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early view

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Health assessment of fan mussels in the southern part of the Sea of Marmara

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Abstract

The fan mussel (*Pinna nobilis* Linnaeus, 1758), a critically endangered species in the Mediterranean Sea, has experienced mass mortality events (MME) due to various pathogens, particularly the protozoan parasite *Haplosporidium pinnae*. Despite these challenges, the Sea of Marmara (SoM) still has significant populations of fan mussels, with no infection-related MME reported so far. This study aimed to assess the general health status of fan mussels in the SoM and understand the factors that contribute to their survival. The samples were collected on September 25, 2023, around the Kapıdağ Peninsula in the southern part of the SoM, a critical area for fan mussel populations, where a total of 20 live specimens were included in advanced laboratory analysis from four stations. Findings revealed that *Haplosporidium* and *Mycobacterium* species were present in *P. nobilis* across all stations, with higher oxidative stress indicators in the digestive gland tissues. Results also observed different life stages of *Haplosporidium* in the mantle and digestive gland. Molecular analyses revealed the presence of haplosporidian parasites, *Perkinsus* sp., and *Mycobacterium* sp. in some samples. Phylogenetic analysis showed that *Haplosporidium* isolates form a distinct clade separate from *H. pinnae*, suggesting they belong to a different species. The detected *Haplosporidium* species in the SoM displayed genetic differences from *H. pinnae* found in the Mediterranean Sea, potentially indicating a less virulent variant. This study raised the question of whether interactions between the detected *Haplosporidium* parasite and other pathogens or region-specific ecological characteristics were effective in the absence of a mass mortality in fan mussels in the SoM.

Keywords: *Pinna nobilis*; critically endangered; mass mortality event; Haplosporida; Mediterranean Sea.

Introduction

The fan mussel (*Pinna nobilis* Linnaeus, 1758) is the largest bivalve species inhabiting the Mediterranean Sea basin (Vicente, 1990). Its range goes from the coast of Spain to the Sea of Marmara (SoM) (Vázquez-Luis *et al*., 2017; Karadurmuş *et al*., 2024), and it can be found in many different types of marine habitats, such as seagrass meadows, rocky substrates, and sandy, muddy, and rhodolite beds (Katsanevakis, 2006; Kersting & García-March, 2017). Individuals can attain considerable shell heights of up to 120 cm and live for up to five decades (Zavodnik *et al*., 1991; Rouanet *et al*., 2015). Capable of filtering high volumes of seawater (Trigos *et al*., 2014), the fan mussel contributes to the removal of suspended particles, including detritus and planktonic organisms, thereby enhancing water clarity and nutrient cycling within its habitat (Basso *et al*., 2015). The extensive surface area of its shell provides a substratum for a diverse array of sessile organisms, fostering local biodiversity and marine ecosystems (Davenport *et al*., 2011).

A mass mortality event (MME) that started in 2016 was found to have affected fan mussels in Spain and subsequently spread throughout the Mediterranean Sea basin (Vázquez-Luis *et al*., 2017; Kersting *et al*., 2019; Katsanevakis *et al*., 2021). Due to the spreading of MMEs and significant reductions in stock sizes, the World Union for Conservation of Nature (IUCN) has classified the species as "Critically Endangered" (Kersting *et al*., 2019). A protozoan species, *Haplosporidium pinnae*, has been reported to be involved in the initial stages of the MME (Vázquez-Luis *et al*., 2017; Catanese *et al*., 2018). However, it has been suggested that bacterial agents such as *Mycobacterium* and *Vibrio* may be responsible for in-

fections in addition to *H. pinnae* (Catanese *et al*., 2018; Grau *et al*., 2022; Carella *et al*., 2023). Additionally, recent studies indicate that a viral infection impairing the immune system in fan mussel populations may be the primary factor contributing to MMEs in the Mediterranean (Carella *et al*., 2023; Carella *et al*., 2024). The MME of fan mussels has been observed in the Mediterranean Sea basin, and both the presence of the parasite and bacterial infections have recently been reported from Çanakkale Strait (Künili *et al*., 2021). In addition to the *H. pinnae* parasite detected by both histopathological and genetic methods, the study also revealed the presence of infections caused by different *Vibrio* species, which had not been previously reported in the region (Künili *et al*., 2021).

The SoM, located in the easternmost part of the Mediterranean Sea basin, still hosts the last known living high-density fan mussel populations (Karadurmuş & Sarı, 2022; Karadurmuş *et al*., 2024; Acarlı *et al*., 2024). Although dead individuals have been reported in various studies (Öndes *et al*., 2020a; Çınar *et al*., 2021; Acarlı *et al*., 2022; Karadurmuş & Sarı, 2022; Karadurmuş *et al*., 2024), there is no evidence of infection-related deaths in the SoM. The main cause of recent deaths has been attributed to extreme weather conditions and anthropogenic impacts (Öndes *et al*., 2020b; Karadurmuş & Sarı, 2022; Karadurmuş *et al*., 2024). This study aims to assess the general health status of critically endangered fan mussels in the SoM and their ecosystem in their natural habitat. Concept of the study provides initial assessments of the infection status of fan mussel samples in critical areas, as well as analysis of physicochemical parameters and microbiological water quality at various stations. This study contributes to a better understanding of the mechanisms driving survival in the SoM, as well as the development of targeted conservation strategies.

