CHAPTER 13

A NEW RECORD OF POTENTIALLY ICHTYOTOXIC HAPTOPHYTE SPECIES, *PRYMNESIUM PARVUM* F. *PATELLIFERUM*, IN THE SEA OF MARMARA DURING A LARGE-SCALE MUCILAGE EVENT IN JUNE 2021

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Abstract

A non-calcifying haptophyte species, *Prymnesium parvum* f. *patelliferum* (Green, Hibberd & Pienaar) Larsen, is reported for the first time in the Sea of Marmara from the mucilage event on 11 June 2021. The species was identified with a light microscope and scanning electron microscope. In addition, the 18S rRNA gene was partially sequenced in this study. Amplified gDNA of the expected size was sequenced and analyzed via bioinformatics applications. Results of the analysis vigorously recommended that a 672 bp PCR product was component of a small subunit ribosomal RNA gene sequence. The isolate was named MEUE1, and the partial 18S rRNA sequence of MEUE1 was submitted to the NCBI database (accession number OQ740296). Sequence analysis of isolated culture also verified that it was *P. parvum* f. *patelliferum*. The species became dominant in a flask that was saturated with sodium bicarbonate to have a basic pH of 8. Although this pH is close to the pH of the culture medium (7-7.5) before the

addition of bicarbonate, all the other species died in this flask, which could be possibly related to toxin production of the species under high pH. It appears that *P. parvum* f. *patelliferum* was introduced to the Marmara Sea, similar to some lakes, ponds, and coastal areas with brackish or freshwater character in/around the Mediterranean Sea. Ichtyotoxicity of prymnesin, produced by this haptophyte, is a risk for fishery and mariculture activities performed in this enclosed sea.

Keywords: Ichtyotoxic microalgae, *Prymnesium parvum* f. *patelliferum*, Marmara Sea, mucilage event

1. Introduction

Prymnesium parvum is a haptophyte species producing a phycotoxin, prymnesin (Yariv and Hestrin, 1961). This toxin has vigorous negative impacts on neurons, cells, fishes, and also has hemolytic property (Manning and Claire, 2010; Seoane et al., 2016). *P. parvum* blooms has a caused fish-killing worldwide, with examples in Scotland (Comin and Ferrer, 1978), Norway (Kaartvedt et al., 1991), Germany (Dietrich and Hesse, 1990), Finland (Lindholm et al., 1999), Bulgaria (Valkanov, 1964), China (Guo et al., 1996), and the U.S.A. (Roelke et al., 2011, 2016). It is a eurythermal and euryhaline species (Baker et al., 2007) growing at salinities of 0.5 to 45 (Brand, 1984; Rashel and Patiño, 2017) and temperatures at 5-30°C (Larsen and Bryant, 1998). There are also previous records of *P. parvum* in the Ebro Delta of Spain (Comin and Ferrer, 1978), in lakes located in Morroco (Sabour et al., 2000), Italy (Mattioli and Simoni, 1999) and Greece (Moustaka-Gouni et al., 2004), as well as in an aquaculture pond in Israel (Gordon and Colorni, 2008). *P. parvum* is recorded as an introduced species in the locations near or along the Mediterranean Sea mentioned above (Roelke, 2016). The alga synthesizes dimethylsulfoniopropionate (DMSP) and an unknown polyol, possibly aiding in the osmoregulation of the species in varying salinity ranges (Dickson and Kirst, 1987). Prymnesin toxicity was reported to be restrained to distinct pH ranges. Hemolysis and ichthyotoxicity increase with high acidity and alkalinity of the environment, respectively (Manning and Claire, 2010).

The organism can grow heterotrophically in the dark and has been observed to be predatory on several types of organisms, such as bacteria, protists, and different zooplankton species in the light (Tillmann, 2003, Liu et al., 2015). High amounts of cellular nitrogen (78%) and phosphorus (45%) may be obtained via predation in *P. parvum* (Carvalho and Granéli, 2010).

P. parvum blooms have been frequently observed since 1963 along Chinese coasts (Guo et al., 1996). In a study performed in two different bays in China, *P. parvum* was detected to have the most toxic effects on fish among three kinds of harmful algae, leading to acute death in five hours (Yang et al., 2018).

