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Metagenomic Characterization of Planktonic Communities During a Mucilage Event in the Çanakkale Strait (Dardanelles), Turkey

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Abstract: The present study investigates the planktonic communities through metagenomics sequencing during a mucilage event in the Çanakkale Strait (Dardanelles), Turkey. Mucilage samples were collected in May 2021 during an intense period of mucilage formation in three different stations of Dardanelles (Station 1: 40°9'8.09"N, 26°24'16.19"E; Station 2: 40°6'21.62"N, 26°22'41.25"E; Station 3: 40°6'42.78"N, 26°23'57.00"E). The dominant planktonic eukaryotes, at the phylum level, were Dinoflagellata (38.57%), Protalveolata (15.03%), Diatomea (12.41%), Nematozoa (8.44%), Apicomplexa (6.79%) and Chlorophyta (5.43%), which constituted 86.68 % of the total number of sequences. The most dominant OTUs (>10%), were *Alexandrium* and Syndiniales Group II. other dominant OTUs (>2%) were *Viscosia* sp., *Lankesteria*, *Arcocellulus*, *Thalassiosira* and *Nannochloris*. This study has clarified the situation of planktonic communities during a mucilage event in the Çanakkale Strait (Dardanelles), Turkey. As a result, the most dominant genus was *Alexandrium*, which has been known to produce mucilage. Some *Alexandrium* species can produce toxins, cause severe impacts on human health, and lead to bivalve, shrimp, and fish mortality. Therefore, a more detailed study is needed to determine the *Alexandrium* toxins in the mucilage structure. In addition, the heavy metal content of the obtained mucilage was investigated, and the concentrations of the As and Cr are above the disposal limits in the landfill sites. Therefore, collected mucilage from the sea surface should be checked before sending it to landfill sites in terms of the heavy metal content.

Keywords: Çanakkale strait (dardanelles), eukaryotic biodiversity, heavy metal, metagenomic sequencing, mucilage.

Çanakkale Boğazı'nda (Dardanel) Müsilaj Oluşumu Sırasında Planktonik Toplulukların Metagenomik Karakterizasyonu, Türkiye

Öz: Bu çalışmada Çanakkale Boğazı'nda (Dardanel) müsilaj oluşumu sırasında planktonik toplulukların kompozisyonları metagenomik yaklaşımla araştırılmıştır. Müsilaj örnekleri, Çanakkale Boğazı'nın üç farklı istasyonunda (İstasyon 1: 40°9'8.09"K, 26°24'16.19"D; İstasyon 2: 40°6'21.62"K, 26°22'41.25"D; İstasyon 3: 40°6'42.78"K, 26°23'57.00"D) yoğun müsilaj oluşumu gözlemlenen Mayıs 2021'de toplanmıştır. Filum düzeyinde toplam sekans sayısının 86.68% ini kapsayan baskın planktonik ökaryotlar, Dinoflagellata (%38,57), Protalveolata (%15,03), Diatomea (%12,41), Nematozoa (%8,44), Apicomplexa (%6,79) ve Chlorophyta (%5,43) olarak belirlenmiştir. En baskın OTU'ler (>%10), *Alexandrium* ve Syndiniales Group II olup, diğer baskın OTU'lar (>%2) ise *Viscosia* sp., *Lankesteria*, *Arcocellulus*, *Thalassiosira* ve *Nannochloris* olmuştur. Bu çalışma, Türkiye'de meydana gelen bir müsilaj olayı sırasında Çanakkale Boğazı'nda planktonik toplulukların durumunu netleştirmiştir. Sonuç olarak, en baskın cinsin müsilaj üretme kabiliyeti olduğu bilinen *Alexandrium* olduğu belirlenmiştir. Bazı *Alexandrium* türleri toksin üretebilir, insan sağlığı üzerinde ciddi etkilere neden olabilir ve çift kabuklu, karides ve balık ölümlerine yol açabilir. Bu nedenle müsilaj yapısındaki *Alexandrium* toksinlerinin belirlenmesi için daha detaylı bir çalışmaya ihtiyaç vardır. Ayrıca elde edilen müsilajın ağır metal içeriği araştırılmıştır. Müsilajın yapısındaki As ve Cr konsantrasyonları atıklar için belirlenen düzenli depolama sahalarına bertaraf etme sınırlarının üzerindedir. Bu nedenle deniz yüzeyinden toplanan müsilajın düzenli depolama sahalarına gönderilmeden önce ağır metal içeriği kontrol edilmelidir.