Materials and Methods

Sampling procedure

The study was conducted from July to December 2023. Samples were collected on September 25, 2023, in the surroundings of the Kapıdağ Peninsula, situated in the southern part of the SoM (Fig. 1). This area was chosen due to its significance as a hotspot for fan mussel populations, as indicated by previous studies (Karadurmuş & Sarı, 2022; Acarlı *et al*., 2024). Three primary criteria guided the selection of study sites:

(1) Düzler: the area that previously hosted the densest fan mussel populations (Karadurmuş & Sarı, 2022),

(2) Erdek: the only station in the SoM where MMEs are known to occur (Karadurmuş & Sarı, 2022),

(3 & 4) Doğanlar and Karşıyaka: Limitation areas of the coastline along the Kapıdağ Peninsula where living individuals are completely absent (Karadurmuş *et al*., 2024).

A total of 20 live fan mussel specimens were collected from four sites within the study area (Fig. 1) by SCUBA diving, with sampling depths ranging from 1 to 9 meters. The sampling strategy covered a variety of habitats inhabited by fan mussels, classified according to criteria established by the International Union for Conservation of

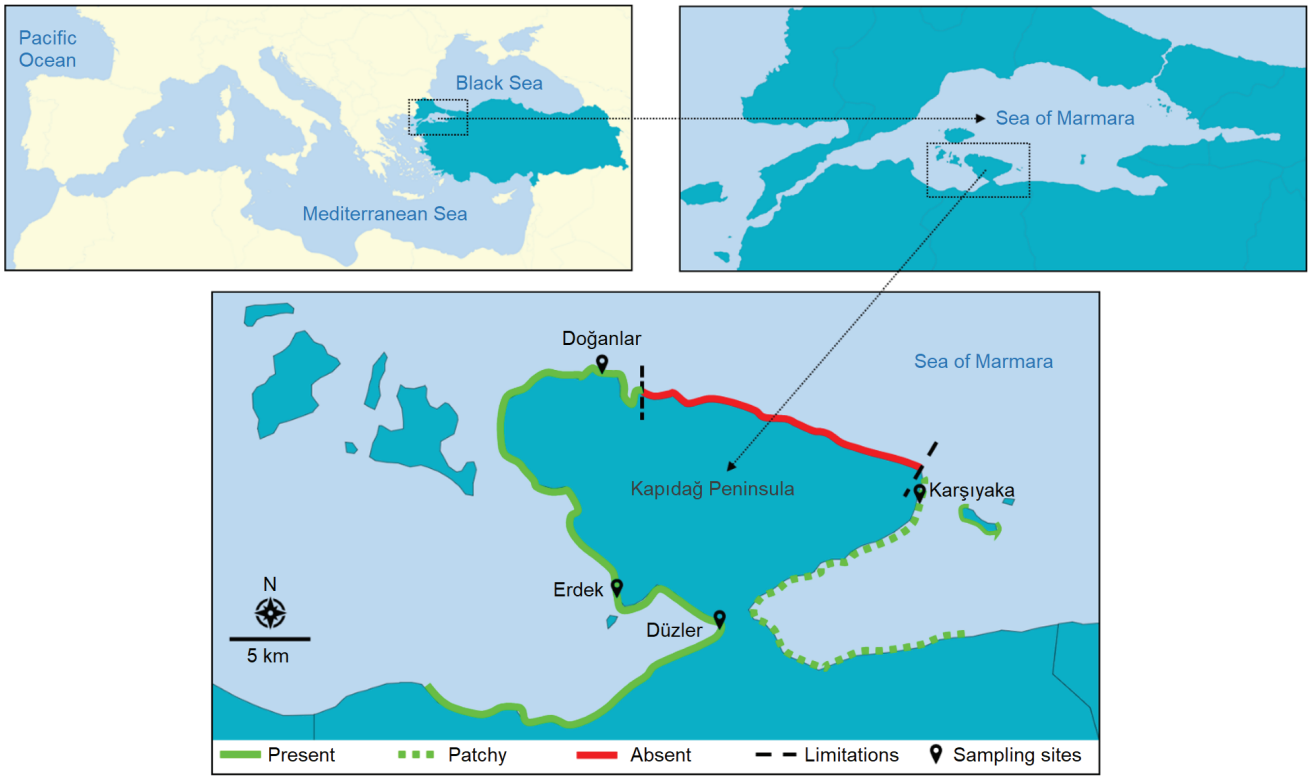

Fig. 1: Map of the study area and sampling points around the Kapıdağ Peninsula (Thick solid and dashed lines give information about the presence of *Pinna nobilis*).

Nature (IUCN, 2024). Adult samples (Richardson *et al*., 1999) were selected within a uniform size range (20< total shell height <24) to standardize diagnostic procedures and protect the juveniles. Juveniles may exhibit higher survival rate against pathogens and better tolerate MMEs (Šarić *et al*., 2020). This study does not include hypotheses about size-dependent health status. The underwater measurements were estimated based on the height and width of the unburied part of the shell (García-March *et al*., 2002), and measurements were made with a long jaw caliper. The sample set comprised fan mussels categorized into two health conditions based on underwater clinical evaluation (Künili *et al*., 2021; Grau *et al*., 2022; Šarić *et al*., 2020):

Asymptomatic (Asx): These individuals exhibited typical healthy responses when approached or touched and had normal mantle appearance.

Sick: This category included individuals that displayed clinically unhealthy/abnormal behavior, characterized by a lack of response to stimuli, along with a pale and retracted mantle and sluggish valve closure.

The inclusion of both clinically Asx and Sick specimens enabled a comprehensive assessment of the infection and/or general health status of fan mussels across the individuals. Environmental parameters crucial for assessing the health and distribution of fan mussels, including sea surface temperature (SST in °C), salinity (SAL in ppt), dissolved oxygen (DO in %), and pH, were measured and recorded using a YSI® ProDss multimeter. Freshly collected specimens were transported to Çanakkale Onsekiz Mart University for subsequent laboratory analyses, including assessments of oxidative stress parameters, microbiological evaluations, and histopathological tests. Transportation was conducted in a cooler box, with specimens separated according to sampling sites. The duration between sampling and the commencement of dissection processes was approximately three hours. Various detailed information about the samples and sampling stations is presented in Table 1.