A total of 24 species belonging to the genus *Prymnesium* is known (Guiry and Guiry, 2018; Sym et al., 2011). Although in the past, *P. parvum, f. parvum* and *f. patelliferum* were assumed as different species; now it was revealed that these are different stages of the *P. parvum* life cycle actually (Granéli, et al., 2012). Scale morphology and/or DNA sequence analyses of 15 different *Prymnesium* species have been reported previously (Edvardsen et al., 2011; Sym et al., 2011; Granéli et al., 2012; Manton and Parke, 1962; Parke et al., 1958; Yoshida et al., 2006; Gayral and Fresnel, 1983).

Table 1. Species of *Prymnesium* genus described based on scale morphology and DNA sequence analyses.

No	Species name
1	*Prymnesium annuliferum* Billard
2	*Prymnesium calathiferum* Chang and Ryan
3	*Prymnesium chiton* (Parke & Manton) Edvardsen, Eikrem & Probert
4	*Prymnesium faveolatum* Fresnel
5	*Prymnesium lepailleurii* Fresnel
6	*Prymnesium nemametecum* Pienaar and Birkhead
7	*Prymnesium neolepis* (M. Yoshida, M. H. Noel, T. Nakayama, T., Naganuma & I. Inouye) Edvardsen, Eikrem & Probert
8	*Prymnesium palpebrale* (Seoane, Eikrem, Edvardsen & Pienaar) Edvardsen, Eikrem & Probert
9	*Prymnesium parvum* Carteer
10	*Prymnesium pigrum* (Geitler) Edvardsen, Eikrem & Probert
11	*Prymnesium pienaarii* (Gayral and Fresnel) Edvardsen, Eikrem & Probert
12	*Prymnesium polylepis* (Manton & Parke) Edvardsen, Eikrem & Probert
13	*Prymnesium radiatum* Sym, Pienaar, Edvardsen & Egge
14	*Prymnesium simplex* (Gayral & Fresnel) Edvardsen, Eikrem & Probert
15	*Prymnesium zebrinum* Billard

There is little information in the literature about the rest of the 9 *Prymnesium* species (*P. czosnowskii* Starmach, *P. gladiociliatum* (Büttner) R. W. Jordan & J. C. Green, *P. kappa* (Parke & Manton) Edvardsen, Eikrem & Probert, *P. laurentianum* (Kling) K. H. Nicholls, *P. minus* (Parke & Manton) Edvardsen, Eikrem & Probert, *P. minutum* N. Carter, P. *neustophilum* (R. E. Norris), *P. papillatum* Chen and Tseng and *P. saltans* Massart) (Guiry and Guiry, 2018; Granéli et al., 2012). *P. parvum* is the most widespread of the *Prymnesium* species, and it is reported to cause harmful blooms together with *P. calathiferum*

The Marmara Sea is an inland sea having a width of 70 km and a length of 250 km and is situated between the European and Asian continents (Beşiktepe et al., 1995). It forms the Turkish strait systems, and the low salinity surface water (0-25 m, 22 psu) originating from the Black Sea flows towards the Marmara Sea, while bottom water which has a high salinity, originating from the Mediterranean Sea passes to the Black Sea through the Marmara Sea.

Especially Istanbul region of the Marmara Sea has been exposed to domestic and industrial pollution since the 1950s with the heavy population influx to this Metropolitan city (Burak, 2008). The great increase in the population in the 1970s and 1990s has caused severe contamination and environmental problems in the bays and coastal areas (İSKİ, 2004, Burak, 2008).

