

RESEARCH ARTICLE

**Diatoms isolated from the marine mucilage in the Sea of Marmara in 2021**

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**Abstract**

This study presents five new records of diatoms based on light and electron microscopy results from the surface water of the Sea of Marmara coast during a large-scale mucilage event in 2021. Three of these diatoms are small nanoplanktic; *Cyclotella cryptica*, *Nitzschia amabilis*, *Thalassiosira pseudonana*, and the two of them are larger diatoms; *Conticribra weissflogii* and *Entomoneis cf. pusilla*. In addition, a species frequently seen in mucilage samples in the Adriatic Sea and the the Sea of Marmara, *Cylindrotheca closterium*, was also isolated and described. The chloroplast rbcL region, SSU, and 18S rDNA of *C. closterium*, *C. cryptica* and *N. amabilis* were amplified using primers in other previous studies. The rbcL, SSU, and 18S rDNA partial sequences were submitted to the NCBI database. Molecular phylogeny (based on 18S, rbcL, and SSU V4 DNA sequences) confirmed that the analysed species were those identified by SEM images.

**Keywords:** Centrales, pennales, nanoplankton, SEM, DNA sequences

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**Introduction**

Mucilage is an aggregated mass of gelatinous organic materials produced by marine organisms such as phytoplankton through the exudation of

photosynthetically derived carbohydrates under stressful conditions (Fonda Umani *et al.* 2005; Degobbi *et al.* 1995), which is also coupled with poor prokaryotic hydrolysis of exopolymers via extracellular enzymes (Muller-Niklas *et al.* 1994; Obernosterer and Herndl 1995; Danovaro *et al.* 2009). The size of aggregates may range from a few millimeters to several meters (Simon *et al.* 2002), and the mucilage may cover an area of over 10,000 km<sup>2</sup> (Malej *et al.* 2003) which negatively affects fisheries, tourism, and maritime operations (Uflaz *et al.* 2021) and benthos (Topçu and Öztürk 2021). Mucilage events have persisted for several months in the northern Adriatic Sea (Vollenweider and Rinaldi 1995; Malej *et al.* 2003). Large-scale mucilage events have occurred in the coastal areas of various seas in history (Lancelot 1995; Degobbi *et al.* 1999; McKenzie *et al.* 2002; Malej *et al.* 2003; Cozzi *et al.* 2004; Najdek *et al.* 2005; Schiaparelli *et al.* 2007; Fukao *et al.* 2009; Fernandes and Frassão-Santos 2011). The frequency of these events increased in the 1980s and 1990s in the Adriatic Sea, probably due to increased nutrient loads and changes in the ratios of limiting nutrients in coastal areas coupled with increased temperatures and irradiances (Degobbi *et al.* 1999).

The Sea of Marmara is a transit basin connecting the Black Sea to the Mediterranean with a surface area of 11,500 km<sup>2</sup>. It has a complex hydrological system with a brackish surface layer (22 ‰ salinity at 0-25 m) originating from the Black Sea and deep water with higher salinity (~38 ‰) coming from the Mediterranean (Unluata *et al.* 1990; Besiktepe *et al.* 1994). Having the characteristics of the Black Sea, the surface layer has relatively low nutrient concentrations associated with photosynthetic activity (Baştürk *et al.* 1990), and primary production is always higher in the upper layer than in the light-limited nutrient-rich intermediate depths (Polat *et al.* 1998).

The Sea of Marmara has been exposed to municipal and industrial pollutants throughout its drainage basins, as well as nutrients and organic inputs from the Black Sea since the 1970s (Orhon *et al.* 1994). Fishermen have observed small-scale mucilage events in this sea since the 1990's. However, except an underwater camera recording of a massive mucilage event in 1992 by a sportsman (Tüfekçi *et al.* 2010), there is no record of such events until mid-autumn 2007 and 2008 (Aktan *et al.* 2008; Tüfekçi *et al.* 2010; Balkis *et al.* 2010, 2013; İşinibilir Okyar *et al.* 2015). Following these events in 2007-2008, the mucilage phenomenon reappeared in the Sea of Marmara in November 2020 and intensified from April-June 2021, which resulted in frightening consequences such as mass mortalities of invertebrates (Karadurmuş and Sarı 2022). The thick density of the mucilage layer, especially in Kocaeli, persisted for several months despite intense cleaning efforts.

Diatom and dinoflagellate increase in abundance was associated with mucilage formation in 2007 and 2008 (Aktan *et al.* 2008; Balkis *et al.* 2010) since these microalgae secrete extracellular polysaccharides (Degobbi *et al.* 1995; Rinaldi

*et al.* 1995). Bacteria also exude similar polysaccharides (Rinaldi *et al.* 1995; Azam and Long 2001). If exudation of bacterial polysaccharides exceeds mineralization of organic matter, these secretions also contribute to mucilage formation. Dominant phytoplankton species of the mucilage phenomenon during 2007 and 2008 were diatoms *Proboscia alata*, *Rhizosolenia* sp., *Pseudosolenia calcar-avis*, *Thalassiosira rotula*, *Ditylum brightwellii*, *Coscinodiscus* spp., *Leptocylindrus minimus*, *Skeletonema costatum*, *Chaetoceros* spp., *Cerataulina pelagica*, *Cylindrotheca closterium*, *Pseudonitzschia* cf. *seriata*, and the dinoflagellate *Gonyaulax fragilis* (Aktan *et al.* 2008; Tüfekçi *et al.* 2010; Balkis-Özdelice *et al.* 2021). *Cylindrotheca closterium* and *Gonyaulax fragilis* were also among the dominant species of mucilage events in the northern Adriatic Sea (Pompei *et al.* 2003; Najdek *et al.* 2005; Pistocchi *et al.* 2005). The nitrogen-to-phosphorus ratio ranged from 0.1 to 11.3, indicating nitrogen limitation of the mucilage-containing seawater in 2008 (Balkis *et al.* 2010).

The species dominating in the early development stage of the mucilage in April 2021 were similar to the composition observed in the previous mucilage event in 2007-2008, that is, *Cerataulina pelagica*, *Cylindrotheca closterium*, *Pseudonitzschia* sp., *Skeletonema costatum*, *Thalassiosira rotula*, *Alexandrium tamarense*, *Dinophysis acuminata*, *Dinophysis caudata*, *Dinophysis fortii*, *Gonyaulax fragilis*, *G. spinifera*, *Gymnodinium* sp., *Gyrodinium* sp., and *Prorocentrum micans* (Ergul *et al.* 2021).