Approval from research ethics committees was not

Table 1. Details on collected *Pinna nobilis* specimens and various features of sampling sites.

Specimen code	Depth & Habitat	Clinical sign*	Station & Coordinate	Water quality parameters**
Sp1	1.5 m – Seagrass	Sick	Karşıyaka 40.43966° N, 27.99791° E	SST: 26.2 °C DO: 95 L% SAL: 23.38 ppt pH: 8.57
Sp2	3.5 m – Seagrass	Sick		
Sp3	5.0 m – Shellfish	Sick		
Sp4	6.0 m – Seagrass	Asx		
Sp5	7.0 m – Seagrass	Asx		
Sp6	9.0 m – Sandy	Sick	Düzler 40.37716° N, 27.87886° E	SST: 24.8 °C DO: 97 L% SAL: 23.82 ppt pH: 8.48
Sp7	6.0 m – Sandy	Sick		
Sp8	4.0 m – Seagrass	Sick		
Sp9	2.0 m – Seagrass	Asx		
Sp10	1.0 m – Sandy	Asx		
Sp11	4.5 m – Seagrass	Sick	Doğanlar 40.51852° N, 27.74888° E	SST: 25.7 °C DO: 96 L% SAL: 24.03 ppt pH: 8.51
Sp12	4.0 m – Sandy	Sick		
Sp13	2.5 m – Sandy	Asx		
Sp14	1.0 m – Sandy	Asx		
Sp15	4.0 m – Seagrass	Sick		
Sp16	3.5 m – Muddy	Sick	Erdek 40.39113° N, 27.79602° E	SST: 24.3 °C DO: 97 L% SAL: 24.40 ppt pH: 8.46
Sp17	6.0 m – Muddy	Asx		
Sp18	4.5 m – Sandy	Sick		
Sp19	2.0 m – Sandy	Asx		
Sp20	2.5 m – Sandy	Asx		

* Asx: Asymptomatic

** SST: Sea surface temperature, DO: Dissolved oxygen, SAL: Salinity

required for this study because it involved experimental work with unregulated invertebrate species. The SoM is a Special Protected Area and biodiversity studies require a special research permit. Due to fan mussels being a strictly protected species (communique on regulation of commercial fishery: No: 2020/20), all samplings were carried out under the permission of national authorities (listed below in Ethics Declarations: Permissions).

Laboratory routines

Oxidative stress parameters

The gill and digestive gland tissues were flash-frozen with liquid nitrogen and preserved at -80°C until further analysis. Prior to assessment, the tissues underwent homogenization using a 50 mM phosphate buffer. Various oxidative parameters were evaluated, encompassing the activities of enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST), along with the determination of lipid peroxidation (MDA). The protein content in the tissues was quantified using the Bradford (1976) method. SOD activity was gauged based on the reduction of nitroblue tetrazolium with a peak absorbance at 550 nm (Flohé & Ötting, 1984). CAT activity analysis involved consecutive measurements every 15 seconds for approximately 90 seconds after the initial tissue readings (Claiborne, 1985). GST activity assessment utilized two measurements taken at 340 nm at different time intervals, with subsequent kinetic calculations (Gibson & Skett, 1986). Lipid peroxidation, indicative of oxidative damage, was determined by quantifying the amount of malondialdehyde (MDA), the final product of this reaction (Sushil *et al*., 1989).

Histological Analyses

The mantles and digestive glands of mussel samples (n = 20) collected from four different stations (Karşıyaka, Düzler, Doğanlar, and Erdek) were fixed in Davidson's fixative for 24 hours. Subsequently, the tissues were dehydrated in a progressive series of ethanol and embedded in paraffin. The tissues were then sliced to a thickness of 5 µm using a Leica rotary microtome. The histopathological sections were stained with hematoxylin-eosin (Bancroft & Gamble, 2008). Histopathological changes were assessed, and micrographs were captured using a CX31 Olympus light microscope equipped with a digital camera and DP2-BSW software.

Microbiological Analyses

Samples were treated by wiping the shells with alcohol-soaked cotton and then opened by cutting the adductor muscles using a sterile knife. From the intravalvular liquid, gill, digestive gland, and mantle, a total of 10 g of sample tissue was collected and transferred to 90 ml of peptone water solution (containing 2% NaCl and 1% bacterial peptone). After homogenization for 4 minutes at 4000 rpm using a Stomacher (Seward, model 400), the samples were decimally diluted, and a 1 ml aliquot from each dilution was spread onto three petri dishes.

For the microbiological analysis of seawater, the membrane filtration method described by American Public Health Association (APHA, 2005) was followed. Three sets of 1:1 dilution (v/v) with filtered sterile seawater were prepared, and then 100 ml of the diluted sample was filtered using a membrane filtration setup with a 0.45 µm pore size filter. The membrane filter was subsequently placed onto petri dishes. The culture media and incubation conditions used for all microbiological analyses were as follows: Marine Agar (Difco) was used at 25°C for 48 hours to enumerate viable total heterotrophic aerobic bacteria (THAB). Thiosulphate Citrate Bile Sucrose (TCBS) Agar (Merck) was used at 30°C for 36 hours to enumerate and isolate *Vibrio* species. Endo-Agar (Merck) was used at 35°C for 24-36 hours for the enumeration of *Enterobacteriaceae*. Plate Count Agar (Merck) supplemented with 2% NaCl was used at 30°C for the passing and short-term storage of bacterial strains isolated before molecular analyses.

