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HALOTOLERANCE LIMITS OF THE BLACK SEA REPRESENTATIVE OF THE GENUS *ENTOMONEIS* EHRENBERG, 1845 (BACILLARIOPHYTA)

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The genus *Entomoneis* Ehrenberg, 1845 is quite rich in species. Underestimated diversity of this genus requires its deeper morphological and molecular study, as well as an investigation of ecological and physiological characteristics of its species – specifically, their tolerance limits to environmental factors. Considering the distribution of *Entomoneis* species in water bodies with various salinity, we aimed at studying the tolerance limits and determining optimal salinity for vegetative growth and sexual reproduction of the diatom *Entomoneis* sp. from the Black Sea. We used reproductively compatible clonal cultures isolated from samples taken on the Crimean and Turkish Black Sea coasts. For *Entomoneis* sp. clone 7.0906-D, the nucleotide sequence of the *rbc*L gene was obtained; it is presented in the GenBank database under the number MT424817. Morphologically, the studied species resembles *E. paludosa*; according to molecular data, it is far from it. In accordance with its ecological and physiological characteristics, this species is a marine one. According to published material available, *E. paludosa*, unlike *Entomoneis* sp., inhabits brackish, slightly saline, and even fresh water bodies. Experiments on halotolerance show the following: the Black Sea clones of *Entomoneis* sp. are viable in a range of at least 40 ‰ (8 to 48 ‰). A salinity range of the medium within which *Entomoneis* sp. revealed sexual reproduction is much narrower – 18 to 36 ‰. Optimal salinity values for vegetative growth and sexual reproduction were determined (27.4 and 26.4 ‰, respectively); those turned out to be higher in both cases than the values in the natural habitat of this species. As salinity of the medium increased, *Entomoneis* sp. initial cells resulting from sexual reproduction tended to decrease in size.

Keywords: diatoms, sexual reproduction, vegetative growth, salinity, *Entomoneis* sp.

Limits of salinity tolerance (halotolerance) are one of the key ecological and physiological characteristics of species. Those determine the possibility of species existence in a particular water area (Brand, 1984). High halotolerance is one of the indispensable conditions for the species to spread everywhere and become cosmopolitan. On the contrary, stenohalines are much less likely to be widely distributed. The results obtained in the previous studies show that clones from populations of several Black Sea species have much higher salinity optima than salinity of their habitats (Davidovich & Davidovich, 2020).

The diatom genus *Entomoneis* Ehrenberg, 1845 includes species with unique morphology: those have a two-lobed keel elevated above the valve surface, a sigmoid curvature of the raphe, and numerous perforated copulae. The genus is quite rich in species. To date, AlgaeBase (2020) contains 36 species and 21 intraspecific names of its representatives. Out of all the species names, 28 are considered as taxonomically accepted. In some cases, opinions on the validity of species names differ among authors.

As established, most of the species belonging to this genus are brackish or marine; only a few of them have been recorded in fresh or highly desalinated water bodies (Liu et al., 2018 ; Round et al., 1990). The Black Sea representative of the genus *Entomoneis* chosen by us for the study is morphologically similar to *Entomoneis paludosa* W. Smith, 1853 (Fig. 1).

