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Morphology, Molecular Genetics and Potential Importance for Mucilage Events of the New Coccolithophorid *Ochrosphaera neapolitana* in the Sea of Marmara

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Abstract: The coccolithophorid *Ochrosphaera neapolitana* was reported for the first time from samples obtained during a large-scale mucilage event in the Sea of Marmara in May 2022 in a previous study. We also found this species in our samples obtained about a year ago (i.e., in June 2021). In our study, *O. neapolitana* was further isolated and produced in the laboratory as a monoculture for further investigations using electron microscopy and molecular methods. *Ochrosphaera neapolitana* was identified using a small sub-unit (SSU) rRNA sequence and subsequent phylogenetic analysis. During the laboratory experiments, *O. neapolitana* was surprisingly observed to produce conspicuous levels of mucilage as a skim layer in mono- or multi-species cultures, mainly comprising other diatom species. This observation could be a significant milestone in understanding the reasons and mechanisms of mucilage events that occur in the Sea of Marmara.

Keywords: coccolithophorid; haptophyte; Marmara; mucilage event; *Ochrosphaera*

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1. Introduction

Marine mucilage is a type of gelatinous organic (mainly polysaccharides) material produced by marine organisms such as phytoplankton, presumably to protect them from fluctuations in environmental conditions [1–3]. Phytoplankton excretes heteropolysaccharides [4] and their fibrils bond to each other, gradually forming larger macroaggregates such as marine snow [5]. As a result of these macroaggregates sticking to detritus and suspended particles, mucilage formation may be extended [5–7]. Marine mucilage may also contain specific microbial flora [7,8]. Mucilage formation has adverse effects on fisheries, tourism, maritime operations [9], and benthos [10]. Its occurrence frequency is reported to increase with elevated nutrient loads and sudden alteration in the ratios of limiting nutrients in coastal areas, which could be also associated with rising temperatures and irradiances [1,11,12].

The Marmara Sea has a surface area of 11,500 km^2 and is located at the intersection point between the Black Sea and the Mediterranean Sea. Together with its two narrow channels, it forms the Turkish strait system. The Marmara Sea has a brackish surface layer (0–25 m) with a salinity of 22 originating from the Black Sea and a higher salinity (~38) of deep water inflowing from the Mediterranean Sea [13]. This enclosed sea has been exposed to extreme levels of municipal and industrial pollutants and nutrients throughout

its drainage basins associated with high population, especially in Istanbul (about 15 million inhabitants), and inflow of particulate/dissolved pollutants from the Black Sea since the 1970s [14,15]. Approximately five million cubic meters of wastewater including high levels of dissolved nutrients (such as nitrogen and phosphorus) discharge into the Marmara Sea daily [16]. Vardar et al. [17] reported that on a daily basis almost 3 billion microplastic particles were released from the effluent of a single wastewater treatment plant (i.e., Ambarlı) to the Sea of Marmara. Such a high number of microplastics could also act as a substrate for the bottom-dwelling or other pelagic algae and for their dormant stages in addition to the provision of a larger area of surface for the attachment of the mucilage layer.

The first reports of large-scale marine mucilage events in the Marmara Sea correspond to the 2007 period [18–21]. The mucilage phenomenon reappeared in the Marmara Sea in November 2020 and intensified during April–June 2021, causing serious environmental problems [22,23] and with public attention in the national media continuing for weeks. Although neither the organisms responsible nor the triggering mechanism is known, one or more species of diatoms and dinoflagellates were thought of as responsible organisms for the events in that period [18–20,24].

Since 2021, ecological studies trying to determine organisms responsible for producing mucilage in the Sea of Marmara have been intensified, resulting in the finding of new plankton species in this sensitive ecosystem [25–27]. Very recently, Durmuş et al. [25] reported a total of new 65 taxa of microalgae for the first time in the Sea of Marmara during and after an intense mucilage period from March 2021 to June 2022. The coccolithophorid *Ochrosphaera neapolitana* was also included in the list of new records for the Sea of Marmara obtained from samples in May 2022, which is the first reporting of this species [25]. The genus *Ochrosphaera* (most probably *O. neapolitana*) was also mentioned in İmamoğlu et al. [28]'s study as a species in the culture collection of microalgae at Ege University in Ege, Izmir, Turkey.

The littoral coccolithophorid genus *Ochrosphaera* Schussnig is not frequently reported to form visible blooms [29] and an insignificant role is attributed to this genus in the biogeochemical context of long-term carbonate fixation. It has been reported in the coastal waters of the North Atlantic, the Indian Ocean, the Mediterranean Sea [30], and Japan [31].

Since the other two species (i.e., *O. verrucosa* Schussnig and *O. rovignensis* Schussnig) were suppressed [29], *O. neopolitana* appears as the only species of this genus. Typical coccoliths of *Ochrosphaera* are named tremaliths (these are vase-shaped coccoliths calcified only on the rim of the baseplate scale). *Ochrosphaera* has a heteromorphic life cycle [31] comprising diploid coccolith-bearing cells and a haploid non-calcifying phase, which produces mucilage layers [29]. This genus has the specific feature of progressive extracellular calcification of the old coccoliths instead of their renewal [29].

Using *Ochrosphaera neapolitana* samples obtained from the Kalamis region of Istanbul city, the aims of the present study are (1) the description of its detailed morphology using scanning electron microscopy (in addition to light microscopy), (2) disclosing its molecular genetics, and (3) the evaluation of its potential role in mucilage formation events in the Sea of Marmara.

2. Materials and Methods

A seawater sample was obtained using a polyethylene bottle on 11 June 2021 from the surface, which had a thick mucilage layer, from the Sea of Marmara, located at Kalamis, Istanbul (40°58′39.99″ N, 29°02′18.37″ E, Figure 1). The temperature and salinity of the seawater were 22 °C and 23 PSU, respectively (Hanna HI98319 salinity tester).