Anahtar kelimeler: Ağır metal, çanakkale boğazı (çanakkale), metagenomik dizileme, müsilaj, ökaryotik biyoçeşitlilik.

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INTRODUCTION

The Dardanelles (Çanakkale Strait), a strait that separates Asian and European continents and connects the Aegean Sea and Sea of Marmara, is a significant watercourse for the international vessels. There are two major currents in the strait. The first one is a surface current flowing from the Black Sea to the Sea of Marmara through the Bosphorus and the Agean Sea through the Dardanelles. The second one is the undercurrent which flows from the Agean Sea to the Black Sea (Artüz et al., 2007; Yücel & Tarhan 2019). The pollution load of the Sea of Marmara increased incrementally, thus affecting the Dardanelles. The growth of population and industrialization on the seaside, insufficient treatment of domestic and industrial wastewater (Artüz et al., 2007; Okus et al., 2008), climate change, and global warming (Balkis et al., 2011) led to severe biologic problems in the marine environment. Consequently, intense mucilage has been found in Turkish seas in recent years for the first time. The mucilage problem, which was recorded in the Sea of Marmara (Aktan et al., 2008; Tüfekçi et al., 2010; Balkis et al., 2011) and the Dardanelles (Yentür et al., 2013) between 2007-2008 for the first time, reached a critical level to affect fishery, tourism, and social life in the first two quarters of 2021.

It has been reported that mucilage remarkably decreased the density of zooplankton groups, which are critical nutritional sources for larvae of marine species both in the Bosphorus (Okyar et al., 2015) and the Dardanelles (Yentür et al., 2013). Besides, research conducted between December 2020 and March 2021 reported the impacts of mucilage on endangered coral *Cladocora caespitosa*, which are essential for the Dardanelles and coral-rich habitats in Eceabat and Nara regions, which result in coral mortalities (Özalp, 2021).

It was reported that *Chrysophaeum taylorii*, which spreads through tropical and subtropical Atlantic and Western Pacific shores and produces mucilage in benthic areas, produced mucilage for the first time in the benthic area in the Aegean Sea in May 2011 (Aktan & Topaloğlu 2011). In another study conducted on the shores of Büyükada Island (the Marmara Sea) in 2008, it was detected that *Clindrotheca closterium, Pseudo-nitzschia sp., Skeletonema costatum, Thalassiosira rotula,* and *Gonyaulax fragilis* species were the dominant species in the mucilage structure (Balkis et al., 2011). In June 2007, when mucilage structure is observed in the north-eastern Sea of Marmara, it was observed that *Gonyaulax hyalina* and *Thalassiosira gravida* species, which are known to cause mucilage, were dominant (Tas et al., 2020). Moreover, the research conducted in the Sea of Marmara and the Gulf of İzmit between October 2007 and February 2008 reported that *Gonyaulax fragilis*, *Skeletonema costatum* and *Cylindrotheca closterium* were the dominant species of the mucilage structure (Tüfekçi et al., 2010). Yet information on the diversity of planktonic communities in the mucilage structure from the Sea of Marmara ecosystem is limited only to microscopic descriptions.

This study researches metagenome analysis of the mucilage structure reaching a critical level in the Dardanelles in the first two quarters of 2021, distribution of planktonic communities, and heavy metal content of the mucilage.