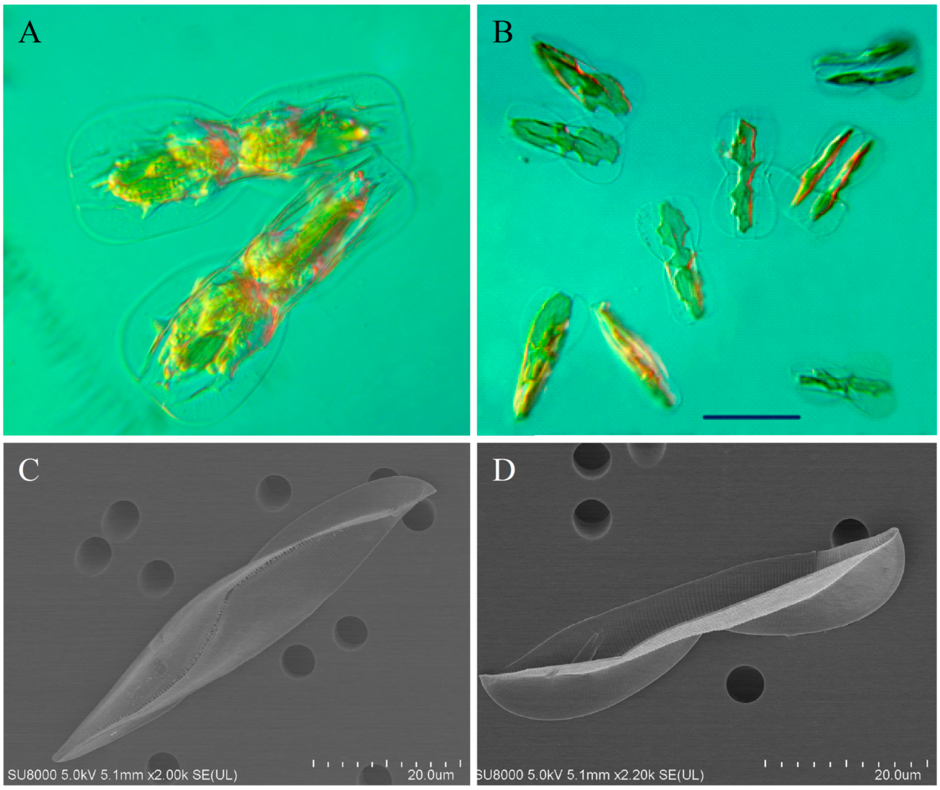

Fig. 1. *Entomoneis* sp. A, B, alive dividing cells, light microscopy (differential interference contrast), scale bar 50 μm; C, D, valves of the frustule, scanning electron micrographs

The latter inhabiting benthos and plankton of seas and brackish waters was recorded in the Black Sea as well (Ryabushko, 2006). Morphologically, the species studied in our experiments resembles *E. paludosa*; according to preliminary molecular data, it is far from it in the phylogenetic tree constructed. Undoubtedly, we should agree with the opinion of Mejdandžić *et al.* (2018): underestimated diversity of *Entomoneis* requires a deeper morphological and molecular study of this genus, as well as an investigation of ecological and physiological characteristics of its species – specifically, their tolerance limits to environmental factors. Given the wide distribution of *Entomoneis* species in water bodies with various salinity, we aimed at studying the tolerance limits and determining optimal salinity for vegetative growth and sexual reproduction of *Entomoneis* sp. from the Black Sea.

MATERIAL AND METHODS

Clones used in the experiments were isolated by micropipette from samples taken on the Crimean and Turkish coasts of the Black Sea. The clone 7.0906-D was derived from the population near Akçakoca (Düzce-Akçakoca, Turkey, 41°05′25″N, 31°07′26″E); clones 8.0727-A, 8.0727-B, 8.0727-D, and 8.0727-E were derived off the Tarkhankut Peninsula (45°19′50″N, 32°34′36″E). At the time of the experiments, mean length of vegetative cells in the clones was as follows: 8.0727-A, 31 μm; 8.0727-B, 31 μm; 8.0727-D, 42 μm; 8.0727-E, 42 μm; and 7.0906-D, 21 μm. For *Entomoneis* sp. clone 7.0906-D, the nucleotide sequence of the *rbc*L gene was obtained which is presented in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) under the number MT424817.

A reproductively active pair of the Black Sea clones 7.0906-D + 8.0727-D was used in the experiment aimed at studying salinity effect on the sexual reproduction of the diatom *Entomoneis* sp. A culture in the exponential growth stage was used as the inoculum. *Prior* to the experiment, the cultures were kept in glass Petri dishes at a temperature of (20 ± 2) °C under natural light from a northern window in the ESAW medium (Andersen et al., 2005) with modifications (Polyakova et al., 2018) adjusted to salinity of 18 ‰. The experiments were carried out under similar light and temperature conditions. Each Petri dish (5 cm in diameter) was filled with 10 mL of the medium with different salinity: 4.5; 8; 12; 18; 24; 30; 36; 42; 48; and 54 ‰. Reduced salinity was obtained by diluting the ESAW medium (36 ‰) with distilled water – similarly to that described by other authors (Karaeva & Dzhafarova, 1993 ; Bagmet et al., 2017). A medium with salinity above 36 ‰ was prepared by adding the required amount of sodium chloride to the ESAW medium. The salinity level was measured with a handheld refractometer (model RHS-10ATC, China). Then, the mixture of reproductively compatible clones (30 μL) was added to each Petri dish. In the experiments on salinity effect on vegetative growth, 20 μL of each clonal culture were inoculated into Petri dishes with media with different salinity. The results of sexual reproduction were evaluated on the third, fourth, and fifth days after mating. The number of vegetative and generative cells was counted in 20 microscope fields of view. The number of cells that entered the sexual process was estimated as the ratio of the number of generative cells to the total number of cells on average over three days of the experiment. Gametes, zygotes, auxospores, and developing initial cells were considered generative cells.