Seawater samples were transferred into 100 mL Erlenmeyer flasks in the laboratory and enriched with F/2 Medium. Cells that increased in the top layer of the flask were chosen with thinned Pasteur Pipettes and grown in tubes. Then, they were transferred into 100 mL flasks and kept under 30–40 µmol photon m^{-2} s^{-1} irradiance, 20 °C temperature, and 12:12 light–dark cycle conditions in a climate chamber.

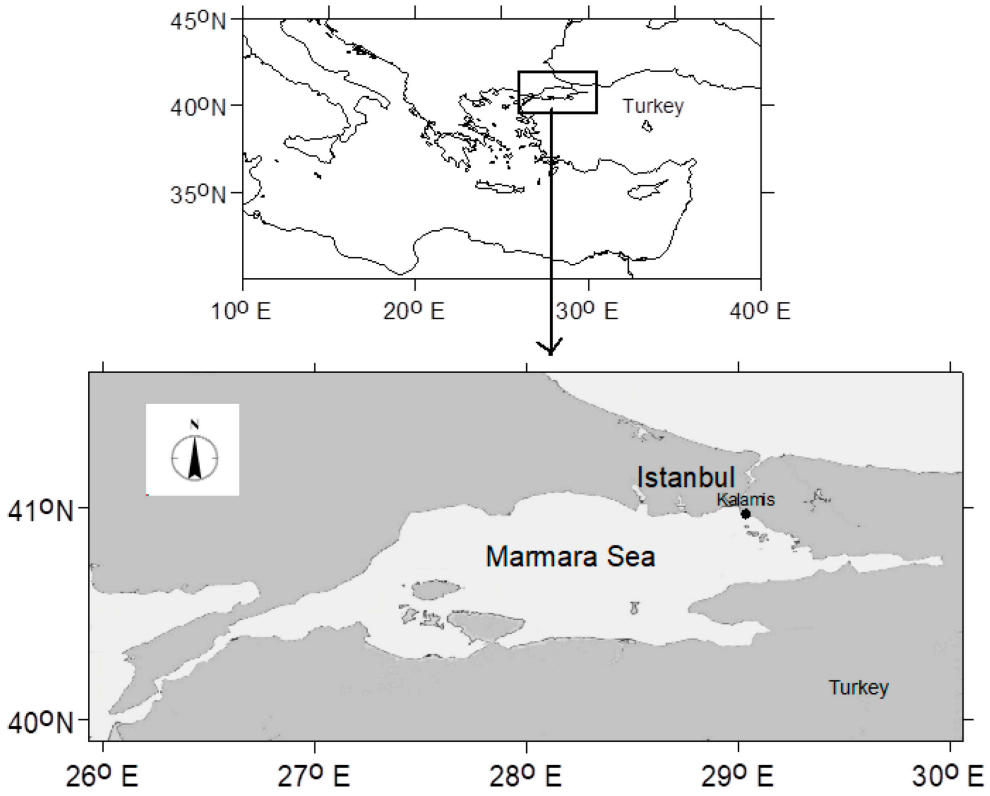


Figure 1. Sampling region for the coccolithophorid *Ochrosphaera neapolitana* in the Sea of Marmara on 11 June 2021.

2.1. Microscopy

Live cells of *Ochrosphaera neapolitana* were investigated under the light microscopes Nikon/Eclipse E100 and Nikon/Eclipse TS100 with 1000× and 400× magnifications. For the scanning electron microscopy, culture cells were fixed with 2% glutaraldehyde solution (Grade I, Sigma G-5882, St. Louis, MO, USA) for a few minutes at 22 °C temperature and filtered on 0.2 µm pore size polycarbonate filters. It is worth noting that we have used high-grade glutaraldehyde (SEM grade), which is always kept in the deep freeze when not used. We believe that we obtained high-quality SEM photographs because of this well-preserved SEM-grade glutaraldehyde. This glutaraldehyde was added to the liquid sample directly, and after a few minutes, the sample was filtered and then covered with iridium without additional washing with distilled water or ethanol for photographing using the SEM. After coating the filters with iridium using the Leica EM ACE600 Sputter Coater instrument, samples were examined with a Zeiss GeminiSEM 500 field emission scanning electron microscope, in Germany. In pursuing good quality photography, cells were also separately fixed with 1% osmium tetraoxide and passed through an ethanol series of 25%, 50%, 75%, and 90% with polycarbonate syringe filters and investigated with SEM as above.

2.2. DNA Isolation, PCR Analysis, and Phylogenetic Tree Construction

Following the manufacturer's instructions, DNA was extracted from about 300 mg of the material using a commercially available kit (QIAamp DNA Minikit (Cat#51304, Qiagen, Valencia, CA, USA). For this, the algal cell culture in a 15 mL falcon tube was centrifuged and then the supernatant was discarded, obtaining an algal cell pellet (~300 mg). Two-milliliter tubes containing the ~300 mg sample with three-millimeter tungsten carbide beads were then frozen in liquid nitrogen. The tubes were put into the adapter sets, with the Tis-sueLyser's clamps fastened. TissueLyser was used to disturb frozen plant tissue samples to homogenize them. Steps of 30 Hz shaking lasting two minutes were used to

create the disturbance. Pellets were washed with PBS to a final volume of 200 µL. Then, 20 µL QIAGEN proteinase K (Qiagen Inc., Valencia, CA, USA) and 200 µL Buffer AL were added into the microcentrifuge tube and mixed by pulse-vortexing for 15 s. The mixed sample was incubated at 56 °C for 10 min. An aliquot of 200 µL ethanol (96–100%) was added to the sample and remixed by pulse-vortexing for 15 s before being centrifuged at 6000× *g* for 1 min. The supernatant was transferred to the QIAamp Mini spin column in a clean 2 mL collection tube, and the tube containing the filtrate was discarded. A 500 µL Buffer AW1 was added to the collection tube, well mixed, and centrifuged at 6000× *g* for 1 min. Afterward, 500 µL Buffer AW2 was added to the collection tube and centrifuged at 20,000× *g* for 3 min. The QIAamp Mini spin column was placed in a clean 1.5 mL microcentrifuge tube. In total, 200 µL Buffer AE was added and incubated at room temperature (15–25 °C) for 1 min, and then centrifuged at 6000× *g* for 1 min.