In this study, phytoplankton samples collected from the surface layer of the sea, including mucilage aggregates, were cultured and isolated in the laboratory and investigated with scanning electron microscopy and molecular analyses. The study aimed to identify diatom species correctly, which are difficult to determine based on light microscopy in the fixed samples due to their small size and delicate ornamentation.

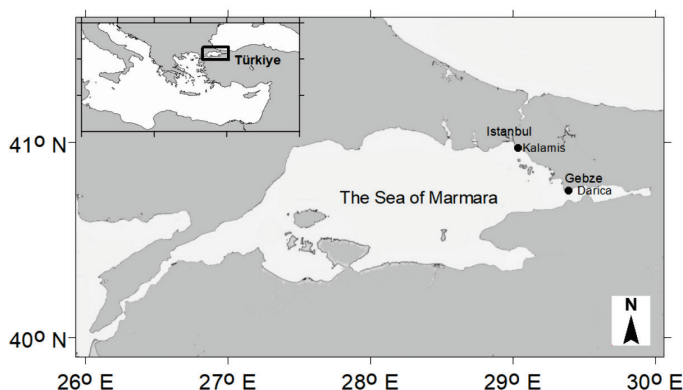
## **Materials and Methods**

### *Sampling and cultures*

Seawater samples were taken from the coastal surface water of the Sea of Marmara on 11 June 2021 from Kalamis Marina and on 22 June 2021 from Darica, Gebze (Table 1, Figure 1). The temperature and salinity of the sample area seawater were 23.2°C and 23.7 ‰ (Hanna HI98319 salinity tester), respectively. Samples were taken into polyethylene bottles from the coast and transferred into 100 ml Erlenmeyer flasks by adding nutrients (F/20 medium) to increase cell numbers. Then, cells were isolated with thinned glass Pasteur pipettes. Cultures were kept in a climate chamber within an F/2 medium under a 12:12h light-dark cycle, 20°C temperature, and ~10-30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  irradiance.

**Table 1.** Location information of the sampling area for phytoplankton species on 11 and 22 June 2021

| Sample no | Latitude | Longitude | Field          | Date       |
|-----------|----------|-----------|----------------|------------|
| 1         | 40° 75'  | 29° 39'   | Darıca, Gebze  | 22.06.2021 |
| 2         | 40° 58'  | 29° 02'   | Kalamış Marina | 11.06.2021 |



**Figure 1.** Sampling areas for phytoplankton species on 11 and 22 June 2021

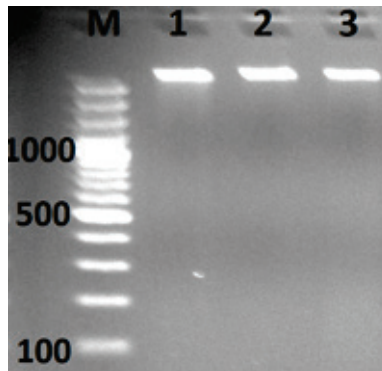
#### *Electron microscopy*

For diatoms, samples were washed with 100 ml distilled water, filtered through 0.45  $\mu\text{m}$  cellulose acetate (CA) filters, and resuspended with 5 ml distilled water. After washing, concentrated sulphuric acid and nitric acid (1:1) were added to cells and boiled in a water bath for 30 minutes. Cells were rewashed with 100 ml distilled water and filtered on 0.45  $\mu\text{m}$  CA filters. Then, filters were coated with iridium using the Leica EM ACE600 Sputter Coater instrument and examined with a Zeiss GeminiSEM 500 field emission scanning electron microscope (SEM).

#### *Genomic DNA isolation from microalgae*

In the study, live cultures were studied for gDNA isolation. Care was taken to ensure that algal samples were axenic. Since three species could be isolated as single cell cultures during the molecular studies, genomic DNA was isolated from the three algal samples using QIAamp Mini DNA Kit (Qiagen) following the protocol recommended by the company.

Two methods determined the purity of the isolated DNA: spectrophotometric (NanoDrop ND 1000) and electrophoresis. The quantity and quality of the isolated DNAs were measured in the Nanodrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA), and its integrity was checked with a 1.5% agarose gel stained with ethidium bromide. The 260/280 ratio was determined to be between 1.8 and 2.0, indicating high quality (Figure 2). Isolated stock samples were kept at  $-80^{\circ}\text{C}$ .



**Figure 2.** 1.5% agarose gel electrophoresis results of gDNAs isolated from algae samples (M: DNA Ladder; 1: Isolate MAR1 (*Cylindrotheca closterium*); 2: Isolate MAR2 (*Nitzschia amabilis*); 3: Isolate MAR3 (*Cyclotella cryptica*)

#### *PCR analysis and agarose gel electrophoresis*

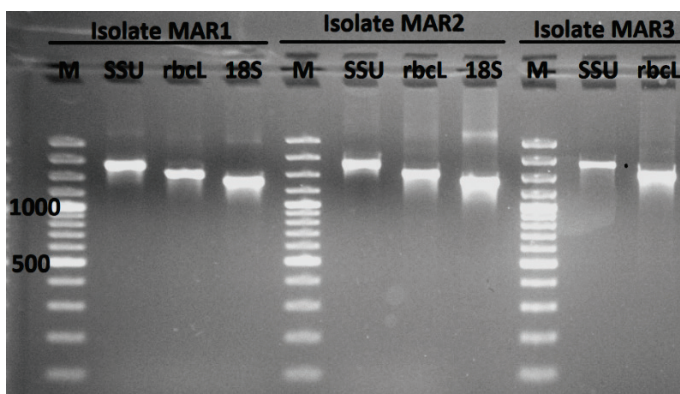
Two nuclear 18S rDNA markers, 18S and SSU, and the chloroplast *rbcL* gene were used to represent the three eukaryotic genomes in phototrophs. In PCR analysis, the method of Gastineau *et al.* (2012, 2016) were applied by modifying the protocol, and the *rbcL*, SSU and 18S primers (Table 2) determined by the researchers were used. Gradient PCR was performed using a thermal cycler (Applied Biosystems-Perkin Elmer instrument) to determine the optimum temperatures at which the primers bind to DNA to obtain the amplification product from the primers. PCR procedures were carried out using a Solis BioDyne 5x FIREPol® Master Mix kit. For a final volume of 20 µl, mixtures were done with 5x FIREPol® Master Mix (1x), 0.2 µM, DNA template (5 ng/µl), 0.2 µM of primers, and finally, DNase and RNase free water was added to make up to 20 µl. For PCR, thermal cycler conditions were as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min. These cycles were followed by a final extension step at 72°C for 10 min. The best DNA binding temperature of each primer applied to gradient PCR was determined according to the clearest band yield on 2% agarose gel. The gel was visualized on the imaging device (Bio-Rad Laboratories, Segrate, Milan, Italy) to detect the presence of amplification products.