Molecular Analyses

Genomic DNA extraction was performed from pure bacterial isolates and ethanol-fixed tissue samples, following different protocols. The pure isolates were incubated overnight in broth culture medium (Tryptic Soy Broth supplemented with 1% NaCl) and the extraction was performed using the PureLink Genomic DNA Mini Kit (Invitrogen). The ethanol-fixed tissue samples (approximately 50 mg wet weight) were first washed thoroughly with PBS (phosphate buffered saline), and then the extraction was performed with the Quick-DNA Fecal/Soil Microbe DNA Miniprep Kit (Zymo Research) using a tissue homogenizer (Bullet Blender Storm, Next Advance Inc.). The manufacturer's instructions were followed in the extractions, and the resulting genomic DNA was used as a template for PCR.

We used a universal primer set to identify the bacterial isolates through partial 16S rRNA gene sequencing. All sampled fan mussel tissues were also screened for microorganisms previously reported to be isolated from diseased mussels in the scientific literature, including *H. pinnae*, *Mycobacterium* sp., *Perkinsus* sp., and *Marteilia* sp., regardless of histopathology findings. All primer sequences and target organisms used in this study are given in Table 2.

The PCR mixture was the same in all reactions: approximately 40–50 ng of template DNA, and 0.4 µM each of primer, DreamTaq PCR Master Mix (2X) (Thermo Scientific), and nuclease-free water (Thermo Scientific). All amplifications were performed in a thermal cycler (Biometra TAdvanced - Analytik Jena AG) and programmed according to the primer pair used.

For the bacterial isolates, the cycler was programmed as initial denaturation at 95°C for 3 min, followed by 30

Table 2. The primer sequences and target organisms used in this study.

Primer name	Primer sequence (5'- 3')	Target organism	Ref.
S-D-Bact-0008-a-S-20	AGAGTTTGATCCTGGCTCAG	domain Bacteria	a
S-*-Univ-0536-a-A-18	GWATTACCGCGGCKGCTG	Universal	
HPN-F3	CATTAGCATGGAATAATAAAACACGAC	*Haplosporidium pinnae*	b
HPN-R3	GCGACGGCTATTTAGATGGCTGA	*Haplosporidium pinnae*	
mycgen-f	AGAGTTTGATCCTGGCTCAG	*Mycobacterium* sp.	c
mycgen-r	TGCACACAGGCCACAAGGGA	*Mycobacterium* sp.	
PerkITS-85	CCGCTTTGTTTGGATCCC	*Perkinsus* sp.	d
PerkITS-750	ACATCAGGCCTTCTAATGATG	*Perkinsus* sp.	
Pr4	CCGCACACGTTCTTCACTCC	*Marteilia* sp.	e
Pr5	CTCGCGAGTTTCGACAGACG	*Marteilia* sp.	

Ref. a: Suau *et al*., 1999; b: Catanese *et al*., 2018; c: Böddinghaus *et al*., 1990; d: Moss *et al*., 2008; e: Le Roux *et al*., 2001

cycles of amplification (denaturation at 95°C for 30 s; annealing at 56°C for 1 min; extension at 72°C for 1 min), and a final extension step of 72°C for 4 min. The cycler was programmed for *H. pinnae*-specific amplification as initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. For *Mycobacterium* screening, the following program was used: initial denaturation at 95°C for 10 min, followed by 35 cycles of amplification (denaturation at 95°C for 30 s; annealing at 50°C for 1 min; extension at 72°C for 1 min) and a final extension step of 72°C for 10 min. Likewise for *Perkinsus* sp., initial denaturation at 95°C for 4 min, followed by 40 cycles of amplification (denaturation at 95°C for 30 s; annealing at 55°C for 30 s; extension at 72°C for 30 s) and a final extension step of 72°C for 5 min. The cycler was programmed for *Marteilia* sp. screening as initial denaturation at 95°C for 5 min, followed by 40 cycles of amplification (denaturation at 95°C for 1 min; annealing at 55°C for 1 min; extension at 72°C for 1 min) and a final extension step of 72°C for 10 min. All PCR amplicons were loaded on a 1.4% (wt/vol) agarose gel in TAE buffer containing ethidium bromide (0.5 ug/ml). After electrophoresis, the size of the products was estimated against GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific) on a UV transilluminator. All amplicons were purified and sequenced bidirectionally by a sequencing company (Istanbul, Türkiye). Bioedit (v 7.0.5.3) software was used for sequence editing and analysis, and the Basic Local Alignment Search Tool (BLAST+ v 2.7.1) was used for sequence similarity calculation (Camacho *et al*., 2009). The nucleotide sequences obtained in this study were aligned using the ClustalW algorithm (Larkin *et al*., 2007) with the sequences in the GenBank database for phylogenetic analysis. The analysis was performed in MEGA7 software (Kumar *et al*., 2016) using the Maximum Likelihood method, based on the Tamura-Nei model (Tamura & Nei, 1993), with 1000 bootstrap replicates.

Statistical analyses

Statistical analyses were performed using SPSS 21.0 software. The normal distribution of the data was assessed with the Kolmogorov-Smirnov test, and the homogeneity of variances was tested using the Levene test. Parametric One-Way ANOVA and/or non-parametric Kruskal-Wallis tests were employed to compare enzyme analyses and MDA levels. Distinct letters or numbers were used to indicate significant differences among concentrations. The significance level (α) was set at 0.05 for all analyses.