Five clones were involved in the experiments on environmental salinity effect on vegetative growth to obtain biological replicates and process statistical data. Salinity effect on alga vegetative reproduction was assessed by the rate of change in the number of cells. Specifically, the number of cells in 15 microscope fields of view was counted on the third, fourth, and fifth days after the inoculation. Then, the rate of cell division (r, day^{-1}) was determined based on the equation of exponential population growth:

$$N_t = N_0 \exp(r\Delta t) ,$$

where N_t and N_0 denote mean number of cells in the field of view at time t and at initial time t_0, respectively;

Δt denotes a time interval between t and t_0.

The values of the coefficient r were calculated using a least squares method, with the capabilities of Microsoft Excel. In order to switch to a unit of measurement "divisions·day^{-1}", obtained r values were divided by ln2. Salinity values optimal for the growth were determined from the position of the maximum of the parabolic function used to fit the data. The position of the approximating function maximum was found from the value of the first derivative equal to zero.

Cell sizes were determined under a Biolar PI microscope (PZO, Poland) equipped with an eyepiece ruler calibrated with an object micrometer that has a unit value of 1.60 μm – with an eyepiece magnification of 12× and a water immersion objective magnification of 40×. The photographs were obtained with a Canon PowerShot A640 digital camera.

For examination under a scanning electron microscope, diatom cells were purified from organic material by boiling in 35 % hydrogen peroxide (H_2O_2) in a sand bath for two days for three hours; then, cell suspension was centrifuged and washed with distilled water; the operation was repeated 7–8 times. A few drops of the resulting suspension were placed on aluminum stub, dried in air, and covered with gold. The electron micrographs were obtained on a Hitachi SU8020 scanning electron microscope.

RESULTS

The experiments showed that the investigated diatom of the genus *Entomoneis* is not viable in the medium with salinity of 4.5 and 54 ‰: cells died on the second day after the inoculation. Obviously, the sharper the change in salinity, the stronger the stress experienced by the alga. To avoid hypoosmotic shock, we carried out an experiment with a gradual decrease in salinity. Within a week, *Entomoneis* sp. clones were adapted to the medium with 8 ‰ salinity; then, those were transferred to the medium with 4.5 ‰ salinity. The results of the experiment showed that the cells remained alive for two days after inoculation in the medium with 4.5 ‰ salinity; on the third day, chloroplasts began to break down, and the alga lost its ability to divide and finally died.

In the medium with salinity of 8 and 12 ‰, the cells reproduced vegetatively, and the division rate for five clones averaged 0.48 and 0.96 divisions·day^{-1}, respectively. With an increase in medium salinity up to 42 ‰, the rate of cell division remained high (0.90 divisions·day^{-1}); however, at this salinity level, there was no auxospore formation. In the medium with 48 ‰ salinity, a positive dynamics of population growth was recorded, but with a low division rate – as few as 0.28 divisions·day^{-1}. The maximum vegetative growth was observed at 27.4 ‰ (Fig. 2A).