The integrity of isolated gDNA was checked by visualization of ethidium bromide-stained DNA separated on 1.5% (*w*/*v*) agarose gel. The DNA sample was stored at −80 °C until it was used. The isolated gDNA was used as the template for PCR-based amplification using Small Sub-Unit (SSU) primers to identify the algal species isolated from marine mucilage events. According to the manufacturer's protocol, PCR was performed using the Solis BioDyne 5× FIREPol® Master Mix kit (Solis BioDyne, Tartu, Estonia). The SSU rRNA gene was amplified using the forward primer 5′ CTGCCCTATCAGCTTTGGATGG -3′ (18SF) [32] and the reverse primer 5′ CCATTCAATCGGTAGGTGCG -3′ (18SR). For PCR amplification, thermal cycler (Applied Biosystems™ MiniAmp™ Thermal Cycler, Thermo Fisher Scientific Inc., Waltham, MA, USA) conditions were as follows: initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 1 min., annealing at 58 °C for 1 min., and extension at 72 °C for 90 s. These cycles were followed by a final extension step at 72 °C for 5 min. The DNA band around the predicted size was removed from the agarose gel (1%) using the Qiagen Gel Extraction Kit according to the manufacturer's protocol. PCR products were sequenced by Sentebiolab, Ankara, Turkey (https://sentebiolab.com.tr/; accessed on 29 December 2022). The raw DNA sequence data were manually examined. Using the submission portal (https://submit.ncbi.nlm.nih.gov/subs/genbank/; accessed on 11 January 2023), the identified *O. neapolitana* SSU rRNA sequence was deposited in the NCBI GeneBank (https://www.ncbi.nlm.nih.gov/; accessed on 11 January 2023) under accession number OQ220476. Phylogenetic analysis was performed by MEGA7 software (https://www.megasoftware.net/; accessed on 12 January 2023) [33]. The evolutionary history was inferred using the Maximum Likelihood method based on the Jukes–Cantor model [34]. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The proportion of sites where at least one unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. The analysis involved eight nucleotide sequences. The codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. Fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There was a total of 78 positions in the final dataset.

2.3. Mucilage Observation

Observations were performed on the extracellular foamy organic material (mucilage produced) by both single-cell cultures of *Ochrosphaera neapolitana* and co-cultures with two other diatom species. These two diatom species are *Nitzschia* sp. and *N. amabilis*, which were observed to occur together with *O. neapolitana* in the original sample. Cultures were made daily following inoculation in the culturing flasks and lasted for at least 3–4 months (>10 inoculations). The same mucilage production observations were observed in all inoculations consistently (i.e., more than 10 inoculations) for both mono- and multi-species cultures with *Ochrosphaera neapolitana*.

3. Results

3.1. Morphology from Light and SEM Microscopy

O. neapolitana shows a heteromorphic life cycle with an alternation between diploid coccolith-bearing cells and a haploid non-calcifying phase. The coccolith-bearing phase can be either non-motile or motile with two slightly unequal flagella. Coccolith-bearing flagellated cells do not have haptonema. The size range of the cells was 6–15 µm (Figures 2 and 3).

Ochrosphaera coccoliths are found in the typical tremalith structure (Figure 2a–c). Tremaliths are vase-shaped muroliths that have a rim with a sub-vertical wall. Unfortunately, we do not have a close-up picture of the liths. Pulley-shaped coccoliths have not been observed in the samples. Unlike other coccolithophore species, scales are not shed in this species (Figure 2a–c). Another type of scale belonging to motile S-cell (different from C cells) is shown in Figure 2d.