**Table 2.** Primers used in PCR analysis (F: forward/sense, R: reverse/antisense)

| Target   | Name   | Sequence(5' - 3')     | Definition             | Product size (bp) |
|----------|--------|-----------------------|------------------------|-------------------|
| rbcL     | rbcL-F | AAGGAGAAATHAATGTCT    | rbcL-F/sense primer    | 1000-             |
|          | rbcL-R | AARCAACCTTGTGTAAGTCTC | rbcL-R/antisens primer | 1200              |
| SSU      | SSU-F  | AACCTGGTTGATCCTGCCAG  | SSU-F/sense primer     | 1000-             |
|          | SSU-R  | CCTTCTGCAGGTTACCTAC   | SSU-R/antisens primer  | 1200              |
| 18S rRNA | 18S-F  | CTGCCCTATCAGCTTGGATGG | 18S-F/sense primer     | 1000-             |
|          | 18S-R  | CCATTCAATCGGTAGGTGCG  | 18S-R/antisens primer  | 1200              |

### *Sequencing, sequence, and phylogenetic analysis*

The amplified and expected-size PCR products were purified from the gel using the QIAquick Gel Extraction Kit (Qiagen) according to the protocol recommended by the company (Figure 3). Care was taken to ensure that the samples to be transmitted to the sequence gave clean and single bands. Extracted DNA bands from an agarose gel were sequenced using the same primers used for PCR amplification. The sequencing service was commercially provided by Medsantek, Ankara, Türkiye (<https://medsantek.com.tr/>). The Pairwise Sequence Alignment (Nucleotide) Tool developed by EMBL-EBI ([https://www.ebi.ac.uk/Tools/psa/emboss\\_needle/](https://www.ebi.ac.uk/Tools/psa/emboss_needle/)) was used to match forward and reverse complement sequences after personally reviewing the raw DNA sequence data. Using the BLAST (Basic Local Alignment Search Tool; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) search program, the consensus sequence was searched for similar sequences in GenBank at the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>), Bethesda, USA (Altschul *et al.* 1990). MEGA7 (<https://www.megasoftware.net/>) was used to conduct the phylogenetic analysis (Kumar *et al.* 2016). Aligned 18S rDNA, SSU rDNA, and *rbcL* sequences were evaluated with bootstrap analysis (1000 replicates) (Felsenstein 1985).



**Figure 3.** Images of 1.5% agarose gel of the samples purified from the gel and sent to Medsantek <http://www.medsantek.com.tr/> for sequence analysis

### *NCBI GenBank data transfer*

The identified DNA sequences were submitted to the NCBI GenBank database using the submission portal (<https://submit.ncbi.nlm.nih.gov/subs/genbank/>), and an accession number was obtained for each submitted sequence. The 18S sequences of the isolates MAR1, MAR2 and MAR3 were submitted to GenBank under accession numbers ON942237, ON942239, and ON942241, respectively. The sequences of *rbcL* of the isolates MAR1, MAR2, and MAR3 were deposited in the GenBank database with the following accession numbers: ON959499, ON959500, and ON959501.

## Results and Discussion

### SEM Analysis

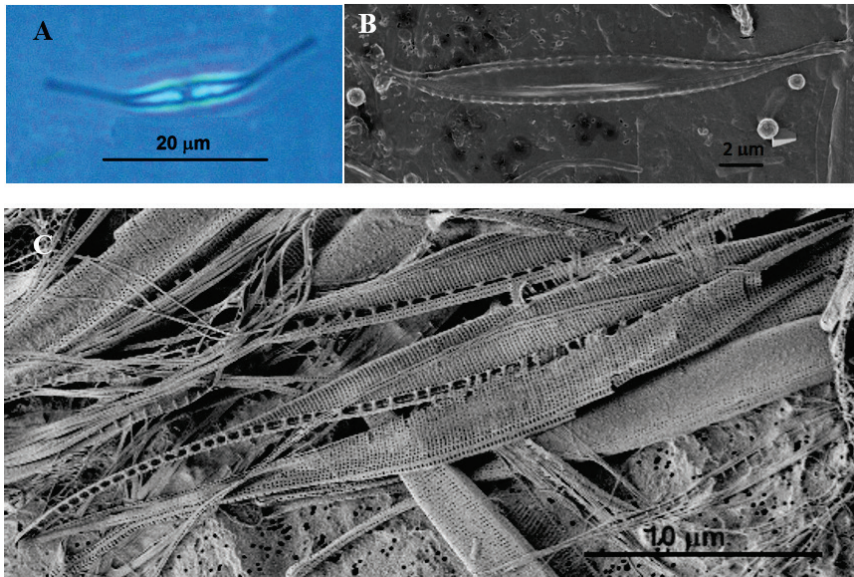
Descriptions of a total of six diatom species isolated from the mucilage samples taken from the Sea of Marmara on June 2021 were presented in this study. Five of these species; *Cyclotella cryptica*, *Nitzschia amabilis*, *Thalassiosira pseudonana*, *Conticribra weissflogii* and *Entomoneis* cf. *pusilla*, are new records for this sea.

