones

The salinity optimum for vegetative growth of the Black Sea *T. undulatum* population was of 27.8 ‰, while for vegetative growth of the Canary clones – of 32.4 ‰. The optimal salinity for sexual reproduction of the Black Sea clones was of 27.2 ‰ (Fig. 4).

**DISCUSSION**

Morphology and way of life of four genera – *Ardisonea* De Notaris, *Climacosphenia* Ehrenberg, *Syne-drosphenia* (H. Peragallo) Azpeitia Moros, and *Toxarium* J. W. Bailey (family Toxariales Round), named also toxariids, – are in evident disagreement with data on genosystematics, which places them among polar centric diatoms [19, 20, 21, 24, and others]. Distinction and evolutionary uniqueness of this group have recently been confirmed by results of study of *Ardisonea crystallina* (C. Agardh) Grunow reproductive biology. The mode of reproduction of this alga was not oogamous, which is typical for all centric diatoms studied by far, but was similar to that of pennates [13]. Judging by molecular phylogeny [21],
Fig. 4. Relative number of generative cells in mixtures of reproductively compatible *Toxarium undulatum* clones depending on a salinity level of a medium. Approximation was performed by a second-degree polynomial. A dash-dotted line indicates an optimum position.

it can be concluded, that evolutionary line of toxariids separated from its ancestors at the turn of the Cretaceous and Jurassic periods about 150 million years ago; 35 million years later, *Toxarium* separated from *Ardissonea* and *Climacosphenia*. It should be noted, that there are only two species in the genus *Toxarium* [22]. This testifies a significant conservativeness of its genetic composition: for more than 100 million years, it has changed a little. At least, those changes have not resulted in occurring new, morphologically distinguishable species. Therefore, it can be assumed that inhabiting new places that differ in salinity level, *Toxarium* retains its features developed by millions of years of evolution, which at the physiological level are shown in certain physiological optima. With evolutionary adaptation to new salinity, a shift in tolerance limits and physiological (ecological) optima is inevitable; the question is the tempo of such changes.

Are 7–8 thousand years a sufficient period for evolutionary changes that may lead not only to emergence of new properties but also to emergence of new diatom species? The answer can be positive, if we take a complex of cryptic species of the genus *Haslea* as an example [17, 18]. *H. karadagensis* recently described by us [16] is a Black Sea endemic [18]. Moreover, this species has tolerance limits and physiological optima proving its oceanic origin: for its growth, 25–30 ‰ were optimal, and sexual reproduction did not occur in the media with salinity below 17 ‰ [14].

*T. undulatum* is widely tolerant in relation to salinity. The cells remained viable (in varying degrees) in salinity range 12 to 42 ‰; sexual reproduction occurred in a narrower range. Such a wide range with an optimum of about 30 ‰ proves an assumption that it was the relative euryhalinity that allowed the species to inhabit new place after appearance of straits connecting the Black and the Mediterranean seas. It is unlikely that the ancestral Mediterranean (oceanic) population was stenohaline, and the tolerance range of the Black Sea population has expanded significantly due to evolutionary changes over a relatively short period (7–8 thousand years). This can also be confirmed by results obtained while studying the position
of physiological optima and tolerance limits of the oceanic clones sampled by us from Gran Canaria coast and determined morphologically as *T. undulatum*. Unlike the salinity of the Black Sea, the salinity of this part of the World Ocean has been unchanged for many millions of years; however, the selected clones had similar wide range of tolerance as the Black Sea ones, and their optima for growth has not differed significantly.

Any conclusions regarding possible reproductive barriers between the Black Sea population of *T. undulatum* and populations from other parts of the World Ocean are too premature before crossing experiments are performed.

The size of cells is known to determine a phase of their life cycle [22]. *A priori*, before position of the upper limit of size range of auxospore formation is identified, one can claim, based on the general principles [12], that cells of clones 8.0822-D and 8.0920-E were in the generative phase, while cells of clone 8.1227-A did not reach the size that allows entering sexual process. Correlation between resistance to a change of the medium salinity and cell size was observed in four studied clones from the Black Sea: small cells turned out to be more resistant to abrupt salinity changes (Fig. 2). However, final conclusion about the dependence of salinity tolerance on a life cycle phase is premature, first of all, due to small amount of data obtained, and, in addition, due to a possible clone-specific response. In many experiments, we faced clone specificity; this concerned a wide range of characteristics, from an ability to synthesize and accumulate certain substances (for example, pigments in species of the genus *Haslea*) to an ability to enter sexual process and an intensity of auxosporulation. It should be noted, that in the closely related species, *A. crystallina*, on the contrary, larger cells adapted more quickly to a transfer from a medium with salinity of 20 ‰ to a medium with salinity of 12 ‰ [15].

With these differences, it is important to note the following general pattern: for all Black Sea diatoms investigated to date, salinity optima for vegetative growth and sexual reproduction were higher than salinity of 17–18 ‰ typical for the Black Sea near the Crimean coast (Table 1). An excess was of 8–12 ‰ or more. An evidence, that Black Sea diatoms divide faster at salinity levels higher than Black Sea one, is not new (for example, [4, 8, 10]). Such a discrepancy between physiological (ecological) optimal values and conditions, typical for population habitats of the species under study (however, see different opinion [10]), clearly shows that these species entered the Black Sea but an evolution rate has not allowed them to bring their physiological and ecological characteristics into full agreement with environmental conditions.