The first Master Plan for wastewater management in İstanbul was finalized in the 1970s, and Istanbul Water and Sewerage Administration (İSKİ) was established in 1981 (Mutailifu, 2019). However, Istanbul municipality started to discharge the wastes under the pycnocline–thermocline layer (to the Mediterranean deep water) without pre-treatment in the Marmara Sea to transport it to the deep Black Sea water in the 1980s (Artüz, 2007). Unfortunately, only 30% of this discharge reaches the Black Sea, while the rest remain at the bottom (Yalçın, 2017). Combined with industrial and agricultural wastes, municipal wastewater originating from different sources contributes to environmental contamination problems and mucilage events in this enclosed sea (Orhon et al., 1994). The first record of mucilage in the Sea of Marmara was in 2007 (Aktan et al., 2008). During this event, hypoxia, anoxia or fish deaths were not reported (Karadurmuş and Sarı, 2022). On the contrary, the mucilage event devastated the ecosystem in 2021. A new haptophyte species for the Marmara Sea, *Ochrosphaera neapolitana* Schussnig was observed in high numbers within the mucilage layer on 11 June 2021 in Kalamis coastal water (Eker-Develi, et al., 2023). Isolated cells of this species also produced a mucilaginous sheath at the upper side of the cultures in the laboratory (Eker-Develi, et al., 2023). This species was also found in the samples taken outside of the Kucukcekmece Lake and near Bosphorus in May 2022 and reported as a new record for the Marmara Sea (Durmus et al., 2022). In addition to this species, 64 other new species were presented in the study of Durmus et al. (2022). This event negatively influenced the maritime activities, fisheries, and benthic organisms (Uflaz et al., 2021; Yıldız and Gönülal., 2021; Topçu and Öztürk, 2021) in the Marmara Sea. Mass death of invertebrates on the coast and deep waters was observed (Karadurmuş and Sarı, 2022). The impact of the mucilage was lethal to fishes and crustaceans.

Global warming, environmental pollution, and change in the ratios of nutrients may cause an imbalance in the ecosystem triggering the occurrence of nuisance and harmful algae. This study recorded *Prymnesium parvum* f. *patelliferum*, an ichtyotoxic haptophyte species, in the Marmara Sea for the first time. Since the species has non-calcifying scales and a sensitive cell wall, it is possible that it was missed in routine samplings in the time series studies performed using fixatives such as formaldehyde or Lugol since they destroy cells.

2. Material and Methods

A sample of seawater was taken with a polyethylene bottle from the surface water of Kalamıs coast, Istanbul, in the Marmara Sea (40.58 N, 29.02 E; Fig. 1), which has a dense mucilaginous sheath on 11 June 2021. Using a salinity tester (Hanna Instruments-HI 98319) with an electrode, the temperature and salinity of the sample were determined as 22 and 23, respectively. pH of the samples was measured using pH indicator strips (Merck,

1.09535.0001).

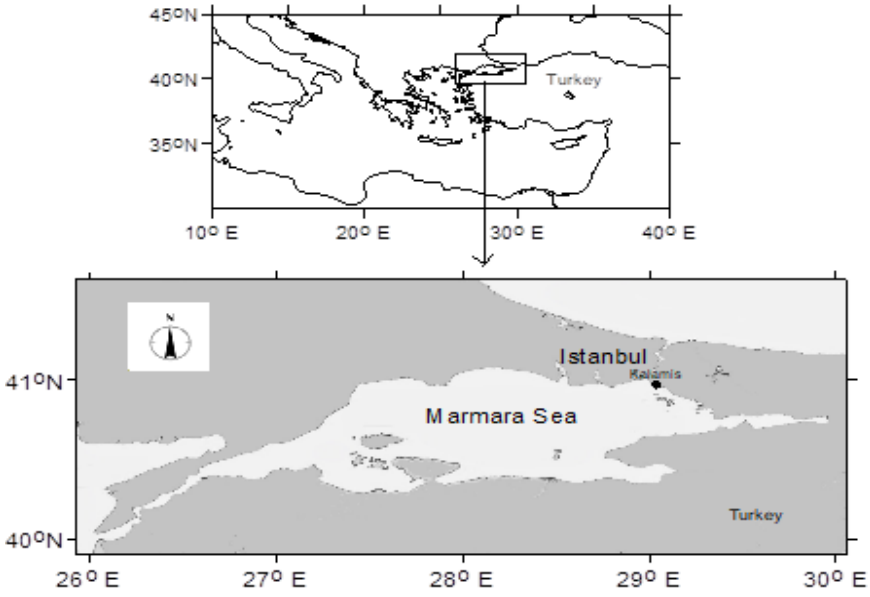


Figure 1. Sampling region for *Prymnesium parvum* f. *patelliferum* on 11 June 2021.

Mucilage containing seawater was put into 100 ml flasks and supplemented with F/2 Medium. They were kept in a climate chamber under the conditions of 20°C temperature, 30-40 µmol photon m^{-2} s^{-1} irradiance, and 12:12 light-dark cycle. Without selecting any cells, sodium bicarbonate was added to a flask by mixing until the saturation point (pH= 8). *P. parvum* f. *patelliferum* was observed to survive in the flask while the other species died. The clonal culture was obtained by serial dilution method of the isolated culture (Vaulot, et al. 2023).