### *Cylindrotheca closterium* (Ehrenberg) Reimann & Lewin

Synonyms: *Nitzschia closterium* (Ehrenberg) W. Sm., *Nitzschiella closterium* (Ehrenb.) Rabenh

Basionym: *Ceratoneis closterium* Ehrenberg

Description: Cells are unicellular, raphide-shaped with a swollen centre, tapering to very long projection (Figures 4A-C). They are twisted along the apical axis and weakly silicified (Figure 4A, B). Each cell has two equal chloroplasts (Figure 4A). There are 14 fibulae in 10  $\mu\text{m}$  (Figure 4C) (Jahn and Kusber 2005; Hilaluddin *et al.* 2011). The length and width are 29-35  $\mu\text{m}$  and 1.4-3.2  $\mu\text{m}$  (20 cells), respectively.



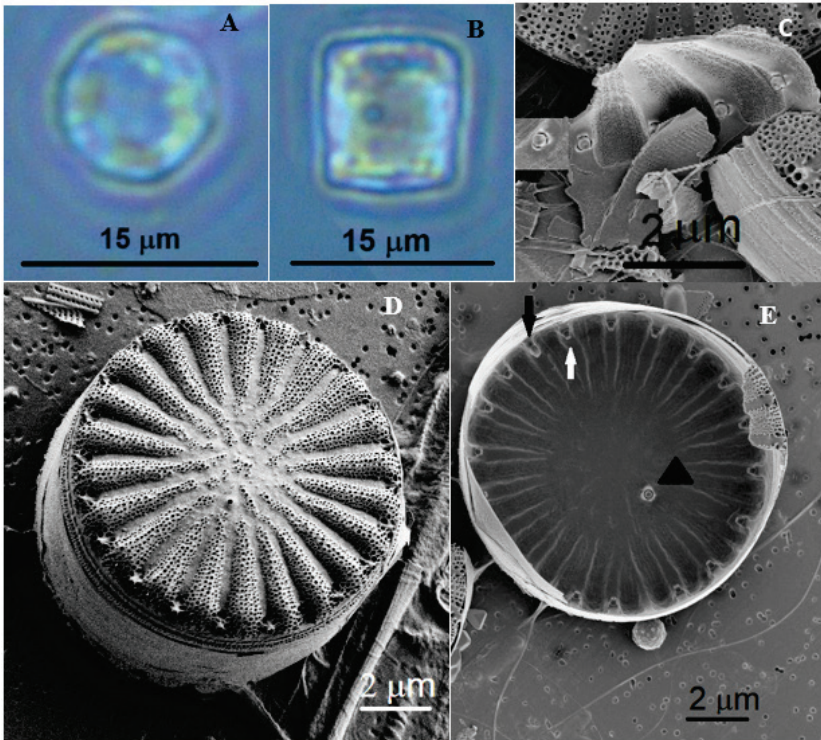
**Figure 4.** Light microscope (A) and SEM (B-C) pictures of *Cylindrotheca closterium*. (A) a live cell, (B) formaldehyde-fixed material, (C) acid-cleaned cells.

*Cylindrotheca closterium* is a widespread species in coastal and estuarine waters (Round *et al.* 1990; Vanormelingen *et al.* 2013, Table 3). They are mostly benthic but sometimes also planktic (Vanormelingen *et al.* 2013).



*Cyclotella cryptica* Reimann, Lewin & Guillard

Description: Valves are drum-shaped, and the central area is not isolated from the marginal radiated and raised striae, separated by costae (Figures 5A-E). There are 9-10 striae per 10  $\mu\text{m}$ , and each stria is formed by 9-11 rows of areolae which decrease in number towards the centre of the valve (Figures 5C, D) (Hoops and Floyd 1979; Cavalcante *et al.* 2013; Hevia-Orube *et al.* 2015). Marginal fultoportulae are present on each costa, and they are externally surrounded by a thick elevated silica wall and by three satellite pores internally (Figure 5C). One-two central fultoportulae is positioned (Figure 5E). In the internal view, the rimoportula is located near the valve margin and appears as a short stalk (Figure 5E). The length and diameter of the cells are 3.5-12.5  $\mu\text{m}$  and 7-14  $\mu\text{m}$  (20 cells), respectively. Each cell has numerous chloroplasts.



**Figure 5.** Light (A, B) and scanning electron microscope (C, D, E) images of *Cyclotella cryptica* (A) valve view (B) girdle view (C) internal valve view showing marginal fultoportulae with three satellite pores (D) valve-girdle view displaying raised striae and marginal fultoportulae (E) internal view, black arrow shows rimoportula, white arrow marginal fultoportulae, and the arrowhead shows central fultoportula.



This planktonic species was found in brackish waters and marine environments (Bilous *et al.* 2021; Table 3). Morphologically, this species is similar to *C. meneghiniana* (Hevia-Orube *et al.* 2015). It was shown that *C. cryptica* displayed morphologically similar features to *C. meneghiniana* when growing at salinities less than 1.4 ‰ but exhibited morphological properties typical of *C. cryptica* at salinities above 4.3 ‰ (Schultz 1971). Although these two species were suggested as conspecific by Houk *et al.* (2010), the study of Beszteri *et al.* (2007) reported that strains of *C. cryptica* were genetically homogeneous, and they diverged from *C. meneghiniana* genetically.

*Cyclotella cryptica* grows both autotrophically using NO<sub>3</sub> as the source of nitrogen and as heterotrophically by using glucose in the bottom water or mud (Pahl *et al.* 2010; Roberts *et al.* 2020; Bilous *et al.* 2021). It also produces high lipids, making it an attractive species for use as a resource in biofuel production studies and nutraceuticals (Slocombe *et al.* 2015; Roberts *et al.* 2020). In the laboratory, the viscosity of the *C. cryptica* culture was higher than the *N. amabilis*, *T. pseudonana*, *C. closterium* and *E. cf. pusilla* cultures under the same growth conditions in the present study. This species was reported to produce β-Chitin fibrils (Herth and Barthlott 1979), which are most plentiful polysaccharides containing nitrogen in the oceans (Cheng *et al.* 2023).