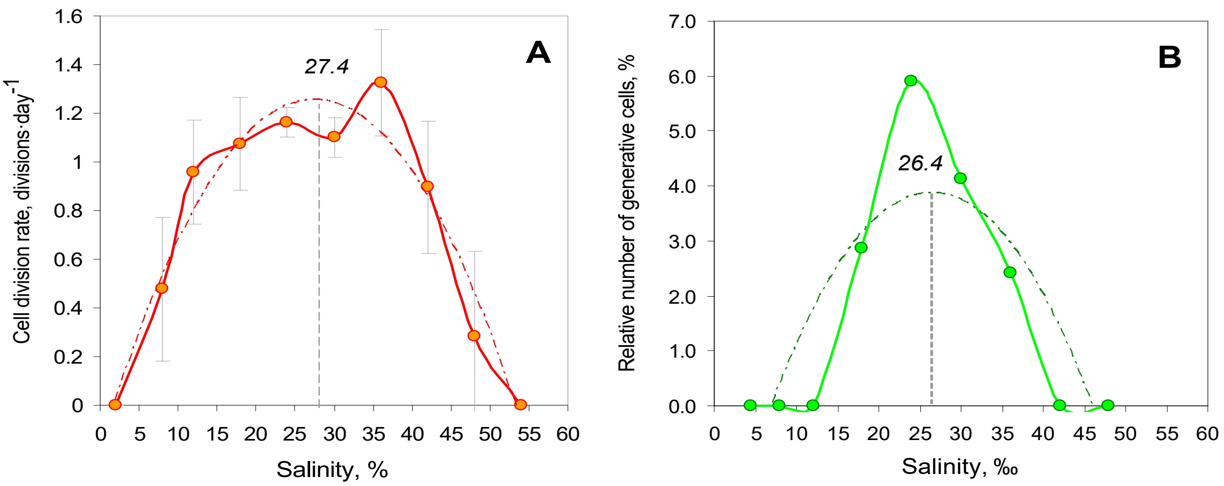

Fig. 2. A, dependence of the cell division rate of *Entomoneis* sp. on salinity of the medium; B, relative number of generative cells in mixed cultures of reproductively compatible *Entomoneis* sp. clones depending on the salinity level of the medium. The approximation was performed by a second-order polynomial (dashed-dotted line). The dashed line indicates the optimum position

Sexual reproduction with formation of initial cells occurred with the salinity level gradations of 18, 24, 30, and 36 ‰. An increase in salinity favorably affected the sexual reproduction of the alga. When transferred – after preliminary acclimation to 18 ‰ – to the medium with higher salinity, the ratio of cells participating in the sexual process increased significantly. Specifically, in the medium with salinity of 18 ‰, the relative number of *Entomoneis* sp. cells that entered the sexual process was two times less than in the medium with salinity of 24 ‰. For the Black Sea clones studied, optimal salinity for auxospore formation turned out to be 26.4 ‰ (Fig. 2B).

We studied the dependence of the length of initial cells on the salinity level of the medium as well (Fig. 3). The size range of the initial cells obtained in the experiment varied from 106 to 139 μm.

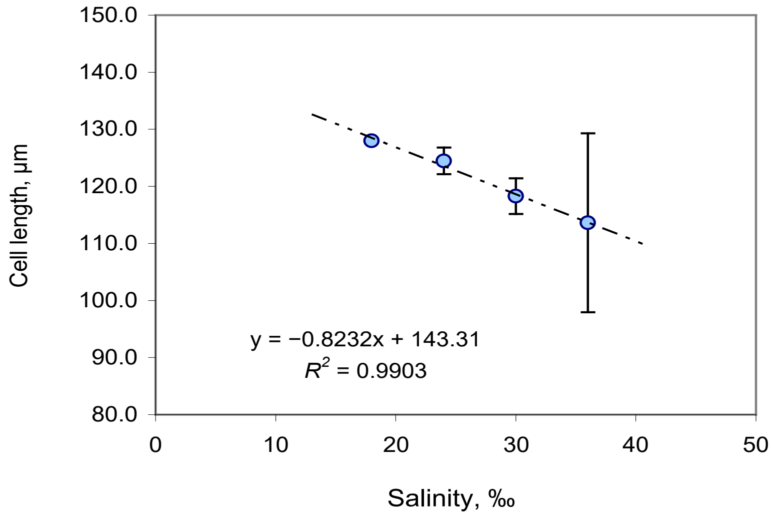

Fig. 3. Dependence of the length of initial cells on the salinity of the medium at which mixtures of *Entomoneis* sp. clones were maintained in mating experiments