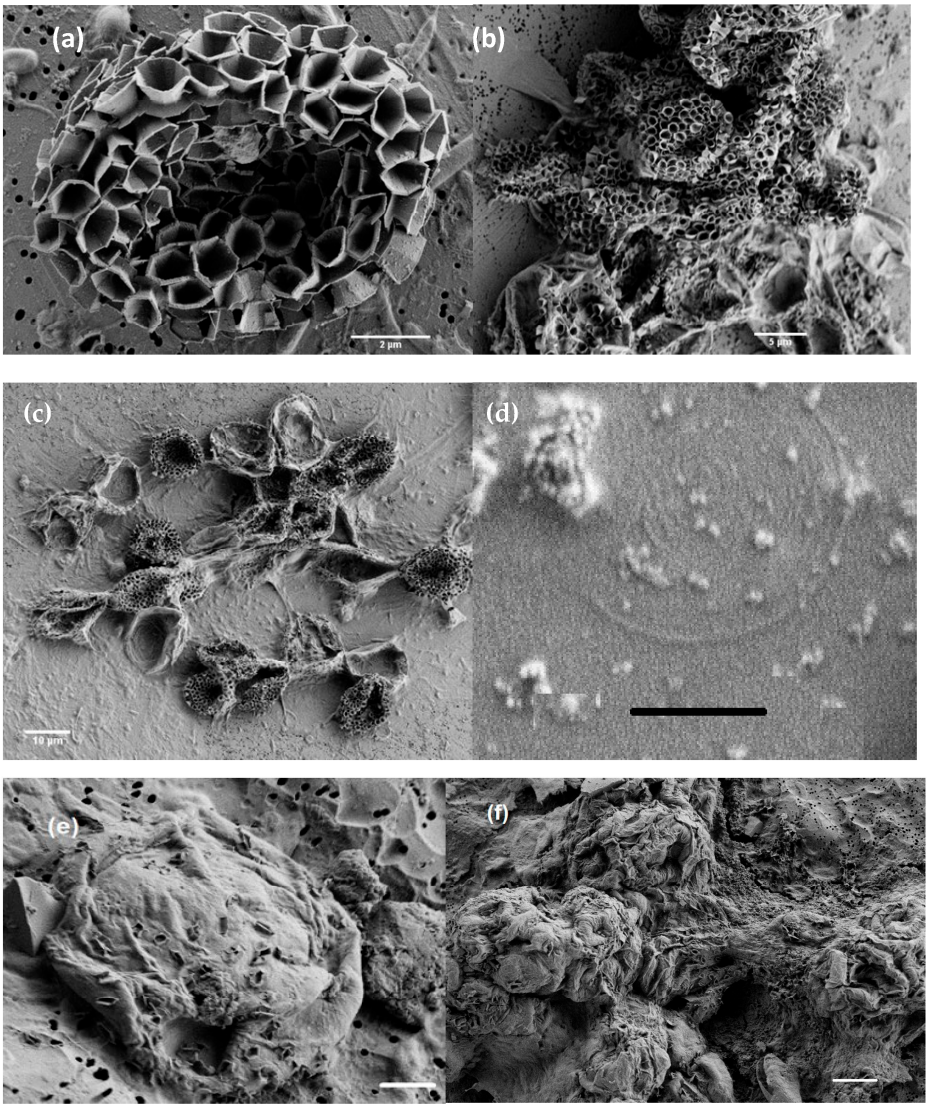


Figure 2. *Ochrosphaera neapolitana* images from the SEM: (**a**) a coccolith-bearing diploid cell scale bar; scale is 2 µm, (**b**) a group of coccolith-bearing cells; scale is 5 µm, (**c**) an aged colony, scale 10 µm, (**d**) an organic base-plate scale of an S-cell; scale is 300 nm, (**e**) a coccolith-bearing cell fixed with 1% osmium tetraoxide; scale 1 µm, and (**f**) deformed cells after fixing with osmium tetraoxide; scale 4 µm.

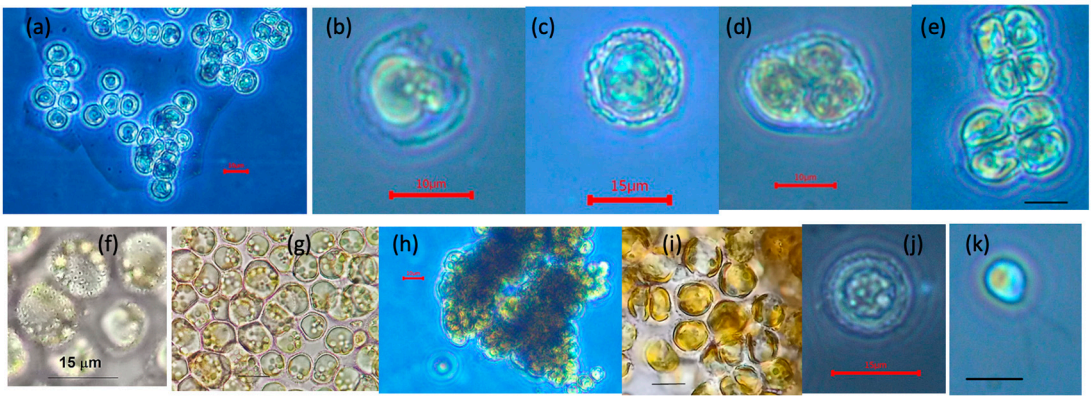
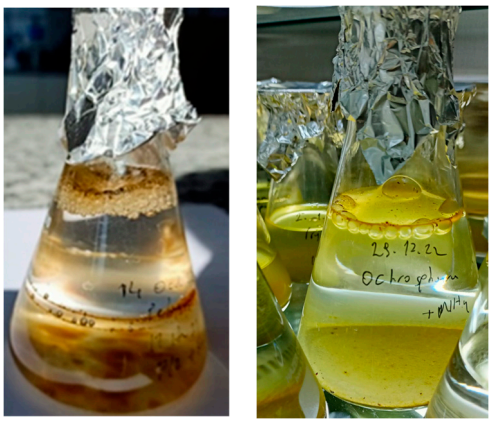


Figure 3. *Ochrosphaera neapolitana* images from the light microscope: (**a**) light microscope view of non-calcifying cells; scale 10 µm (**b**) a coccolith-bearing cell; scale 10 µm, (**c**) another coccolith-bearing cell; scale 15 µm, (**d**) a divided coccolith-bearing cell; scale 10 µm, (**e**) tetrad (meiocytes) before the reappearance of S-cells; scale 10 µm, (**f**) colony of coccolith-bearing cells under the light microscope; scale 15 µm, (**g**) image showing insides of coccolith-bearing cells; scale 15 µm, (**h**) a colony under the light microscope; scale 10 µm, (**i**) two chloroplasts seen in each cell; scale 10 µm, (**j**) over-calcified pseudocysts; scale 15 µm, and (**k**) a coccolith-bearing flagellated cell; scale 15 µm.

It is worth noting that the osmium tetraoxide fixation method used here damaged the cells, causing deformation or loss of the majority of the scales. The damage could also be partly due to the pressure in the syringe filtration or the dehydration process with ethanol series (Figure 2e).