2.1. Microscopy

Samples were investigated alive under a light microscope (Nikon/Eclipse E100) with 1000X magnification. For scanning electron microscopy (SEM), cells were fixed with 2% osmium tetraoxide for 10 min. and filtered through 10 mm Ø, 0.2 µm pore size polycarbonate syringe filters. Following this procedure, filters were passed through 25%, 50%, 75%, and 95% ethanol series for ca. 10 min, coated with iridium (Leica EM ACE600 Sputter Coater) and investigated with a Zeiss GeminiSEM 500 FeSEM, Germany (Zingone et al., 1995; Zingone et al., 1999).

2.2. DNA isolation, Sequencing, and Phylogenetic Analysis

The company's suggested methodology was followed while isolating DNA from isolated microalgal samples using the QIAamp Mini DNA Kit (Qiagen). The quality of the extracted DNA was evaluated using the spectrophotometric and electrophoresis techniques. The Nanodrop ND-1000 Spectrophotometer (Wilmington, DE, USA) was employed to evaluate the quantity and quality of the isolated DNAs, and a dye for DNA, ethidium bromide, incorporated into 1.5% agarose gel was used to affirm their integrity. The 18S primers chosen by the researchers were utilized in PCR analysis by Gastineau et al. (2016), with the methodology modified. Applied Biosystems-Perkin Elmer's thermal cycler performed gradient PCR to find the ideal temperatures at which the primers bind to DNA and produce the amplification result. A kit called the Solis BioDyne 5x FIREPol® Master Mix was used for the PCR processes. Mixtures were made with 5x FIREPol® Master Mix (1x), 0.2 M, DNA template (5 ng/l), 0.2 M primers, and 20 µl of DNAse and RNAse free water. The total volume was 20 µl. For PCR, the following conditions were used in the thermal cycler: first denaturation at 95°C for 4 min, then 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. After this process, the ultimate extension step lasting 7 minutes at 72°C was performed. The gel was viewed using an imaging device (Bio-Rad Laboratories, Segrate (Milan), Italy) to determine the existence of amplimers. The amplified and expected-size PCR products were extracted from the gel utilizing the QIAquick Gel Extraction Kit (Qiagen) in agreement with the manufacturer's manual. The same primers employed for PCR amplification were utilized to sequence extracted DNA bands from an agarose gel. Medsantek, Ankara, Türkiye (https://medsantek.com.tr/), supplied the sequencing service commercially. The phylogenetic analysis used MEGA7 (https://www.megasoftware.net/) (Kumar et al., 2016). The aligned 18S rRNA sequence was evaluated with bootstrap analysis (1000 replicates) (Felsenstein, 1985). The phylogenetic tree was obtained by applying the Maximum Likelihood Estimation method according to the Kimura 2-parameter model (Kimura, 1980). The percentage of phylogenetic trees showing the taxa clustered is exhibited beside of the branches. Preliminary tree(s) for the heuristic analysis were attained by using the Neighbor-Joining approach to a matrix of pairwise distances assessed by the Maximum Composite Likelihood (MCL) technique. The tree is drawn to scale, with branch lengths expressed in the units of substitutions per site. The analysis included 20 nucleotide sequences. Codon positions involved were 1st+2nd+3rd+Noncoding. All positions having <95% site coverage were disregarded. < 5% alignment gaps, missing data, and ambiguous bases were retained at any position. A total of 671 positions were present in the ultimate dataset. The 18S sequence of isolate MEUE1 was deposited in GenBank with the accession number OQ740296.