DISCUSSION

The data on environmental salinity effect on *Entomoneis* representatives are quite contradictory. Specifically, in the literature, one can find information that *E. paludosa* is relatively abundant in the environment with salinity lower than 1 ‰ (Dalu et al., 2015). Considering this, the species could be assigned to inhabitants of fresh and slightly brackish waters. Other authors describe the species as widespread in brackish-water bodies (Kulikovskiy et al., 2016 ; Weckstrom & Juggins, 2006). At the same time, many publications report *E. paludosa* presence in a typical marine environment (Morant-Manceau et al., 2007 ; Rech et al., 2005 ; Ryabushko et al., 2019). The results of our experimental studies indicate that the investigated representative of the genus *Entomoneis* is viable in quite a wide salinity range – 8 to 48 ‰, but it cannot be assigned to freshwater or brackish-water inhabitants. When placed in a medium with salinity of lower than 8 ‰, the cells died soon, even if it was preceded by gradual acclimation. Salinity of the Black Sea water off the Crimean coast is 17–18 ‰. Under conditions of lower salinity, existence of the studied *Entomoneis* representative is hardly possible: according to the results of our experiments, auxospore formation is limited to a salinity range 18 to 36 ‰ (perhaps, the range is somewhat wider, taking into account the gradations adopted in the experiments). Sexual reproduction in this species, as well as in other diatoms studied, turned out to be more sensitive to environmental conditions (Davidovich & Davidovich, 2020). According to references in the literature (an extensive list is presented in AlgaeBase), *E. paludosa* should be considered as a cosmopolitan inhabiting water bodies with different salinity – from freshwater to typical marine ones. However, the question arises whether all those diatoms, that are found under completely different salinity conditions and are assigned to *E. paludosa*, in fact belong to this species. As is often in diatomology, the problem of species identification comes to the fore. Assuming that in all cases the species was identified correctly, another question arises whether it is possible to relate such a wide range of halotolerance to one species. Indeed, there are some species with a very high salinity tolerance. Specifically, *Nitzschia palea* (Kützing) W. Smith can develop at salinity 0 to 22 ‰ (Bagmet et al., 2017 ; Trobajo et al., 2011). *Tabularia tabulata* (C. A. Agardh) Snoeijs is capable of developing in a wider salinity range – 0.5 to 49 ‰ (possibly, even wider), with sexual reproduction occurring within a very wide range – 8 to 49 ‰ (Davidovich, 2017). As noted above, halotolerance range of the *Entomoneis* representative studied by us is relatively narrower. With regard to *E. paludosa* salinity tolerance, it is premature to draw any conclusions until reliable data on this species are obtained.

Of interest is the question of salinity effect on the size of initial cells resulting from sexual reproduction. The life cycle duration of clones of the new generation depends on the initial cell size. As established in experiments with the centric diatom *Coscinodiscus wailesii* Gran, smaller initial cells formed under conditions of higher salinity (Nagai & Imai, 1999). In *T. tabulata*, the size of initial cells did not depend on medium salinity (Davidovich, 2017). These examples show that the response of organisms to environmental salinity is species-specific. According to the data obtained, size of *Entomoneis* sp. initial cells tended to decrease with salinity increase. However, since the resulting initial cells differed slightly in size (by about 10–15 %), the potential duration of the existence of new generation clones will not differ noticeably.

Summarizing, *Entomoneis* sp. inhabiting the Black Sea can be assigned to euryhaline organisms with environmental salinity tolerance ranging from 8 to 48 ‰ for vegetative growth and from 18 to 36 ‰ (perhaps, a little wider) for sexual reproduction. Optimal salinity for vegetative growth is 27.4 ‰, and for sexual reproduction, it is 26.4 ‰, which significantly exceeds salinity of the Black Sea water in its habitats. A more detailed study of the Black Sea representative of the genus *Entomoneis* might provide grounds for describing a new species.