MATERIALS AND METHODS

Samples and Heavy Metal Analysis: Sampling was performed as part of three nearly simultaneous expeditions to different regions of the Dardanelles (Station 1: 40°9'8.09"N, 26°24'16.19"E; Station 2: 40°6'21.62"N, 26°22'41.25"E; Station 3: 40°6'42.78"N, 26°23'57.00"E) in May 2021. Sampling points in the Dardanelles is shown in Figure 1. Samples were collected using a 5L Niskin bottle at the maximum depth of 2 m according to the ISO 5667-9 method for the analyses by means of the Turkish Coast Guard ship in Çanakkale (ISO 5667-9, 1992). The mucilage samples were concentrated after centrifuging (Hettich, Rotofix 32A, Germany) at 6000 rpm for 10 min and then filtered through a pre-weighed glass fiber filter (Merck Millipore, AP40, Germany). The filter was heated to constant mass at 70 ± 1º C for 24 h and then weighed to determine the dry solid. The filters and a blank filter with 9 ml HNO_3 and 1 ml of H_2O_2 were digested in pressurized digestion vessels using a microwave oven (CEM, MARS-6, USA). The temperature was ramped to 210 °C within 15 minutes and held for 20 minutes at a constant microwave digestion temperature. Digested samples were diluted after cooling and centrifuging for soluble metal analysis. Soluble heavy metals in the diluted samples were analyzed by an inductively coupled plasma-optical emission spectrometry (ICP-OES / Agilent 5110 Dual View, USA).

DNA Extraction, PCR Amplification and Bioinformatics Analysis: Total DNA was extracted from the mucilage samples using GeneMATRIX Kit (EURx Poland) according to the manufacturer's instructions. The eukaryote nuclear 18S rRNA gene was amplified by PCR, using the eukaryotic-specific primers 18S-566F CAGCAGCCGCGGTAATTCC and 18S-1200R CCCGTGTTGAGTCAAATTAAGC (Hadziavdic et al., 2014).

Figure 1. Sampling points in the Dardanelles (Google Earth Map).

The first PCR amplification was performed in 25 μL volume, containing 10 μL of 2X KAPA HotStart ReadyMix (Roche, Switzerland), 5 μL of each primer (1 μM), and 2.5 μL of template DNA. Thermal cycling conditions were: 95 °C for 3 min followed by 25 cycles of 95 °C for the 30s, 55 °C for 30 s, 72 °C for 30 s, and a 5 min extension at 72 °C and a final hold at 4 °C. The second PCR amplification was performed in 50 μL volume, and reactions contained 25 μL of KAPA HiFi HotStart ReadyMix, 5 μL Nextera XT1 (N7xx), 5 μL Nextera XT2 (S5xx), 5 μL of cleaned PCR product and 10 μl PCR Grade water. The second thermocycling conditions were: 95°C for 3 min followed by 8 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a 5 min extension at 72°C and a final hold at 4°C. The sequencing (2 × 250 bp) was performed on the MiSeq platform. The processing and quality control was conducted using DADA2 (Callahan et al., 2016). Chimera check was conducted with DADA2. Amplicons with a quality score of more than 20 were retained, and amplicons were filtered and trimmed with DADA2. Taxonomic classification was performed against the SILVA 138 ribosomal RNA gene database (Quast et al., 2012).

RESULTS AND DISCUSSION

***Planktonic community compositions*:** The dominant planktonic eukaryotes, at the phylum level, were Dinoflagellata (38.57%), Protalveolata (15.03%), Diatomea (12.41%), Nematozoa (8.44%), Apicomplexa (6.79%) and Chlorophyta (5.43%), which constituted 86.68 % of the total number of sequences (Figure 2).

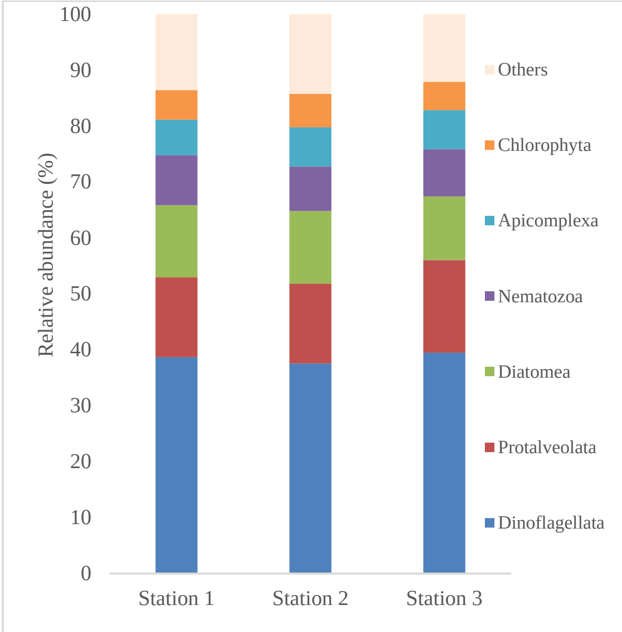

Figure 2. Planktonic community compositions during the mucilage event.