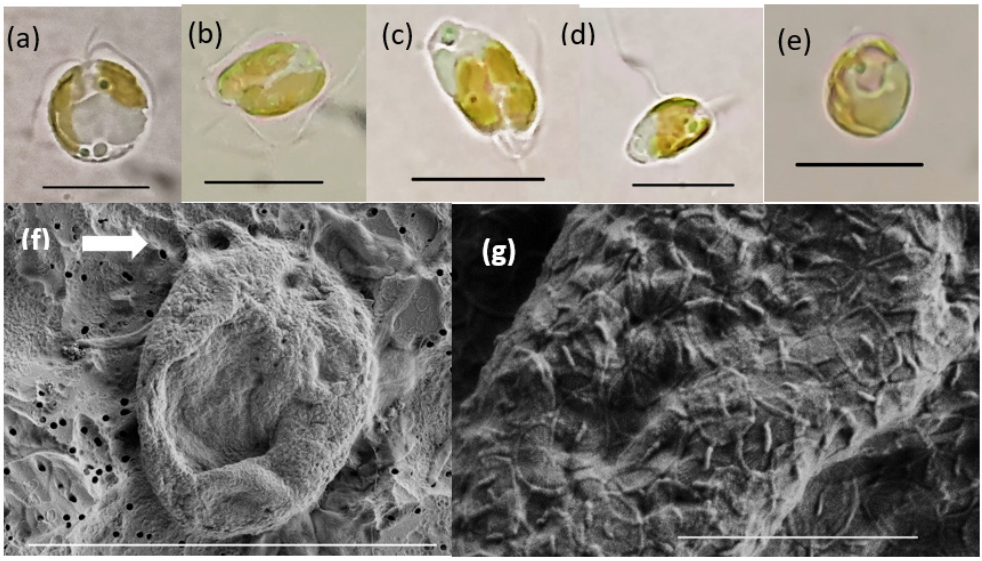
3. Results

Prymnesium parvum f. *patelliferum* became dominant in a flask with a basic pH of 8. The seawater's pH before adding bicarbonate was ~7.5. The abundance of this species was not high in the mucilage layer. It was noticed in the samples after it became dominant in the high-pH flask. The pH of the isolated culture was 9. Cells were more spherical in the late stationary phase cultures.

***Prymnesium parvum* f. *patelliferum* (Green, Hibberd & Pienaar) Larsen**

Cells are unicellular, spherical to ellipsoidal (Fig. 2 a-i). The length is 8-11 µm, and the width is 4-6 µm. Cells are sometimes mildly flattened. The posterior end is rounded or tapered, while the anterior is obliquely truncated (Fig. 2b-d). Cells possess one well-developed and non-coiling haptonema and two flagella (Fig. 2a-d). Body scales are oval with radial fibers on the surface and distal body scales have tall upright rims (Fig. 2g). Cells have two yellow-green chloroplasts (Fig. 2a-c). A contractile (pulsatile) vacuole is occasionaly present either near to nucleus or at the anterior tip of *P. parvum* f. *patelliferum* (Fig. 2e). There are no flagellar hair or flagellar scales (Fig. 2i).

The flagella are used for providing movement and stirring the periphery layer of the cells, and the haptonema is used for attachment to the substratum or phagotrophy (McLaughlin, 1958; Prescott, 1968; Tillmann, 2003).