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REFERENCES

1. Davidovich O. I. Vliyanie solenosti na vegetativnyi rost i polovoe vosproizvedenie diatomovoi vodorosli *Tabularia tabulata* (C. A. Agardh) Snoeijs. In: *Aktual'nye problemy bioraznoobraziya i prirodopol'zovaniya* : materialy Vserossiiskoi nauchno-prakticheskoi konferentsii, Kerch, 26 September – 1 October, 2017. Simferopol : ARIAL, 2017, pp. 38–43. (in Russ.)
2. Karaeva N. I., Dzhafarova S. K. Experimental investigations of polygalobic *Bacillariophyta* in connection with medium salinity. *Al'gologiya*, 1993, vol. 3, no. 2, pp. 97–105. (in Russ.)
3. Kulikovskiy M. S., Glushchenko A. M., Genkal S. I., Kuznetsova I. V. *Opredelitel' diatomovykh vodoroslei Rossii.* Yaroslavl : Filigran', 2016, 804 p. (in Russ.)
4. Polyakova S. L., Davidovich O. I., Podunai Yu. A., Davidovich N. A. Modification of the ESAW culture medium used for cultivation of marine diatoms. *Morskoj biologicheskij zhurnal*, 2018, vol. 3, no. 2, pp. 73–78. (in Russ.). https://doi.org/10.21072/mbj.2018.03.2.06
5. Ryabushko L. I. *Microalgae of the Black Sea Benthos (Check-list, Synonyms, Comment)*. Sevastopol : EKOSI-Gidrofizika, 2006, 143 p. (in Russ.)
6. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway / Guiry M. D., Guiry G. M. (Eds) : [site], 2020. URL: http://www.algaebase.org [accessed: 27.05.2020].
7. Andersen R. A., Berges J. A., Harrison P. J., Watanabe M. M. Recipes for freshwater and seawater media. In: *Algal Culturing Techniques*. Amsterdam : Elsevier Academic Press, 2005, pp. 429–538.
8. Bagmet V. B., Abdullin Sh. R., Kuluev B. R., Davidovich O. I., Davidovich N. A. The effect of salinity on the reproduction rate of *Nitzschia palea* (Kützing) W. Smith (Bacillariophyta) clones. *Russian Journal of Ecology*, 2017, vol. 48, iss. 3, pp. 287–289. https://doi.org/10.1134/S1067413617030043
9. Brand L. E. The salinity tolerance of forty-six marine phytoplankton isolates. *Estuarine, Coastal and Shelf Science*, 1984, vol. 18, iss. 5, pp. 543–556. https://doi.org/10.1016/0272-7714(84)90089-1
10. Dalu T., Taylor J. C., Richoux N. B., Froneman W. A re-examination of the type material of *Entomoneis paludosa* (W. Smith) Reimer and its morphology and distribution in African waters. *Fottea*, 2015, vol. 15, iss. 1, pp. 1–25. https://doi.org/10.5507/fot.2015.002
11. Davidovich N. A., Davidovich O. I. Salinity optima for vegetative growth and sexual reproduction of the diatom *Toxarium undulatum*. *Morskoj biologicheskij zhurnal*, 2020, vol. 5, no. 1, pp. 20–28. https://doi.org/10.21072/mbj.2020.05.1.03
12. Liu B., Williams D. M., Ector L. *Entomoneis triundulata* sp. nov. (Bacillariophyta), a new freshwater diatom species from Dongting Lake, China. *Cryptogamie, Algologie*, 2018, vol. 39, iss. 2, pp. 239–253. https://doi.org/10.7872/crya/v39.iss2.2018.239
13. Mejdandžić M., Bosak S., Nakov T., Ruck E., Orlić S., Gligora Udovič M., Peharec Štefanić P., Špoljarić I., Mršić G., Ljubešić Z. Morphological diversity and phylogeny of the diatom genus *Entomoneis* (Bacillariophyta) in marine plankton: Six new species from the Adriatic Sea. *Journal of Phycology*, 2018, vol. 54, iss. 2, pp. 275–298. https://doi.org/10.1111/jpy.12622
14. Morant-Manceau A., Nhung Nguyen T. L., Pradier E., Tremblin G. Carbonic anhydrase

activity and photosynthesis in marine diatoms. *European Journal of Phycology*, 2007, vol. 42, iss. 3, pp. 263–270. https://doi.org/10.1080/09670260701425522