Mucilaginous extracts of the non-calcifying cells are observed under the phase contrast microscope (Figure 3a). Calcifying cells under the light microscope are also seen (Figure 3b–k). These calcifying cells are also observed in high quantities in the mucilage layer at the top of the flasks, indicating that in addition to non-calcifying cells, they also contribute to mucilage formation. Meiocytes are observed in Figure 3d,e. While there is a cover of coccoliths surrounding the quadrate cells, no coccoliths surround each meiocyte. Coccolith-bearing colonies are seen under low magnification (Figure 3b) and high magnification (Figure 3f) with light microscopy. Chloroplasts are observed under the light microscope (Figure 3i). A calcareous 'pseudocyst' is formed due to the extracellular over-calcification of coccoliths (Figure 3j). A coccolith-bearing motile cell with two flagella is seen in Figure 3k. Culturing flasks of *O. neapolitana* with the two diatom species or as the sole species are seen in Figure 4a,b, respectively.



(**a**) (**b**)

Figure 4. (**a**) Mucilage formation at the surface layer of the flask when *Ochrosphaera neapolitana* was cultured together with two different diatom species (2 weeks old) and (**b**) mucilage formation in monospecific *Ochrosphaera neapolitana* culture (2 weeks old).

3.2. Molecular Analysis

In the present study, axenic and living cultures were analyzed for gDNA isolation; gDNA was isolated successfully and the integrity of extracted gDNA isolated with agarose gel (Figure 5A) was checked, at which time it was determined that they were of high quality for PCR analysis. Isolated gDNA was used as the template for PCR-based amplification using 18S primers to identify the algal species isolated from marine mucilage events. The PCR product obtained after PCR analysis was visualized on agarose gel (1.5%) (Figure 5B), and it was isolated and directly sequenced by the Sanger method.

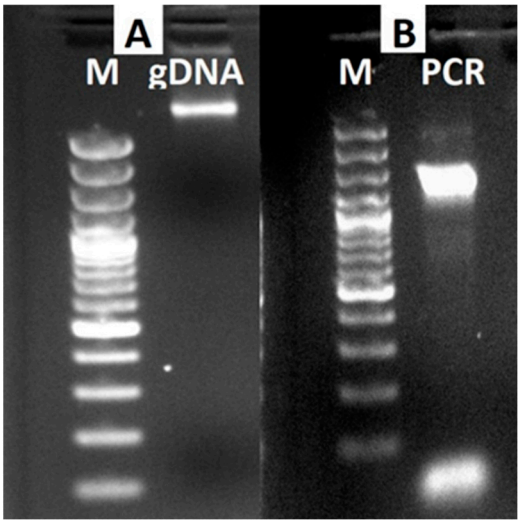


Figure 5. (**A**) Agarose gel (1.5%) electrophoresis results of genomic DNA of *O. neapolitana* isolated from mucilage (M: Marker, DNA: gDNA of *O. neapolitana* isolate TR2 (OQ220476)) and (**B**) agarose gel (1%) electrophoresis result in which 18S primers were used (M: Marker, PCR: amplification product of *O. neapolitana* isolate TR2 (OQ220476)).

The SSU sequence of the mucilage-isolated algal species was compared to the SSU rRNA sequencing identified in the GenBank database using the BLAST algorithm for searching sequences. The sequence obtained through PCR was verified to be 205 bp in length. Using BLAST and phylogenetic analyses (Figure 6), the species was verified, and the identified sequence were observed to have a high similarity to the SSU gene from other strains of *Ochrosphaera.* To investigate the relationships among the *Ochrosphaera* species based on their shared SSU rRNA sequences, the sequence data for the isolate were also employed to build a phylogenetic tree. By comparing the sequences discovered from the SSU rRNA PCR profiles, the 29 isolates received from the database at NCBI as a result of the comparison of SSU rRNA sequence similarities were clustered into four branches. In total, 22 isolates were pooled together into the same branch, but seven isolates were grouped into different branches (Figure 6). Pairwise sequence alignment of the identified sequence from *O. neapolitana* isolate TR2 (OQ220476) with the known 18S rDNA gene indicated that the maximum sequence identity (98.04%) was between *O. neapolitana* isolate TR2 (OQ220476) and *O. neapolitana* culture collection CCAP 932/1 (FR865767). Although defined as different species in different studies, all the species of *Ochrosphaera* are now suppressed as O. neapolitana by Fresnel et al. [29].