#### *Conticribra weissflogii* (Grunow) StachuraSuchoples & Williams

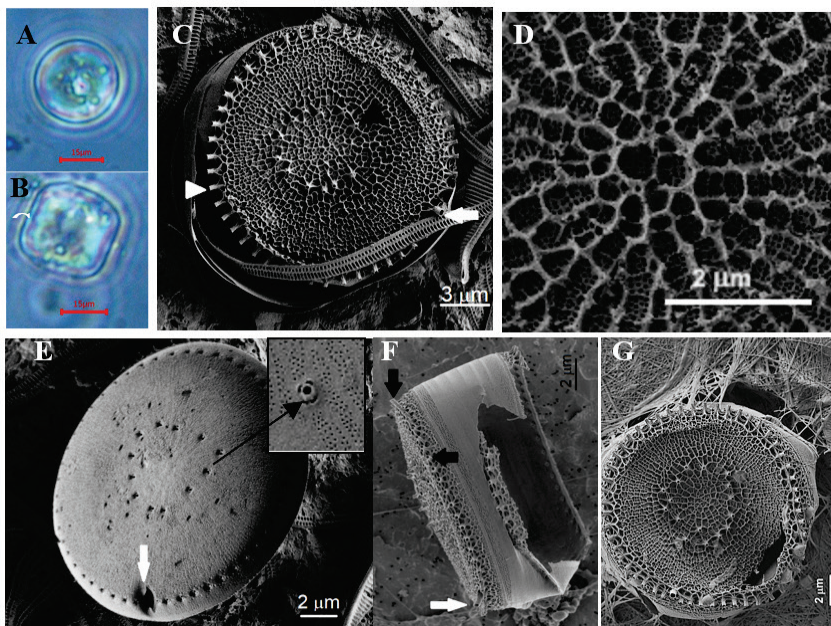
Basionym: *Micropodiscus weissflogii* Grunow in Van Heurck

Synonyms: *Eupodiscus weissflogii* Grunow in Van Heurck, *Micropodiscus weissflogii* Grunow in Van Heurck, *Eupodiscus weissflogii* (Grunow in Van Heurck) De Toni, *Thalassiosira fluviatilis* Hustedt, *Thalassiosira weissflogii* (Grunow) G. Fryxell and Hasle

Description: Valves are round with a flat surface (Figures 6A-H). Irregularly shaped areola are radially striated (Figure 6C). Areolae are loculate and open to the outside by irregular foramina (Figures 6C and D). Internally, continuous cribra occlude the areolae (Figure 6E). Pervalvar axis is generally shorter than the diameter (Figure 6F). There is one ring of marginal fulcportulae: 13-15 in 10 μm. One prominent rimoportula is evident on the margin of the valve (Figures 6C, F) (Hevia-Orube *et al.* 2015; Stachura-Suchoples and Williams 2009; Vona *et al.* 2018). Ten to fifteen central fulcportulae are arranged in a circle or randomly near the centre (Figures 6C, E). The cribra is composed of circular and linearly arranged pores. Internally, the cribra are bi- to multiseriate and extends towards the mantle (Figure 6E). Marginal and central fulcportulae have four satellite pores (Figures 6E). β-Chitin fibrils produced by mainly marginal fulcportulae were observed in the samples without acid cleaning (Figure 6G), (Herth and Barthlott 1979; Jeffries *et al.* 2015).

This species inhabits freshwater, brackish water and marine water (Roberts *et al.* 2007; Stachura-Suchoples and Williams 2009, Table 3). *Conticribra weissflogii*

is a species complex based on rDNA with divergent populations in the east and the western Atlantic Ocean, California, Hawaii, and Indonesia (Sorhannus *et al.* 2010). It was reported to use the C<sub>4</sub> pathway of photosynthetic carbon assimilation to increase its photosynthetic efficiency (Roberts *et al.* 2007). *Conticribra weissflogii* was suggested to induce sexual reproduction when ammonium was supplied (Moore *et al.* 2017). However, images shown by Moore *et al.* (2017) do not correspond to auxospores but to dead cells. In the present study, a dense mucilaginous layer was present at the top of the culture flasks of this species. Viscosity of the cultures was considerably high, which indicates exudation of exopolysaccharides.  $\beta$ -Chitin fibrils observed in the SEM images of this species have already been polysaccharides.



**Figure 6.** Light and scanning electron microscope images of *Conticribra weissflogii* (A-G). (A) valve view and (B) girdle view with light microscopy, (C) external valve view, SEM, white arrow shows the rimoportula, black arrowhead show central fultoportulae, white arrowhead shows marginal fultoportulae, (D) loculate areolae on the external face of valve, (E) internal view, white arrow shows the rimoportula, continuous cribra on the valve face obscure areolae, marginal and central fultoportulae are also seen, black arrow shows one of the central fultoportula having four satellite pores (F) girdle view, white arrow shows the rimoportula, black arrows show marginal fultoportulae, (G) a dense layer of  $\beta$ -Chitin fibrils originating mainly from marginal fultoportulae.

**Table 3.** Geographic distribution of phytoplankton species encountered in the Sea of Marmara during mucilage event in June 2021

|                                 | Marmara Medit. | Black Sea | Atlantic | Pacific | Baltic | Bug River, Ukraine | Rivers, Türkiye |   |
|---------------------------------|----------------|-----------|----------|---------|--------|--------------------|-----------------|---|
| <b>Centrales</b>                |                |           |          |         |        |                    |                 |   |
| <i>Conticribra weissflogii</i>  | new            | -         | -        | 1       | 2      | 3                  | 4               | 5 |
| <i>Cyclotella cryptica</i>      | new            | 6         | -        | 7, 8    | 9      | 10                 | 4               | 5 |
| <i>Thalassiosira pseudonana</i> | new            | 11        | 12       | 13      | 14     | 3                  | -               | - |
| <b>Pennales</b>                 |                |           |          |         |        |                    |                 |   |
| <i>Cylindrotheca closterium</i> | 15             | 16        | 12       | 17      | 18     | -                  | -               | - |
| <i>Entomoneis cf. pusilla</i>   | new            | 19        | -        | -       | -      | -                  | -               | - |
| <i>Nitzschia amabilis</i>       | new            | 20        | 21, 22   | 24      | 23     | -                  | -               | - |

1. Hendey (1954), 2. Berg *et al.* (2017), 3. Hällfors (2004), 4. Bilous *et al.* (2021), 5. Solak *et al.* (2018), 6. Ignatiades *et al.* (1983), 7. Cavalcante *et al.* (2013), 8. Hevia-Orube *et al.* (2015), 9. Liu (2008), 10. Plinski and Witkowski (2020), 11. Urbani *et al.* (2005), 12. Zotov (2018), 13. Wolnik and Carter (2014), 14. Aké-Castillo *et al.* (1999), 15. Deniz and Taş (2009), 16. Güreşen and Aktan Turan (2014), 17. Ojeda *et al.* (2005), 18. Gárate-Lizárraga (2014), 19. Mejdandzic *et al.* (2018), 20. Kaleli *et al.* (2020), 21. Kaleli *et al.* (2017), 22. Dirican *et al.* (2022), 23. Suzuki *et al.* (2009), 24. Hustedt (1939).