Results

Oxidative stress parameters

The lowest SOD values were measured in the digestive gland tissues of samples collected from the Düzler and Doğanlar stations (mean 9.2 and 9.3 U/mg.protein, respectively). The highest values were observed in the gill tissues of samples from the Karşıyaka station (mean 20.9 U/mg.protein). The differences in SOD values among stations were not statistically significant ($F = 0.9$, $df = 3$, $P_{value} > 0.05$). In terms of SOD levels measured in gill and digestive gland tissues, it was consistently observed that the values in gill tissues were higher, and this difference was statistically significant ($F = 6.2$, $df = 1$, $P_{value} < 0.05$) (Fig. 2A).

The lowest CAT levels were observed in the samples from the Doğanlar station, particularly in the digestive gland (127 µmol/ mg.protein), and in the samples from the Erdek station, especially in the gill tissue (138 µmol/ mg.protein). Furthermore, when considering CAT levels, the observed differences between stations ($F = 2.69$, $df = 3$, $P_{value} > 0.05$) and tissues ($F = 2.75$, $df = 1$, $P_{value} > 0.05$) were not statistically significant either (Fig. 2B).

The enzyme levels of GST were found to be signif-

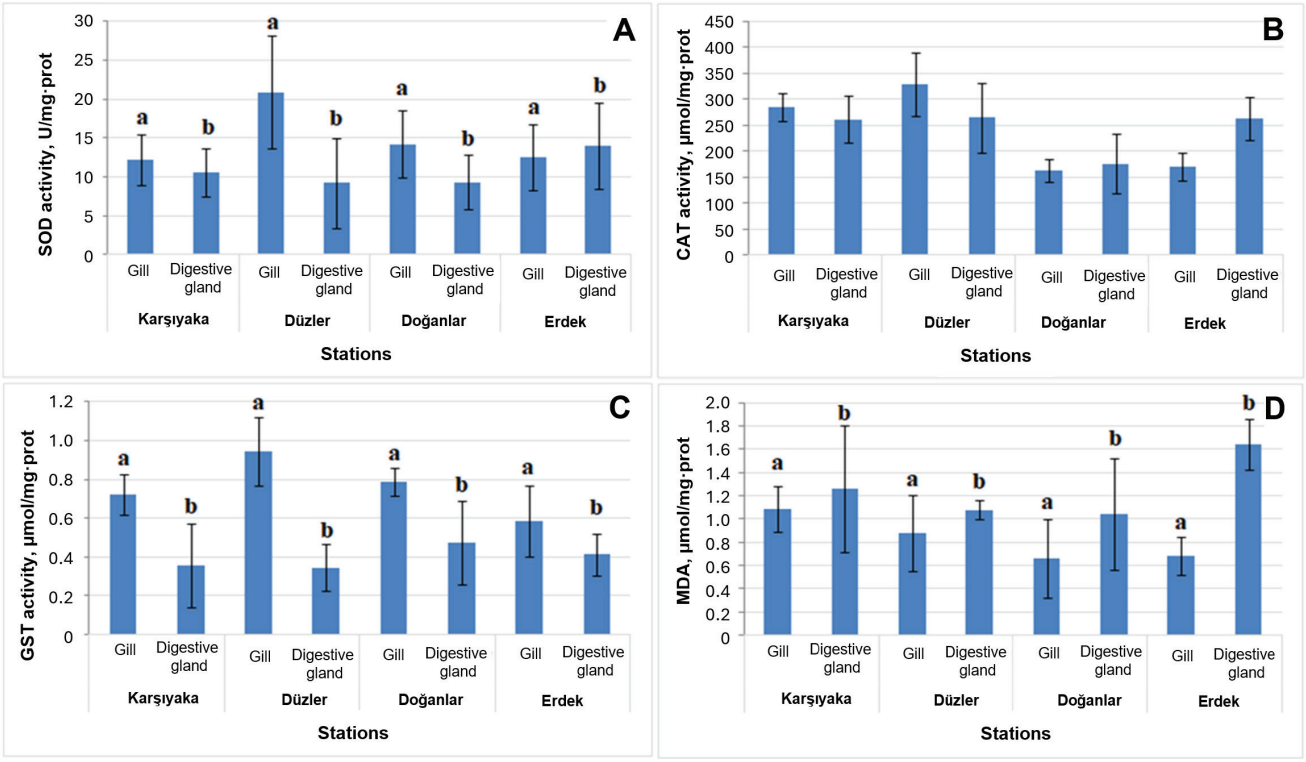

Fig. 2: SOD (A), CAT (B), GST (C) activities, and MDA (D) level in the gill and digestive gland tissues of *Pinna nobilis* from different stations of the Kapıdağ Peninsula (different letters represent statistical difference, $P_{value} < 0.05$).

icantly lower in the digestive gland tissues of samples across all stations, and this inter-tissue difference in GST was statistically significant (F = 42.42, df = 1, $P_{value} < 0.05$). In the gill tissues, the highest GST levels were observed at the Düzler station (mean 0.95 µmol/ mg.protein), while the lowest levels were recorded at the Erdek station (mean 0.59 µmol/ mg.protein). However, this fluctuation in GST levels was not statistically significant at the station level (F = 0.76, df = 3, $P_{value} > 0.05$) (Fig. 2C).

In contrast to antioxidant enzyme activities, the results of lipid peroxidation showed that MDA values measured in the gill tissue were lower than those measured in the digestive gland tissue. The differences observed in MDA levels among tissues were statistically significant (F = 13.38, df = 1, $P_{value} < 0.05$). The highest MDA levels were found in the digestive gland tissue of samples collected from Erdek (mean 1.64 µmol/ mg.protein). MDA values measured in both tissues of samples collected from Karşıyaka were notable (mean 1.1 µmol/ mg.protein in gill tissue and mean 1.26 µmol/mg.protein in digestive gland tissue); however, this difference was not statistically significant (F = 1.4, df = 3, $P_{value} > 0.05$) (Fig. 2D).