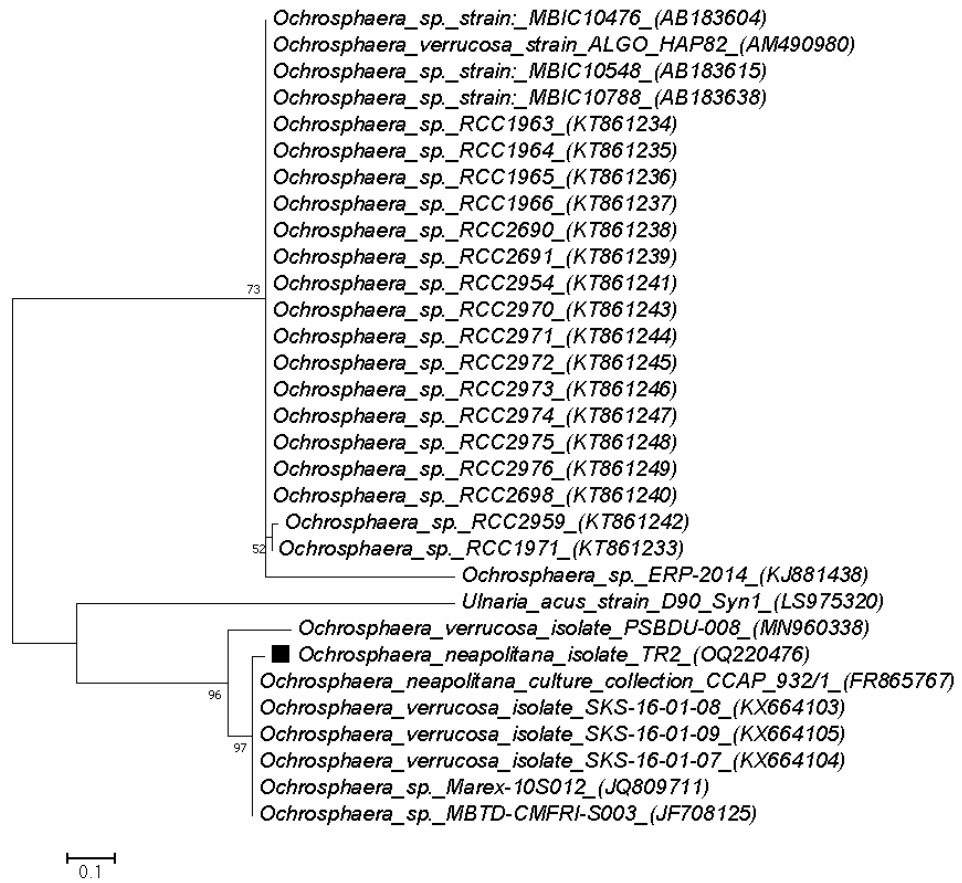


Figure 6. Phylogenetic tree based on SSU rRNA gene sequence, showing the position of the *O. neapolitana* isolate TR2 (OQ220476) (shown in the filled square). The evolutionary history was inferred using the Maximum Likelihood method based on the Jukes–Cantor model [34]. As stated in the NCBI GenBank, names and accession numbers are provided. The percentage of trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The diatom *Ulnaria acus* was used as an outgroup.

3.3. Mucilage Observation

In the original field sample, a dense presence of *Ochrosphaera neapolitana* cells within the mucilage was noted (Figure 7).

In the laboratory, the mono-species *O. neapolitana* culture, (including both calcifying and non-calcifying phases) was observed to produce conspicuous amounts of amorphous mucilage following 2 weeks from its inoculation in culture flasks. The mucilage layer at the surface of the culture flasks was visible for several weeks in both the *O. neapolitana* monoculture as well as mixed culture with *Nitzschia* sp. and *Nitzschia amabilis*. They were also observed to occur together in the sea when they were sampled (Figure 8). They also lived together and produced a dense layer of mucilage in these culture flasks, consistently lasting several months (Figure 4a,b). The density of the mucilage produced by *O. neapolitana* in the monoculture was visibly lower than that when it was cultured with *Nitzschia* sp. and *Nitzschia amabilis* (both species together). These two species, *Nitzschia* sp., and *N. amabilis*, did not produce any mucilage after their isolation from *O. neapolitana*.

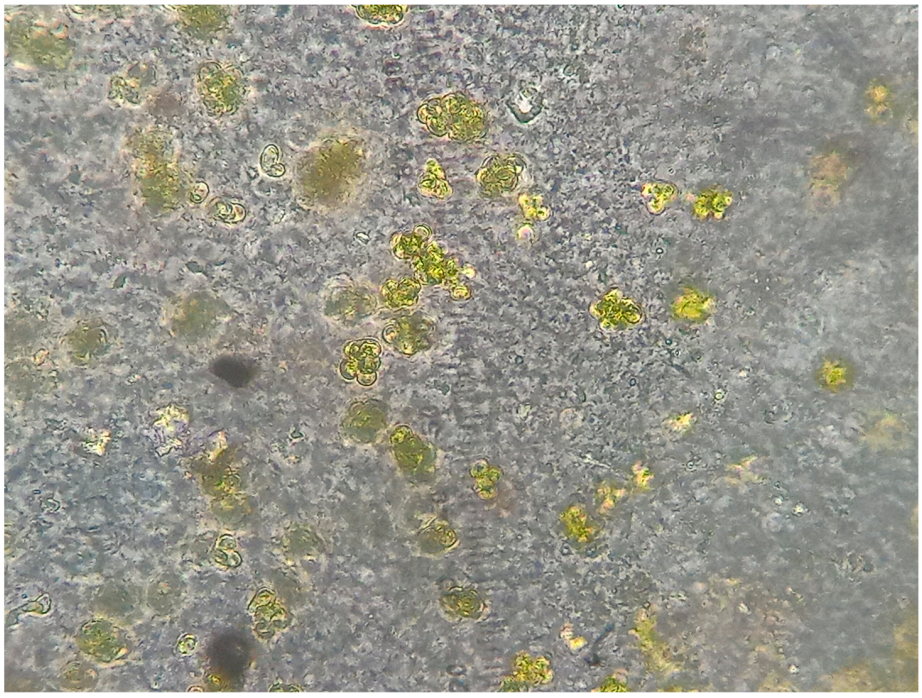


Figure 7. High concentration of *Ochrosphaera neapolitana* cell groups in the original samples taken from Kalamis, Istanbul, on 11 June 2021; the scale is 50 μm.