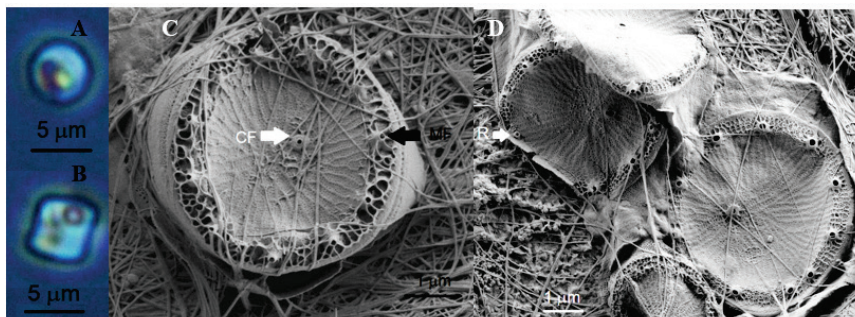
*Thalassiosira pseudonana* Hasle & Heimdal

Synonym: *Cyclotella nana* Hustedt

Description: Valves are circular, with a diameter ranging from 4 to 7  $\mu\text{m}$  (Figures A-D). Frustules are weakly silicified. Cells have irregularly arranged radial ribs and irregular biseriolate poroid areolae along the ribs. There is a single central fultoportula near the centre of the valve body (Figures 7C and D). Around 11 marginal fultoportulae are located at the periphery. The rimoportula is represented by a tubular aperture extending from the margin of the valve (Figure 7C, D) (Guillard and Ryther 1962; Hasle and Heimdal 1970; Lowe and Busch 1975; Hevia-Orube *et al.* 2015). The girdle region is extended and not ornamented (Figure 7D).

*Thalassiosira pseudonana* is a marine cosmopolite species (Table 3). It is the first eukaryotic phytoplankton species whose whole genome sequencing was performed. It has a small genome at 34 megabase pairs. It was induced to reproduce sexually by sustaining with ammonium at the stationary phase (Moore *et al.* 2017), while the sexual stage has never been observed in this species before (Chepurnov *et al.* 2008). This species was also reported to produce  $\beta$ -Chitin fibrils (Herth and Barthlott 1979). Chitin fibrils are seen on SEM images (Figures 7C and D). However, majority of these fibrils may belong to *Conticribra weissflogii* since these two species were mixed in the cultures

during SEM analysis. After the isolation of these two species, the viscosity of *C. weissflogii* culture was higher than of *T. pseudonana*.



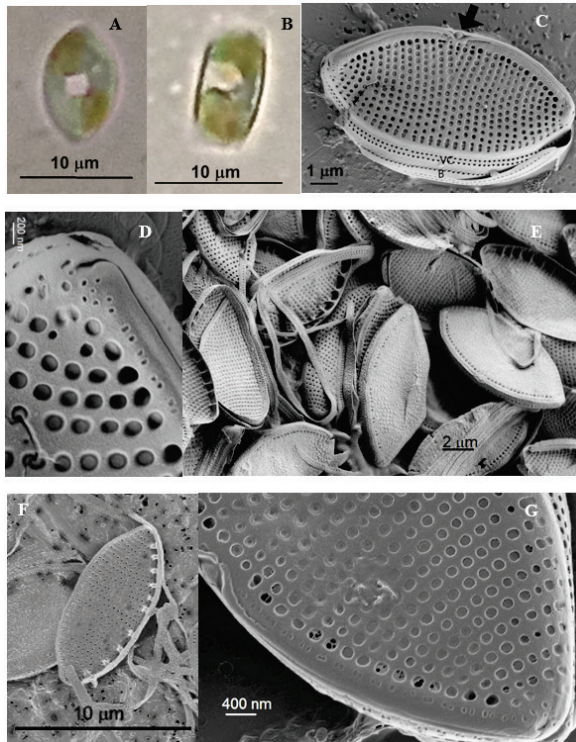
**Figure 7.** *Thalassiosira pseudonana* (A) light microscope image from valve view (B) light microscope image from girdle view (C) valve view showing a single central fultoportula (CF) and marginal fultoportulae (MF) (D) cells from valve and girdle view showing the rimoportula (R).

#### *Nitzschia amabilis* H. Suzuki

Synonym: *Nitzschia laevis* Hustedt

Description: Valves are flat, elliptic to slightly panduriform with apiculate apices (Figures 8A-G). Cells are rectangular in girdle view (Figure 8B). The length and width are 9-10.5  $\mu\text{m}$  and 4-5  $\mu\text{m}$ . There are two chloroplasts at each end. The raphe is eccentric, and in the external view, the terminal fissure is curved toward the valve face in the apex (Figures 8C and D). Externally, the central nodule has a bumpy thickening (Figure 8C). Areolae are occluded by the hymen with perforations. There are 10-14 fibulae per 10  $\mu\text{m}$ , and the median two fibulae are relatively distant. Valvocopulae and girdle bands are distinguishable (Lee *et al.* 1989; Suzuki *et al.* 2009, 2010; Kaleli *et al.* 2020; Dirican *et al.* 2022).

This marine species was previously found in different geographic regions (Table 3). *Nitzschia amabilis* was one of the endosymbiont diatoms of foraminifera harvested from the Red Sea, Mambassa Harbour, Kenya, Indian Ocean, Hawaii, and the Great Barrier Reef, Australia (Lee *et al.* 1989). Based on LSU rRNA, a species found in Malaysian waters, *Nitzschia dentatum* was grouped in the same clade with *N. amabilis* and *N. navis-varingica* isolated from similar locations (Suriyanti and Usup 2017).