The most dominant OTUs (>10%) (Table 1) were *Alexandrium* with 25442 reads (35.36%), Syndiniales_Group_II with 7958 reads (11.06%). Other dominant OTUs (>2%) (Table 1) were *Viscosia* sp with 6305 reads (8.76%), Lankesteria with 4217 reads (5.86%), *Arcocellulus* with 3719 reads (5.17), *Thalassiosira* with 2133 (2.96) and *Nannochloris* with 1753 reads (2.44%).

Table 1. Top seven of planktonic eukaryotes. Results are given at species level or a higher taxonomic level if the sequences could not be assigned to a species.

	Dominant OTUs (%)
Alexandrium (**Dinophyceae**)	35.36
Syndiniales_Group_II (**Protalveolata**)	11.06
Viscosia sp (**Enoplea**)	8.76
Lankesteria (**Apicomplexa**)	5.86
Arcocellulus (**Diatomea**)	5.17
Thalassiosira (**Diatomea**)	2.96
Nannochloris (**Chlorophyta**)	2.44

A plethora of studies has been conducted on planktonic communities in water sources with metagenomic approaches. In the present study conducted in May 2021, when intense mucilage was observed, the dominant group was found to be the *Alexandrium* genus. Among many dinoflagellate species, the *Alexandrium* genus, in particular, produces toxins resulting in problems on human and environmental health as well as tourism economies (Vingiani et al., 2020). It was reported that *Alexandrium* species have the capability to produce mucilage and lead to shrimp and fish mortality in regions with algae bloom (Landsberg et al., 2002; Lewis et al., 2018). *Alexandrium minutum* is the most common toxic species in the western Mediterranean Sea (Vila et al., 2001). It caused larval mortality of some bivalve species and reduced valve gape and clearance rate of large-sized class bivalves (May et al., 2010). The presence of *Alexandrium* species in the water column and sediment in the Gulf of Gemlik (the Sea of Marmara) between 2011-2012 was reported (Balkis et al., 2016; Balkis & Taş 2016). *A. minutum* cysts were determined in the sediments of Izmir Bay (Aydın et al., 2011; Aydın & Uzar, 2013). Moreover, vegetative forms of *A. minutum* were reported from the Marmara Sea (Balkis, 2004; Tüfekçi et al., 2010).

This study reports the second most dominant group as Syndiniales Group_II: endosymbionts of tintinnid ciliates, crustaceans, fish, protozoa, algae, and other dinoflagellates in particular (Luo et al., 2016). All the *Syndiniales* species identified have been reported to kill their hosts, including other protists and metazoans, and then release dinospores (Coats & Park., 2002; Guillou et al., 2008; Clarke et al., 2019). For this reason, *Syndiniales* species can potentially affect plankton population dynamics and biogeochemical cycling. The research in the literature has demonstrated that parasitic *Syndiniales* species assume the role of biological control agent on dinoflagellates that increase by algae bloom (Chambouvet et al., 2008; Mazzillo et al., 2011). As proof of this finding and parallel to the present study, 27.70% dinoflagellata and 12.81% parasitic Syndiniales Group I were dominant groups in polluted waters (Thessaloniki Bay, Greece) (Tsipas, 2020).

In this study, free-living marine nematode *Viscosia* sp. was detected in mucilage structure. In a similar vein, it was found among dominant nematode species in the Ligurian Sea, North-West Mediterranean (Moreno et al., 2009), Strait of Sicily, Central Mediterranean Sea (Sandulli et al., 2015), Aegean Sea, Eastern Mediterranean (Lampadariou & Eleftheriou, 2018) and the Black Sea (Ürkmez et al., 2016).

Gregarines, a large group of Apicomplexans, live in the body cavities of invertebrates, and some species attach to the epithelial tissues of the host, invading the intestinal lumen. In contrast, others invade the reproductive systems (Leander et al., 2016). As a member of this group, Lankesteria was found present in the mucilage structure at a rate of 5.86%. Lankesteria species were isolated in ascidians on the Central California Coast (Levine et al., 1981), and they were reported to cause large-scale infection in the ascidian *Ciona intestinalis* species (Mita et al., 2012). No report of Lankesteria species has been found in the literature for the Sea of Marmara and the Aegean Sea. However, the closest information to the present study area is *Lankesteria metandrocarpae* species in Varna Bay (the Black Sea) (Dzhembekova et al., 2017).