Histological analyses

Uni-nucleate and early plasmodial stage (two cells) of haplosporidian-like parasitic protozoans and brown cells were observed in the mantle sections of mussels collected from different stations (Fig. 3). Haplosporidian-like parasitic findings were frequently encountered in the mantle sections of mussel samples collected, especially from the Düzler station. In this station, we observed diffuse-type inflammation in mantle sections of infected specimens (Fig. 4). Diffuse brown cells were associated with infection. Uni-nucleate stages were observed in the sections.

Microbiological analyses

Microorganism levels in the seawater and *P. nobilis* samples according to the stations are summarized in Figure 5. Mean levels of THAB were determined in the seawater samples of all stations in the range of 4 to 5 log cfu/100 ml. The highest levels were observed in Karşıyaka at 4.54 log cfu/100 ml, and the lowest was in the samples from Erdek at 4.26 log cfu/100 ml. There were no statistical differences between the levels of THAB ($P_{value} > 0.05$). *Vibrio* sp. levels ranged between 3.33 and 3.12 log cfu/100 ml, while Enterobacteriaceae levels ranged between 2.77 and 1.32 log cfu/100 ml. *Vibrio* sp. showed similar levels among stations ($P_{value} > 0.05$); however, Enterobacteriaceae levels were found to be higher at the Karşıyaka (2.77 log cfu/100 ml) and Düzler (2.08 log cfu/100 ml) stations than at the Doğanlar (1.32 log cfu/100 ml) and Erdek (1.34 log cfu/100 ml) stations ($P_{value} < 0.05$) (Fig. 5A). The microorganism levels were found to be higher in *P. nobilis* samples than in the seawater at all stations (Fig. 5B). THAB levels in the samples of all stations were in the range of 6.29–6.22 log cfu/g ($P_{value} > 0.05$). *Vibrio* sp. counts showed similarities among stations and ranged between 4.92–4.40 log cfu/g ($P_{value} > 0.05$), whereas Enterobacteriaceae counts were in the range of 3.91–3.73 log cfu/g ($P_{value} > 0.05$).

The distribution and abundance of identified bacteri-

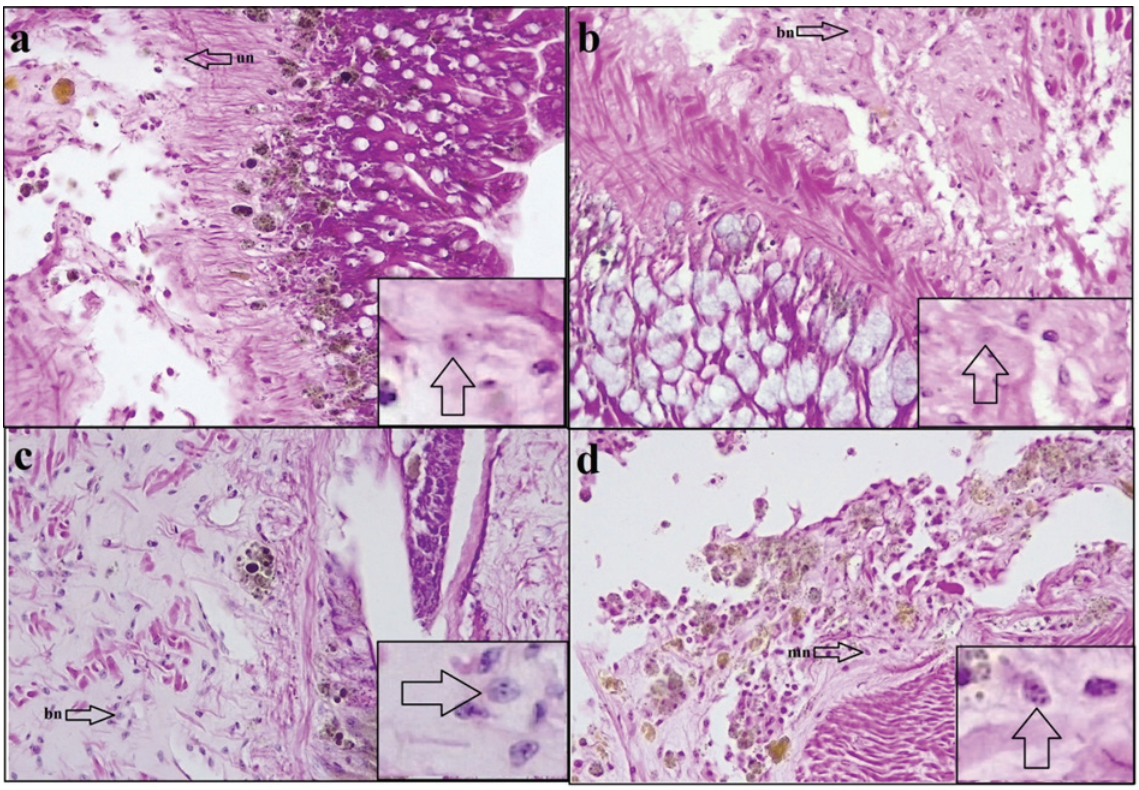

Fig. 3: Histology of the mantle of *Pinna nobilis* infected with haplosporidian-like protozoa collected from four different stations (a. Doğanlar, b. Düzler, c. Erdek, d. Karşıyaka), H&E. un: uni-nucleate stage (arrow and inset), bn: binucleate stage (arrow and inset), mn: multinucleate stage (arrow and inset).