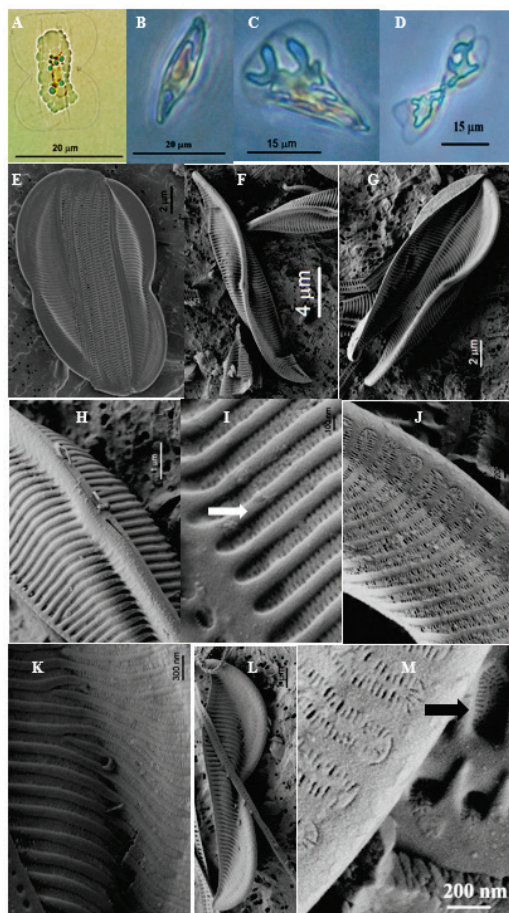
**Figure 8.** Light microscope (A, B) and SEM images (C-G) of *Nitzschia amabilis*. (A) valve view (B) girdle view (C) external valve view showing bunchy thickening of the central nodule, valvocopula (VC), and girdle bands (B) (D) terminal fissure in the apex, (E) internal and external views (F) internal view showing fibulae (G) proximal raphe endings and areolae with hymen.

*Entomoneis cf. pusilla* Bosak & Mejdandzic

Cells have a panduriform shape in girdle view with a significant indentation at half of the frustule length and are frequently twisted around the apical axis (Figures 9A-E). Cell length and width range from 15-30  $\mu\text{m}$  and 8-27  $\mu\text{m}$ . Valves are lanceolate (Figures 9B and F). There is one multi-lobbed chloroplast in each cell (Figures 9A-D). The raphe is sigmoid and situated on an elevated keel (Figures 9F-H). There are dense biseriate transapical striae on the external valve surface (Figures 9I and J). The winged keel is differentiated from the rest of the valve with a straight to arcuate junction line (Figures 7E, 9E, G, K and I). Virgae are straight, slightly elevated from the valve surface, simple and parallel to striae (Figures 9H and I), extending from the valve margin to the keel, and sometimes bifurcated near the junction line and wing (Figure 9K). Basal fibulae are on each keel virga along the valve length, excluding the central area, with 4-5 basal fibulae per 1  $\mu\text{m}$  (Figure 9G). Valve striae 36-43 in 10  $\mu\text{m}$  (Figure 9I).



Wing striae 39-40 in 10  $\mu\text{m}$  (Figure 9J). The hymen is not apparent within the oblong striae of valves. Striae in the valvocopulae are teardrop-shaped and occluded by a hymen with both roundish and rectangular perforations (Figure 9M). The density of perforation is higher in striae on the keel than on the valve body similar to mentioned in Mejdandžić *et al.* (2018). There were 33 areolae in 1  $\mu\text{m}$  near the keel margin and 23-27 areolae in 1  $\mu\text{m}$  near the valve body in this study. However, these numbers are lower than numbers (64-75/49-50 for keel margin/valve margin) reported by Mejdandžić *et al.* (2018).



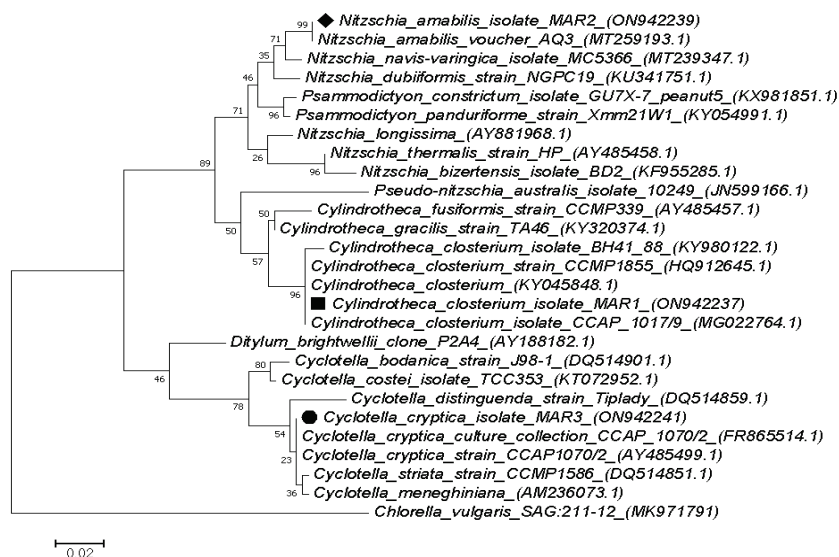
**Figure 9.** Light microscopy (A-D) and SEM images (E-M) of *Entomoneis* cf. *pusilla*. (A) girdle view, (B) valve view, (C) a twisted cell, (D) cells from different views, (E) SEM girdle view, (F) SEM valve view, (G) basal fibulae on the virgae of the keel, (H) proximal raphe endings, (I) biseriate striae near the girdle bands and elevated virga (white arrow) (J) striae on the wing and junction line, (K) bifurcating virgae and the junction line, (L) wings and junction line, (M) teardrop shape areolae (black arrow) in the valvocopula and copula.



The species was first described from the samples collected from the southern Adriatic Sea (Mejdandžić *et al.* 2018). The diversity of this genus was reported to be underappreciated in planktonic habitats (Mejdandžić *et al.* 2018).