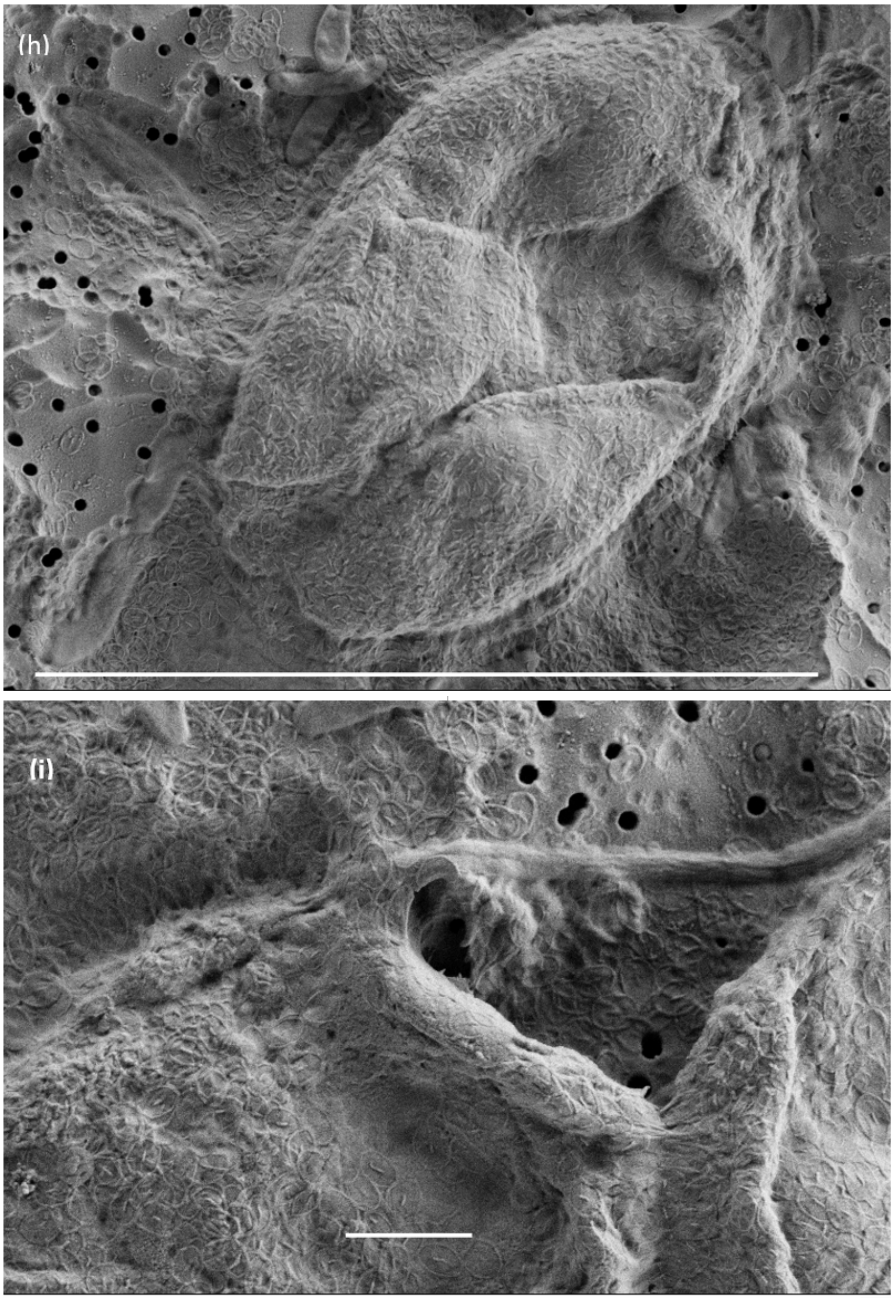


Figure 2. *Prymnesium parvum* f. *patelliferum* light microscopy (a-e) and SEM (f-i) images. (a) A spherical cell in the stationary phase of growth (b) A longer oval cell in the exponential growth phase, (c) Another elongated cell in the early phase of growth (d) Another cell from lateral view (e) A cell with contractile vacuole (black arrow) at the anterior end (f) SEM picture of a cell, white arrow shows one of the flagella (g) close up view of body scales (h) A cell from frontal view (i) close up view of a flagellum and body scales on the filter. Scale bar (a-f, h= 10 µm, g, i= 1 µm).

Molecular Analysis

Living cultures were examined for gDNA isolation in the study. The quality of the gDNAs was assessed using agarose gel electrophoresis at a concentration of 1%, and the 260/280 ratio was found to be between 1.8 and 2.0, suggesting high quality. The 18S rRNA could be successfully amplified from gDNA using the primer pairs. The small subunit rRNA (18S) sequencing of isolated algal species was matched to the sequences found in the GenBank database and through BLAST. The small subunit (SSU, the 40S) 18S rRNA gene is one of the most commonly employed genes in phylogenetic analysis and an essential marker for random target PCR in environmental diversification detection (Meyer et al., 2010); therefore, for phylogenetic analysis of isolated algal species, the 18S rRNA gene was chosen. Phylogenetic analysis and BLAST were used to confirm the species that the SEM images had identified. The MEUE1 isolate's *18S* gene sequences were compared, and the comparison revealed that this isolate was *P. parvum* f. *patelliferum*. In order to examine the similarity amongst the *Prymnesium* species according to shared 18S rRNA sequences, the sequence data for the isolate were also used to form a phylogenetic tree. By comparing the sequences identified from the 18S rRNA, the 18 isolates obtained from the data accessible at NCBI were clustered into two major clades based on rRNA sequence similarities. The isolates were divided into various branches, but isolate MEUE1 was combined into one branch with *P. parvum* f. *patelliferum* isolates (Fig. 3).