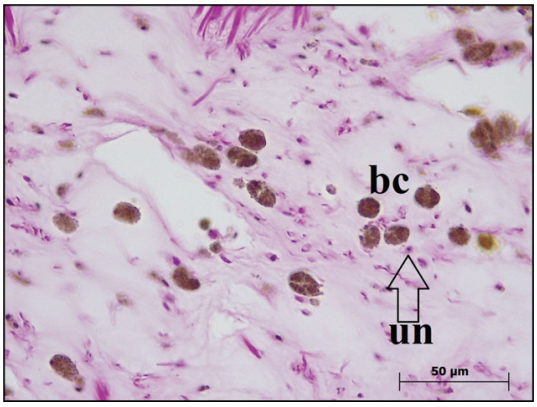

Fig. 4: Histology of the mantle of *Pinna nobilis* infected with haplosporidian-like protozoa collected from the Düzler station. un: uninucleate stage (arrow), bc: brown cells, H&E.

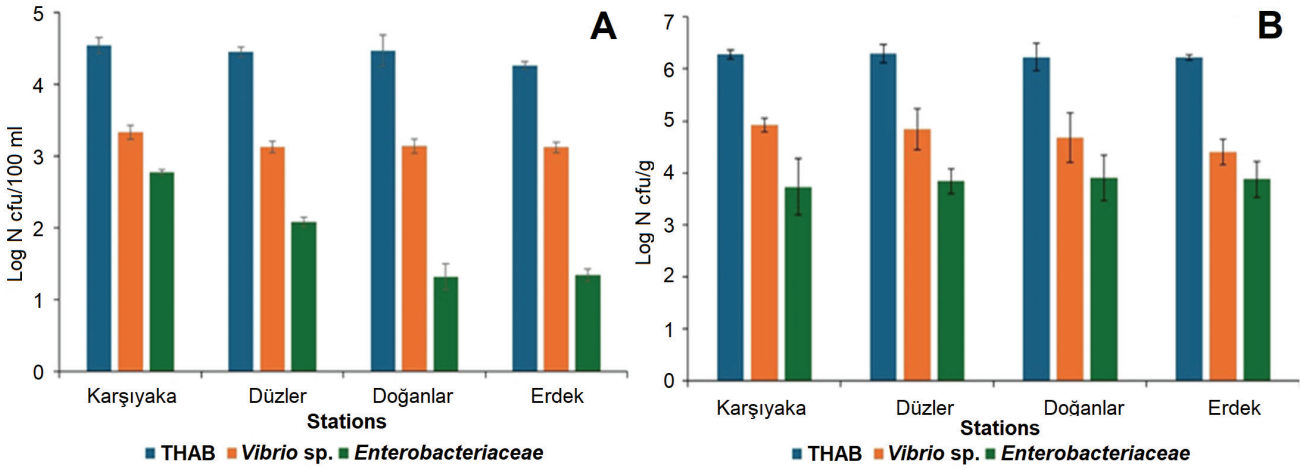

Fig. 5: Levels of viable total heterotrophic aerobic bacteria (THAB), *Vibrio* spp., and Enterobacteriaceae were measured in seawater (A) and *Pinna nobilis* (B).

al isolates from *P. nobilis* specimens across all stations are presented in Figure 6. We identified the isolates using partial 16S gene sequence analysis in the following alphabetical order: *Photobacterium leiognathi* subsp. *mandapamensis* (acc. no. PP864402); *Vibrio harveyi* (acc. no. PP864397, PP864398, PP864399, PP864403, PP864405, PP864409, PP864411, PP864412); *Vibrio mediterranei* (acc. no. PP864410); *Vibrio owensii* (acc. no. PP864396, PP864404); *Vibrio parahaemolyticus* (acc. no. PP864407); *Vibrio tubiashii* (acc. no. PP864413) and *Vibrio sp.* (acc. no. PP864406, PP864408). All isolates identified at the species level showed greater than 99% similarity to the 16S rRNA gene sequences in the GenBank database.

Identified live bacterial species are those capable of being cultured *in vitro*, indicating their ability to actively inhabit their hosts or environments and potentially contribute to compromised health statuses depending on their dominance. Among all *P. nobilis* specimens at all stations, *Vibrio* species were dominant. Specifically, *V. owensii* and *V. harveyi* were the most frequently encountered species, with proportions ranging from 32% to 53% and 12% to 42%, respectively. *P. leiognathi* subsp. *mandapamensis* (*P. mandapamensis*) emerged as the third dominant strain, comprising 14% to 17% of *P. nobilis* specimens at all stations except Doğanlar. Notably, this station exhibited weak bacterial diversity, with almost 100% of the strains composed of *Vibrio* species. The *Vibrio* species depicted twice in Figure 6 are ranked in order of dominance as follows: *V. parahaemolyticus* > *V. mediterranei* > *V. tubiashii* > *V. algoniticus* = *V. calliilyticus* = *V. jasicida*. The most diverse species of this genus were observed at Düzler and Doğanlar stations.