### Molecular Analysis

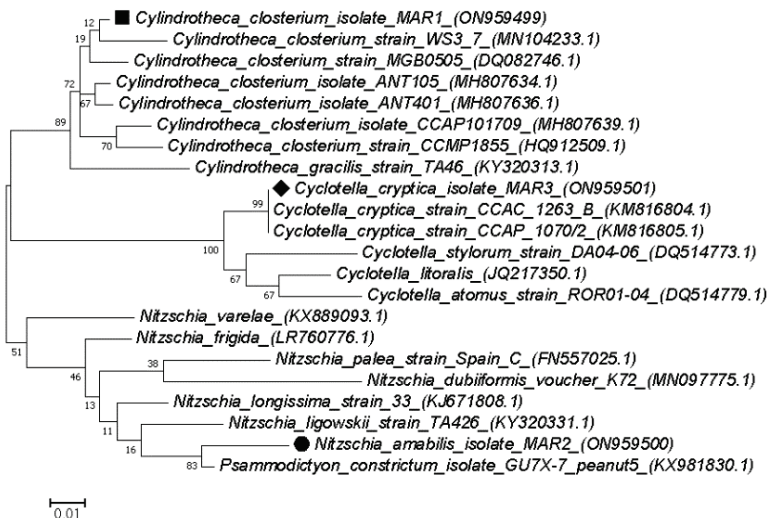
The 18S rRNA and *rbcL* were successfully amplified from gDNA using the primer pairs (18SF/18SR, SSUF/SSUR) and *rbcLF/rbcLR*. A part of the eukaryotic ribosomal small subunit, 18S rRNA is an SSU rRNA. One of the most often utilized genes in phylogenetic analyses is the small subunit (SSU, the 40S) 18S rRNA gene, which is also a crucial marker for random target PCR in environmental diversification detection (Meyer *et al.* 2010). Recently, one of the most used types of genetic markers for molecular investigations has been the chloroplast *rbcL* nucleotide sequence (Meier *et al.* 2008).



**Figure 10.** Neighbor-joining tree based on partial sequences of the small ribosomal subunit RNA of the isolates for phylogenetic inference. The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Therefore, 18S rRNA and *rbcL* genes were preferred for phylogenetic analysis of isolated algal species. The SSU, 18S, and *rbcL* sequences of mucilage-isolated algae were compared to the small subunit rRNA (SSU and 18S) and *rbcL* sequencing identified in the GenBank database and BLAST. Species detected as a result of SEM images were confirmed by BLAST and phylogenetic analysis. As a result of the comparative analysis of the 18S, SSU, and *rbcL* gene sequences of the MAR 1 isolate, it was determined that this

isolate was *C. closterium*. Likewise, as a result of the comparative analysis of the 18S and *rbcL* sequences of the MAR 3 isolate, this isolate was determined to be *C. cryptica*. However, as a result of 18S (MAR2) and SSU (MAR2\_2) analyses performed with the isolate determined to be *N. amabilis* by SEM analysis, it was determined that 18S gave a more reliable result. The species was *N. amabilis* with a high similarity rate (99%) in BLAST analysis (Figure 10). Due to the significant similarities of the SSU sequence with other *N. amabilis* sequences by the BLAST analysis, additional phylogenetic markers for 18S rDNA (18S) were not required to submit for confirmation. The partial *rbcL* sequence identified for *N. amabilis* was the first sequence deposited into the NCBI database, and this explains why the partial sequence of *N. amabilis rbcL* is similar to *Psammodictyon* in phylogenetic analysis (Figure 11). If the *N. amabilis rbcL* sequence had been previously transmitted by different researchers in the NCBI database, the similarity rate with this sequence would probably have been very high.



**Figure 11.** Neighbor-joining tree based on partial sequences of *rbcL* of the isolates for phylogenetic inference. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura 1992). The percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

### Concluding remarks

Five new diatom species, *Cyclotella criptica*, *Nitzschia amabilis*, *Thalassiosira pseudonana*, *Conticribra weissflogii* and *Entomoneis* cf. *pusilla*, were recorded for the Sea of Marmara in this study. Since samples were taken from the Sea of Marmara during the most severe and extensive mucilage event, which has occurred in this sea so far, potential contribution of these species to the mucilage formation appears to be high.

Especially, the relation between mucilage formation and the two diatoms species; *Conticribra weissflogii* and *Cyclotella cryptica* is worth investigating further due to high viscosity (polysaccharides) of these species in the cultures. Lastly, the partial rbcL sequence of *N. amabilis* was first time deposited into the NCBI database with this study.

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**Author contributions:** D.T. methodology, investigation, conceptualization, visualization, and writing original draft. A.E.D. methodology, investigation, conceptualization, review, and editing. E.E.-D. investigation, methodology, conceptualization, data curation, visualization, writing original draft, review, and editing. H.B.Y. investigation, methodology, funding acquisition, review, and editing. All authors contributed to discussion and approved the submitted version.

## 2021 Marmara Denizi müsilaından izole edilen diatomlar

### Öz

Bu çalışma 2021 yılında Marmara Denizi'nde geniş çaplı müsila olayı esnasında kıyı ve yüzey suyunda beş yeni diatom kaydını sunmaktadır. Bu diatomlardan üç tanesi küçük nanoplanktik; *Cyclotella cryptica*, *Nitzschia amabilis*, *Thalassiosira pseudonana*, ve iki tanesi daha büyük diatomlardır; *Conticribra weissflogii* ve *Entomoneis cf. pusilla*. Ek olarak, Marmara Denizi ve Adriatik Denizi müsila olaylarında sık rastlanan *Cylindrotheca closterium* de izole edilmiş ve tanımlanmıştır. *C. closterium*, *C. cryptica* ve *N. amabilis*'in kloroplast rbcL bölgesi, SSU, ve 18S rDNA'sı önceki çalışmalarda kullanılan primerler ile amplifiye edilmiştir. rbcL, SSU ve 18S rDNA kısmi dizileri NCBI veritabanına gönderilmiştir. Moleküler filogeni (18S, rbcL ve SSU V4 DNA sekanslarına dayalı), analiz edilen türlerin SEM görüntüleri ile tanımlanan türler olduğunu doğrulamıştır.

**Anahtar kelimeler:** Centrales, pennaes, nanoplankton, SEM, DNA dizileri

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