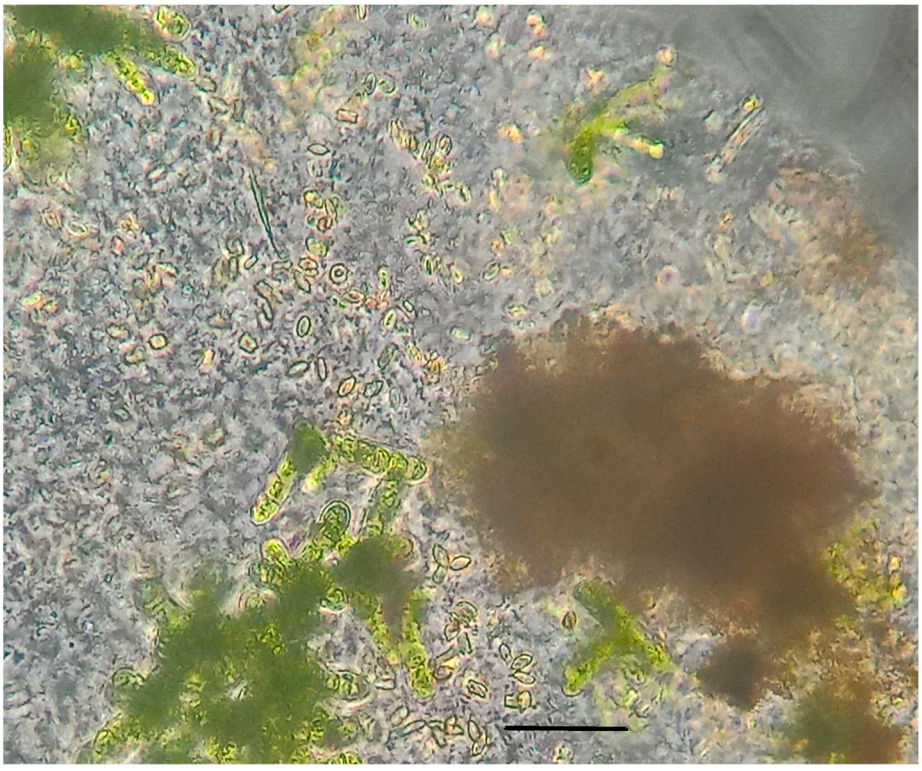


Figure 8. A section of the mucilage sample taken from Kalamis, Istanbul, on 11 June 2021, shows green microalgal species and the pennate diatom *Nitzschia amabilis* (small oval cells); scale is 50 μm.

Except for the diatom *Thalassiosira sp.* and the cyanobacteria *Spirulina sp.*, the coccolithophore *Ochrosphaera neapolitana* was seen as the only species causing the production of mucilaginous organic matter among the 40 different phytoplankton species (belonging to diatoms, dinoflagellates, prasinophytes, haptophytes, dictyochophytes, pelagophytes,

cryptophytes, and eustigmatophytes) cultured (monoculture or multi-species culture) in our laboratory through the years (personal observation by Elif Eker Develi). *Spirulina sp.* also produced a gelatinous structure that generally sticks to the glass surface of the flask. However, its density was not high in the samples collected from the field samples on 11 June 2021 from Kalamis, Istanbul, in the northern Sea of Marmara, wherein *Ochrosphaera neapolitana* was quite dense (Figure 7).

4. Discussion

According to Guiry and Guiry [35], *Ochrosphaera neapolitana* belongs to the order Coccolithales of the Class Prymnesiophyceae under the Phylum Haptophyta. The species *Ochrosphaera neapolitana* was first described by Schussnig [36]. About ten years later, Schussnig [36] described two more species as *O. verrucosa* and *O. rovignensis*, and several investigators in later years supposedly reported the former from different regions [29,37]. Identification of *Ochrosphaera* cells is a particular challenge for the non-calcifying phase of this species. However, Fresnel and Probert [29] suggested that most of these observations may be *O. neapolitana* and further proposed suppression of the two other species in this genus. The range in cell diameter from the Sea of Marmara strain (6–15 µm) is larger than that reported from the cultured organisms in France (5–12 µm, [29]).

O. neapolitana is reported as the sole coccolithophorid that does not continually renew the coccosphere, and due to extracellular over-calcification of coccoliths, a calcareous 'pseudocyst' has been observed in cultures [29]. *O. neapolitana* is known to have a heteromorphic life cycle involving an alternation between (diploid) coccolith-bearing cells and a (haploid) non-calcifying phase [29]. The coccolith-bearing phase may be non-motile or possess flagella as shown in Figure 3k. Although not visible in this figure, this motile phase (S-cells) also bears scales. These types of scales are called base-plate scales, which are non-calcified and different from those of non-motile C-cells, which are mineralized. Fresnel et al. [29] mentioned that haploid S-cells were mainly responsible for mucilage production. In addition to these haploid cells, calcifying non-motile cells observed in our study accumulated in the mucilage layer at the top of the culture flasks, presumably contributing to mucilage formation. Similar to the life cycle of *Ochrosphaera*, another haptophyte genus, *Phaeocytsis,* also has haploid and diploid life cycles, both of which are suggested to be responsible for mucilage events in different regions of the world, such as the Adriatic Sea, Ligurian Sea, North Sea, Japan, and Brazilian coasts [2,11,12,38–44]. Haptophytes generally have a high proportion of lipids in their biomass [45], enabling the floatation of the cells. We have also observed another haptophyte species, *Chrysochromulina alifera,* generally concentrated at the surface layer of the flask in the laboratory culture (personal observation by Elif Eker-Develi).

This is the second report on the haptophyte coccolithophorid *Ochrosphaera neapolitana* in the Marmara Sea. Recently, along with 65 other new species of phytoplankton from the Sea of Marmara, Durmuş et al. [1] published the first presence of *O. neapolitana* from two stations in the Sea of Marmara in samples obtained about one year after (i.e., May 2022) compared to our samples.