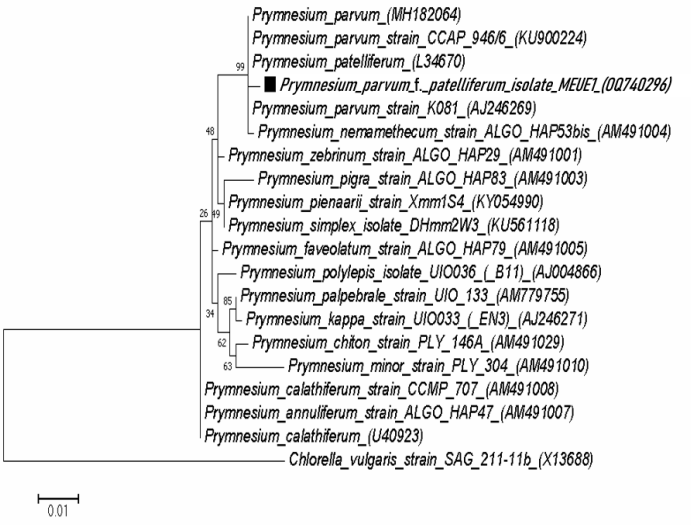


Figure 3. Molecular Phylogenetic analysis by Maximum Likelihood method. Neighbor-joining tree based on partial sequences of 18S rRNA region of the isolate MEUE1 (OQ740296) with each other for phylogenetic inference. Names and accession numbers are presented, as indicated in the NCBI GenBank. The percentage of replicate trees showing the taxa clustered in the bootstrap test (1000 replicates) is exhibited besides the branches. As an outgroup, *Chlorella vulgaris* was used. The square symbol represents the isolate (MEUE1).

4. Discussion

The haptophyte *Prymnesium parvum* f. *patelliferum* is reported first time in the Marmara Sea here. Due to the difficulty in identification of this species under a light microscope, the species, which was fixed with osmium tetraoxide, was identifed with scanning electron microscopy and it was verified with 18S ribosomal RNA. *P. parvum* was recorded in the adjacent Black Sea previously (BSPC Editorial Board, 2014). However, details of the species with electron or light microscopy is not present in this report.

Its presence in the Marmara Sea is a risk for mussel production, capture fishery, and mariculture activities (>4000 tons in Turkish waters; https://data.tuik.gov.tr/Bulten/Index?p=37252&dil=2) since this species may produce toxins (Bertin et al., 2012; Vasas et al., 2012; Luo et al., 2022; Yin et al., 2021).

Prymnesin, the toxin of *P. parvum,* is a collection of substances (Shilo and Sarig 1989) that has properties of ichthyotoxic, hemolytic, cytotoxic, non-dialysable (Yariv and Hestrin, 1961; Ulitzur and Shilo, 1964) in addition to proteinaceous, acid-labile and thermostable (Prescott, 1968). Hemolysin is suggested to be a structural constituent of *P. parvum* membranes and a lipopolysaccharide (Paster, 1968; Padilla, 1970). Prymnesin was also described by Paster (1973) as a glycolipid having high molecular weight witha structure like detergent (Watson, 2001).

Ichtyotoxin of *P. parvum* is harmful to gill-breathing organisms (e.g. mollusks, fish, and arthropods) (Paster, 1973) since it damages the permeability of gill tissues (Watson, 2001).

Since the toxin is acid-labile, it is inactivated in the stomach, intestines and liver. This can be a reason for not having any damaging effects on mammals. In mammals, the toxin has to pass through the digestive system before blood circulation, which is not the case in gill-breathers (Spiegelstein et al., 1969).

The toxicity of the species has not been measured in this study; however, it has been shown that several factors influence the toxicity of the species.

Ichtyotoxin production was found to be inversely correlated with salinity (Reich and Parnas, 1962). Optimal salinity range for toxin secretion in *P. parvum* was 0.3%-5% (Paster, 1973). However, Larsen and Bryant (1998) reported that the salinity did not significantly influence the toxicity. The surface salinity of the Marmara Sea is 23 ±2 psu (Ilıcak et al., 2021). Further studies related to changes in toxicity under different salinity ranges are necessary for this species isolated from the Marmara Sea.