In this study, two Diatomea genus *Arcocellulus* and *Thalassiosira*, were detected in mucilage structure. Similarly, they were dominant groups during algae bloom periods in the Mediterranean Sea (Percopo et al., 2011). *Thalassiosira* species were reported among the most abundant diatom species forming resting stages (Montresor et al., 2013). Parallel to the present study, Tsipas (2020) reported that *Thalassiosira* was found among the dominant groups in polluted Thessaloniki Bay (Greece) at a rate of 2.41%.

It has been reported that the green algae *Nannochloris* genus belonging to the chlorellaceae family, identified among the dominant groups in the mucilage structure in the present study, contains species that lead to ecosystem disruptive algae blooms (Zhang et al., 2015; Mercado et al., 2021). Dzhembekova et al. (2017) detected the presence of *Nannochloris sp.* in Varna Bay (the Black Sea), though not intense, in their research on harmful algae. However, there has been no report of an algae bloom caused by *Nannochloris* species in Turkish waters in the literature.

This study determines planktonic communities in the mucilage structure, which started to be observed in Turkey's water column, surface, and bottom at the beginning of 2021 and reached severe levels as of May. The finding is that the *Alexandrium* genus, a dinoflagellate, and the Syndiniales Group_II, which is known to act as a biological control agent on the species belonging to this genus, are among the dominant groups is particularly significant. *Alexandrium* species have been known the capability to produce mucilage. Some *Alexandrium* species can cause severe impacts on human health and lead to bivalve, shrimp and fish mortality. Therefore, a more detailed study is needed to determine the *Alexandrium* toxins in the mucilage structure.

The heavy metal content of the mucilage**:** There is limited information on the heavy metal concentration of mucilage in the literature. Therefore, the heavy metal content of the obtained mucilage from the Dardanelles was defined in this study. On the other hand, the collected mucilage from the surface of the Marmara Sea was sent to the landfill site for disposal. Thus, the concentration of the heavy metals in the obtained mucilage is crucial for environmental concerns. The microorganisms in the mucilage, such as microalgae and bacteria, have negative functional groups on their surface. These groups are more like to bind positive ions (i.e. heavy metals) in the sea. Due to this reason, the accumulation of heavy metals in the mucilage can occur. The heavy metal concentration of the mucilage and limitations for landfilling are given in the Table 2. The limit concentrations of heavy metals in non-hazardous waste in the Regulation on Landfilling of Wastes by the Republic of Turkey Ministry of Environment and Urbanization are 2, 100, 1, 10, 50, 0.2, 10, 10, 10, 0.5 and 50 mg/kg dry solid for As, Ba, Cd, Cr, Cu, Hg, Mo, Ni, Pb, Se and Zn, respectively (TMEU, 2019).

Table 2. The heavy metal concentration (mg/kg) of the mucilage and limitations for landfilling

Stations	As	Ba	Cd	Cr	Cu	Hg	Mo	Ni	Pb	Ba	Zn
Station 1	**3.10**	0.03	0.18	**10.80**	13.12	0.14	5.59	7.67	2.37	ND	0.77
Station 2	**4.45**	ND	0.12	**21.77**	1.08	0.18	7.96	7.22	2.06	ND	ND
Station 3	ND*	ND	ND	6.72	6.09	ND	4.73	3.71	ND	ND	ND
Limit Conc. **	**2.00**	100.00	1.00	**10.00**	50.00	0.20	10.00	10.00	10.00	0.50	50.00

*ND: under the detection limit

** Limit concentrations of heavy metals in landfilling of non-hazardous waste

The third sampling point (Station 3) is very close to the residential area. The water movement is very limited in that area as well; thus, the heavy metal content of the Station 3 could be lower than the other stations. According to the results, the total concentrations of all heavy metals in the mucilage complied with the regulation, except for As and Cr. Therefore, the collected mucilage from the sea surface should be checked before sending it to landfill sites in terms of the heavy metal content.

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