This species is reported in different regions along the coastal waters of the North Atlantic, Indian Ocean, Mediterranean Sea [29,46,47], and Japan [29]. However, it is either infrequent or occurs in low abundance in the Mediterranean samples. For example, Bonomo et al. [48] did not find *O. neapolitana* collected at 24 marine stations in a coastal area (Gulf of Gaeta, central Tyrrhenian Sea) during a three-year survey (2012–2014). During their intense sampling at the four stations along a north-south transect in the area receiving the inflowing surface Black Sea Water (BSW) in front of the Dardanelles during October 2013, March 2014, and July 2014 within the photic layer (3–75 m), Karatsolis et al. [49] found a total of 95 species of coccolithophores using a scanning electron microscope (SEM); however, none of the *Ochrosphaera* species was present. Similarly, no *Ochrosphaera* was noted among the 98 taxa of coccolithophores identified using SEM from the monthly samples obtained from February 2018–January 2019 in two stations located on the coastal shelf and in the open sea

of the ultra-oligotrophic South-East Levantine Basin, Eastern Mediterranean Sea, during 2018–2019.

The small subunit (SSU) rRNA gene, a critical marker for random target PCR in environmental diversification detection, is one of the most commonly used genes in phylogenetic analysis [50]. For this reason, the SSU rRNA gene was studied in the presented study, and an attempt was made to identify the isolated algae species. There are 58 nucleotide sequences registered in the NCBI database belonging to the genus *Ochrosphaera*. Two species belonging to the genus *Ochrosphaera* were registered: (1) *O. verrucosa* and (2) *O. neapolitana.* Other sequence information was registered as *Ochrosphaera* sp. There are seven records of the *O. verrucosa* species, and the lengths of the sequences entered range from 255 bp to 1722 bp. For *O. verrucosa*, the *18S (SSU)*, *LSU,* and *tufA* genes were identified. In addition, for *O. neapolitana*, 13 pieces of sequence information is available for plastids *atpA* and *atpB*, *ITS*, *16S*, *18S*, *28S,* and *LSU* genes, and the lengths of these sequences are between 255 bp and 4396 bp. Another 38 registered sequences are *18S*, *28S*, *16S,* and *LSU* gene sequences and belong to *Ochrosphaera* sp. The lengths of these sequences range from 255 bp to 1.56 bp. *LSU* sequences are the shortest registered sequences (255 bp) in the database. When the information of the *Ochrosphaera* genus registered in the NCBI database is examined, it is understood that the sources from which the *O. verrucosa* species were isolated are (1) Tuticorin coastal waters, Tamil Nadu and (2) the Atlantic Ocean (Gulf of Mexico, Ewing Bank). Likewise, when the information on *O. neapolitana* is examined, it is seen that the species is isolated from (1) the Mediterranean Sea (French coast) and (2) the Atlantic Ocean (North Atlantic–East Channel waters). *O. neapolitana* isolated from the present study was first isolated from the Marmara Sea, and a mucilage sample was formed. *O. neapolitana* with the SSU gene sequence in the NCBI database is the only strain found in the CCAP932/1 coded culture collection. In the present study, when the phylogenetic tree was performed with the species whose SSU gene sequence was examined, it is seen that the species isolated from the Sea of Marmara mucilage sample shows a high similarity with *O. neapolitana* CCAP932/1.

Although cell numbers were not counted in the original samples, high cell numbers of this haptophyte species were observed in the original samples within the mucilage. They have continued to produce mucilage in the cultures at the top layer of the flasks, especially when they are together with other diatoms. It is most probable that *Ochrosphaera* cells uptake organic matter produced by these diatoms, sustaining further mucilage production. *Ochrosphaera neapolitana* is among the species of haptophytes that utilises organic compounds through osmotrophy [51]. The single-cell cultures were observed to form bubbles at the top of the flasks. A similar finding of mucilage production in the laboratory for the haploid non-calcifying stage is also reported by Fresnel and Probert [29]. This observation is very important as this species could potentially be a strong candidate as the responsible agent for the large-scale mucilage events in the Sea of Marmara observed both in 2007 and 2020/2021. So far, *Phaeocystis pouchetii* (haptophyte), *Skeletonema costatum*, *Cylindrotheca closterium*, *Thalassiosira rotula*, *Proboscia alata*, *Rhizosolenia* sp., *Pseudosolenia calcar-avis*, *Ditylum brightwellii*, *Coscinodiscus* spp., *Leptocylindrus minimus*, *Chaetoceros* spp., *Cerataulina pelagica*, *Pseudo-nitzschia* cf. *seriata* (diatom), and *Gonyaulax hyalina* or *G. fragilis* (dinoflagellate) are suggested as potentially responsible organisms for mucilage production during the events of 2007 and 2020/2021 in the Marmara Sea [18,19,22]. Among these phytoplankton species, *Cylindrotheca closterium* and *Gonyaulax fragilis* were also suggested as organisms causing mucilage events that occurred in the northern Adriatic Sea during the summers between 1999–2002 [12,52,53]. Recently, the cyanobacteria *Trichodesmium erythraeum* has been also suggested to produce mucilaginous blooms in the Bay of Bengal [54]. However, except for *Phaeocyctis antarctica* [55] and *G. fragilis* [52,56], none of the species were shown to produce mucilage in laboratory conditions. In our mono- or mixed-species cultures without *O. neapolitana*, we did not notice conspicuous mucilage production in the laboratory, except on a very small scale in the culture flasks with *Thalassiosira* sp., *Cylotella* sp., and *Spirulina* sp.

Except for the haptophyte *Phaeocystis pouchetii*, all species reported during the mucilage events in the field samples are common and at times abundant organisms in the Sea of Marmara or other regions of the Mediterranean when/where no mucilage events are observed. Due to its small size (6–15 µm), *O. neapolitana* is difficult to identify in field samples using traditional microscopy; therefore, we believe that it never appeared as a responsible organism in the studies published so far. Additionally, it could be suggested that this species somehow found the ideal conditions to contribute to the unprecedented levels of mucilage formation in its new environment, the Sea of Marmara. In any case, following its more detailed morphological description and observation of its conspicuous mucilaginous material in this study, further investigations, we believe, will help in better elucidating its role in the past and in possible future mucilage events in the Sea of Marmara.

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