There are conflicting statements about the impact of light on the toxicity of the species. In one study, the light was reported to augment toxin production in this species (Shilo and Aschner, 1953), while in the other one, toxicity was reported to be increased when the species was grown in the dark (pH: 7.0 - 7.1) than in the light-grown cultures (pH: 8.0-8.1) (Reich and Parnas, 1962). The high pH of light-grown cultures was attributed to photosynthesis. UV light was reported to cause inactivation of the toxin in this species (Parnas et al., 1962).

The toxicity of *P. parvum* was found to be diminished promptly at a pH lower than 7.5 and lost its activity entirely at pH 6 (Shilo and Aschner, 1953, McLaughlin, 1958). However, the toxicity was restored when the solution returned to neutral pH (McLaughlin, 1958). A positive correlation between pH and toxicity was reported in another study as well (Ulitzur and Shilo, 1964). Thus, in the present investigation, survival of only *P. parvum* f. *patelliferum* in the flasks enriched with $NaHCO_3$, which have a higher pH (8) than the culture medium (pH 7-7.5) before the addition of sodium bicarbonate could be possibly related to toxin production of the species with increasing pH. The growth of the natural phytoplankton community taken from the Baltic Sea was either suppressed or inhibited upon adding cell-free filtrate of *P. parvum,* showing an allelopathic effect (Fistarol et al., 2003). Unfortunately, in the present research the toxicity of the species was not measured. However, a pH value of 8 does not appear to be a reason for the death of other species, but the survival of *P. parvum* in the cultures amended with $NaHCO_3$. This pH (8) was generally observed in the Marmara Sea, and the seawater contained a variety of phytoplankton species, in previous studies. For instance, the pH of seawater in the Marmara Sea was reported to change between 7.96 and 9.01 in the monthly samples during June 2020 - May 2021 (Ergül et al., 2021). Yümün and Kam (2021) reported that the pH of the seawater was in the range of 7.97-8.15, while it was between 6.70-6.94 in the mucilaginous layer in Tekirdağ, Marmara Sea in the summer of 2021. The average pH of seawater in İzmit Bay in the seasonal samples between December 2016 and September 2017 was 7.52 (Morkoyunlu Yucel and Altundag, 2020).

Decreased phosphate concentration caused a rise in toxicity in different locations (Dafni et al., 1972; Kaartvedt et al., 1991; Larsen et al., 1993; Simonsen and Moestrup, 1997). This increase was speculated to be related to disruption in the production of membrane phospholipids that may cause transudation (Dafni et al., 1972). A decline in phosphate also coincided with the swelling of the cells. This can be due to leakiness (Dafni et al., 1972). Similarly, our cells were more spherical and swollen in the late stationary phase cultures.

Johansson and Graneli (1999) observed that the toxicity of *P. parvum* rose with both nitrogen and phosphorus limitation. It was suggested that the purpose of toxin production could be due to the requirement to wipe out competition under nutrient deficiency (Johansson and Graneli, 1999).

Potentially toxic microalgal species have previously been reported in the Marmara Sea, especially in Izmit Bay, due to industrial development and intense urbanization (Taş et al., 2016). Blooms of *Noctiluca scintillans* (Artüz and Baykut, 1986), *Prorocentrum scutellum* (Tas and Okus, 2004), *Prorocentrum* spp. (Aktan et al., 2005), *Prorocentrum micans* (Ergül et al., 2014) were reported in this region previously. Another area subjected to heavy pollution since the 1950s is located in the junction between the Marmara Sea and the Bosphorus Strait in the Golden Horn Estuary. Although a restoration in this ecosystem was observed after the remediation efforts performed in the estuary in 1997, harmful algal blooms such as *Pseudo-nitzschia calliantha* and *Heterosigma akashiwo* continue to occur (Taş et al., 2016; Taş, 2019). A recent study reports the presence of a potentially toxic diatom species, *Nitzschia navis-varingica,* in İzmit Bay (Eker-Develi and Kideys, 2022).

Global warming, eutrophication and pollution devastate marine ecosystems in different ways. One result is the large-scale mucilage events, and another is the occurrence of toxic or harmful algae. Both were witnessed in this study. How much the contribution of this species to mucilage development is uncertain. However, *P. parvum* has a mixotrophic and opportunistic character which is a potent risk factor for the gill-breathing organisms in the environment.

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