15. Nagai S., Imai I. The effect of salinity on the size of initial cells during vegetative cell enlargement of *Coscinodiscus wailesii* (Bacillariophyta) in culture. *Diatom Research*, 1999, vol. 14, iss. 2, pp. 337–342. https://doi.org/10.1080/0269249X.1999.9705475
16. Rech M., Mouget J.-L., Morant-Manceau A., Rosa P., Tremblin G. Long-term acclimation to UV radiation: Effects on growth, photosynthesis and carbonic anhydrase activity in marine diatoms. *Botanica Marina*, 2005, vol. 48, iss. 5, pp. 407–420. https://doi.org/10.1515/bot.2005.054
17. Round F. E., Crawford R. M., Mann D. G. *The Diatoms: Biology and Morphology of the Genera*. Cambridge : Cambridge University Press, 1990, 747 p. https://doi.org/10.1017/S0025315400059245
18. Ryabushko L. I., Lishaev D. N., Kovrigina N. K. Species diversity of epilithon diatoms and the quality of the waters of the Donuzlav Gulf ecosystem (Crimea, the Black Sea). *Diversity*, 2019, vol. 11, iss. 7, art. no. 114 (12 p.). https://doi.org/10.3390/d11070114
19. Trobajo R., Rovira L., Mann D. G., Cox E. J. Effects of salinity on growth and on valve morphology of five estuarine diatoms. *Phycological Research*, 2011, vol. 59, iss. 2, pp. 83–90. https://doi.org/10.1111/j.1440-1835.2010.00603.x
20. Weckstrom K., Juggins S. Coastal diatom–environment relationships from the Gulf of Finland, Baltic Sea. *Journal of Phycology*, 2006, vol. 42, iss. 1, pp. 21–35. https://doi.org/10.1111/j.1529-8817.2006.00166.x

ПРЕДЕЛЫ ГАЛОТОЛЕРАНТНОСТИ ЧЕРНОМОРСКОГО ПРЕДСТАВИТЕЛЯ РОДА *ENTOMONEIS* EHRENBERG, 1845 (BACILLARIOPHYTA)

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Род *Entomoneis* Ehrenberg, 1845 достаточно богат видами. Недооценённое разнообразие *Entomoneis* требует более глубокого морфологического и молекулярного исследования этого рода, а также изучения эколого-физиологических характеристик видов, в частности пределов толерантности к факторам среды. Учитывая распространение видов *Entomoneis* в водоёмах с различной солёностью, мы поставили задачу исследовать пределы толерантности и установить оптимальную солёность для вегетативного размножения и полового воспроизведения диатомовой водоросли *Entomoneis* sp. из Чёрного моря. В работе использованы оказавшиеся репродуктивно совместимыми клоновые культуры, которые были выделены из проб, отобранных у крымского и турецкого побережий Чёрного моря. Для клона *Entomoneis* sp. 7.0906-D получена нуклеотидная последовательность гена *rbc*L, которая представлена в базе данных GenBank под номером MT424817. Использованный в экспериментах вид хотя и напоминает по морфологическим критериям *E. paludosa*, но, по молекулярным данным, далеко отстоит от такового и по эколого-физиологическим характеристикам является морским видом. Согласно литературным материалам, *E. paludosa*, в отличие от изученного нами *Entomoneis* sp., обитает в солоноватых, слабосолёных и даже пресных водоёмах. Эксперименты по изучению пределов галотолерантности показали, что черноморские клоны *Entomoneis* sp. жизнеспособны в диапазоне, охватывающем как минимум 40 ‰ (от 8 до 48 ‰). Диапазон солёности среды, в котором *Entomoneis* sp. способен к половому воспроизведению, значительно у́же и находится в пределах от 18 до 36 ‰. Определены оптимальные значения солёности для вегетативного роста и для полового воспроизведения (27,4 и 26,4 ‰ соответственно), оказавшиеся в обоих случаях выше тех значений, при которых вид обитает в природе. У *Entomoneis* sp. по мере увеличения солёности среды отмечена тенденция к уменьшению размеров инициальных клеток, образующихся в результате полового воспроизведения.

Ключевые слова: диатомовые водоросли, половое воспроизведение, вегетативное размножение, солёность, *Entomoneis* sp.