<table>
<thead>
<tr>
<th>Species</th>
<th>Optima for vegetative growth (tolerance limits), ‰</th>
<th>Optima for sexual reproduction, ‰</th>
<th>Source of data</th>
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<tr>
<td><strong>Ardissonea crystallina</strong></td>
<td>29 (9–45)</td>
<td>30</td>
<td>[15]</td>
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<tr>
<td><strong>Climaconeis scalaris</strong></td>
<td>30 (8–48)</td>
<td>30</td>
<td>[3]</td>
</tr>
<tr>
<td><strong>Haslea karadagensis</strong></td>
<td>25–30 (8–45*)</td>
<td>25–30</td>
<td>[14]</td>
</tr>
<tr>
<td><strong>Nitzschia longissima</strong></td>
<td>27 (8–45)</td>
<td>30</td>
<td>[23]</td>
</tr>
<tr>
<td><strong>Tabularia tabulata</strong></td>
<td>30 (2,25–49*)</td>
<td>30</td>
<td>[2]</td>
</tr>
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<td><strong>Toxarium undulatum</strong></td>
<td>28 (8–48)</td>
<td>27</td>
<td>present paper</td>
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</tbody>
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Note: * – higher salinity values were not checked in experiments.

The compliance with these tolerance range conditions, related not only to vegetative growth, but also to sexual reproduction, is extremely important for the species to exist under certain salinity conditions. Sexual reproduction for the overwhelming majority of diatoms is known to be the essential stage
in their life cycle [11, 22]. The next generation may not appear due to inappropriate environmental conditions. *T. undulatum* demonstrated an ability to reproduce sexually in sufficiently wide ranges of salinity. However, in comparison with vegetative growth range, a salinity tolerance range in case of sexual reproduction was narrower. The optimum is much higher than the salinity of the Black Sea and the Donuzlav Lake (the sampling point).

Thus, the data obtained testifies, that *T. undulatum*, along with many other species, populated the Black Sea basin in the post-novoevksinsky period. In its physiological reactions with respect to salinity of the environment, an oceanic origin is seen. An objective of subsequent research will be to study the reproductive relations of the Black Sea and oceanic populations and to determine presence or absence of the reproductive barriers between them.

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REFERENCES


ОПТИМУМЫ СОЛЁНОСТИ ДЛЯ ВЕГЕТАТИВНОГО РАЗМНОЖЕНИЯ И ПОЛОВОГО ВОСПРОИЗВЕДЕНИЯ ДИАТОМОВОЙ ВОДОРОСЛИ TOXARIUM UNDULATUM

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Распространение диатомовых водорослей ограничивается пределами их толерантности по отношению к факторам среды. Род *Toxarium* представлен всего двумя видами, хотя его эволюция продолжается более 100 млн лет. *Toxarium undulatum* широко распространён в тропических и субтропических морях; он встречается и в Чёрном море, солёность которого вдвое ниже океанической. Представляет интерес изучение эколого-физиологических характеристик этого вида с точки зрения его отношения к солёности. Клоновые культуры *T. undulatum* выделены из проб, собранных в озере Донузлав, сообщающемуся с Чёрным морем (юго-запад Крымского полуострова), и в прибрежье острова Гран-Канария (архипелаг Канарских островов). Эксперименты по изучению пределов толерантности по отношению к солёности показали, что черноморские клонсы жизнеспособны в диапазоне, охватывающем как минимум 30 ‰ (от 12 до 42 ‰). Такой же широкий диапазон солёностной толерантности, слегка сдвинутый в сторону больших значений, наблюдали у океанических клонов этого вида. Установлены оптимумы для вегетативного роста и полового воспроизведения. У черноморских клонов они оказались равными 27,8 и 27,2 ‰ соответственно, что заметно выше солёности, которая наблюдается в месте обитания популяции. Аналогичное превышение положения оптимумов в отношении вегетативного размножения и полового воспроизводства по сравнению с теми уровнями солёности, в которых находится природные популяции, было отмечено для ряда других черноморских диатомовых, что свидетельствует об их океаническом (средиземноморском) прошлом. Сделан вывод о том, что *T. undulatum*, наряду с другими видами, начал заселять черноморский бассейн ориентировочно семь тысяч лет назад, после того как в распреснённое Новоэвксинское море-озеро через образовавшийся пролив Босфор стала поступать вода из Средиземного моря. Между тем темпы эволюции не позволили привести физиологические и экологические характеристики изучаемого вида в полное соответствие с условиями окружающей среды. В его физиологических реакциях по отношению к солёности прослеживается океаническое происхождение. Обсуждается возможность виообразования в связи с заселением Чёрного моря океаническими видами.

Ключевые слова: диатомовые, *Toxarium undulatum*, эволюция, солёность, пределы толерантности