Molecular findings

The majority of positive samples were identified in the Düzler and Erdek regions (Table 3). Of the individuals initially assessed as healthy during clinical evaluations, molecular analysis revealed that 7 out of 9 tested positive for various lesions and pathogens. These findings verify that clinical signs are not always reliable indicators of mussel health. Results indicate that the screened microbe groups were investigated in specimens. We also screened for the presence of *Mycobacterium* sp. in all samples using molecular analysis. According to the results, the screened microorganism groups were observed only in some tissues rather than being present in all samples. While some groups coexisted in the same tissue sample, other tissues did not exhibit the presence of any group. The presence of *Mycobacterium* sp. was found in samples 1, 6, 7, 8, 9, 10, 17, and 20 (acc. no. PP868312-PP868319). The *Mycobacterium* species identified in our study did not allow species-level identification, despite the use of *Mycobacterium*-specific primers. However, it is noteworthy that the sequences obtained only matched the species included in the *Mycobacterium simiae* com-

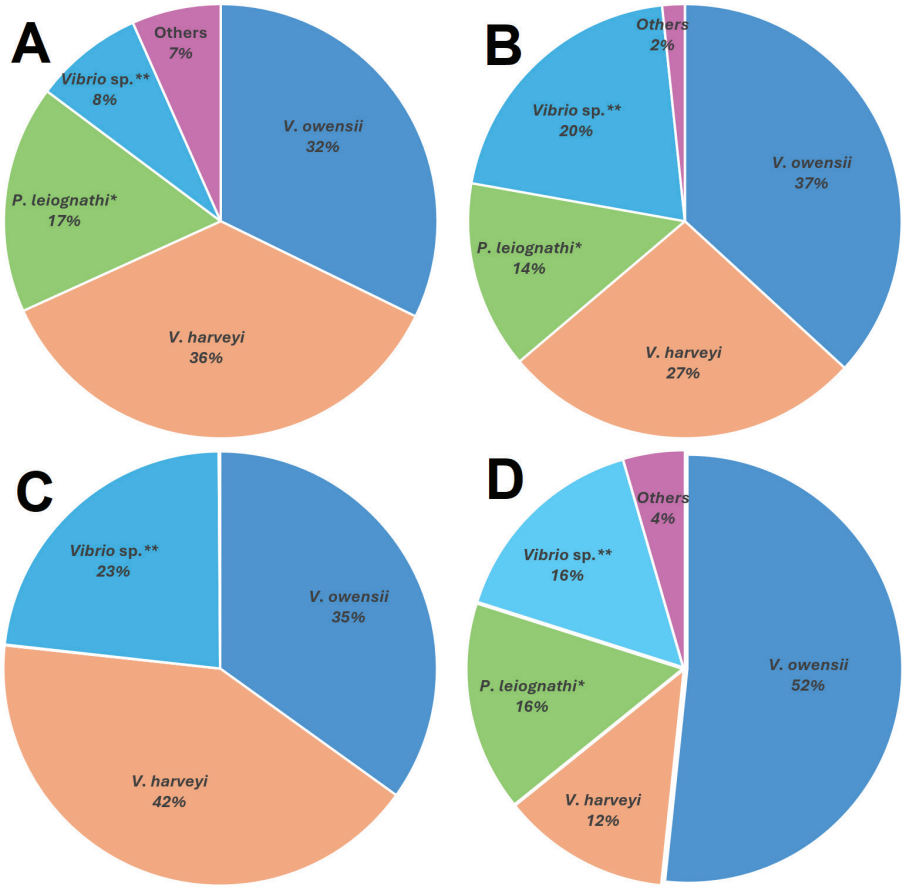

Fig. 6: Distribution of total viable bacterial strains in *Pinna nobilis* according to stations: A) Karşıyaka, B) Düzler, C) Doğanlar, D) Erdek. (Notes: **Photobacterium leiognathi* subsp.

Table 3. Microorganism groups detected in ethanol-fixed tissues by molecular diagnosis.

Stations	Sample no	Clinical sign*	*Haplosporidium* sp.	*Mycobacterium* sp.	*Perkinsus* sp.	*Marteilia* sp.
Karşıyaka	1	Sick	–	+	–	–
	2	Sick	–	–	–	–
	3	Sick	–	–	–	–
	4	Asx	–	–	–	–
	5	Asx	–	–	–	–
Düzler	6	Sick	+	+	–	–
	7	Sick	–	+	–	–
	8	Sick	+	+	–	–
	9	Asx	+	+	–	–
	10	Asx	–	+	+	–
Doğanlar	11	Sick	–	–	–	–
	12	Sick	–	–	–	–
	13	Asx	+	–	–	–
	14	Asx	–	–	–	–
	15	Sick	–	–	–	–
Erdek	16	Sick	–	–	–	–
	17	Asx	–	+	–	–
	18	Sick	–	–	–	–
	19	Asx	+	–	–	–
	20	Asx	+	+	–	–

plex within the non-tuberculous mycobacterium (NTM) group in the GenBank database with 100% similarity. Haplosporidian parasites were identified in samples 6, 8, 9, 13, 19, and 20 (acc. no. PP911424 - 29). In the GenBank database, the ribosomal RNA gene sequences of these parasites consistently matched as *H. pinnae*, according to the BLAST search. However, we identified our isolate as *Haplosporidium* sp. because the 93.86% similarity rate was insufficient for species-level identification. An amplicon of the expected size was obtained with *Perkinsus*-specific primers only from sample number 10, but the product was not sequenced. No members of the genus *Marteilia* were detected in the mussel tissues (Table 3).

Phylogenetic analysis revealed that our isolates were significantly separated from the main clade formed by the *H. pinnae* species. They were instead located within the branch formed by other species within the *Haplosporidium* genus but were clustered together independently in a consistent manner (Fig. 7).

Discussion

MME of *P. nobilis* was first documented in Spain by Darriba (2017), followed by reports of similar events along various Mediterranean coasts (Catanese *et al*., 2018; López-Sanmartín *et al*., 2019; Panarese *et al*., 2019; Katsanevakis *et al.*, 2019)). These studies predominantly attribute the primary cause of mortality to *H. pinnae*. However, additional analyses in some studies have implicated other bacterial agents, such as *Mycobacterium* sp. (Carella *et al*., 2019, 2020; Lattos *et al*., 2020) and *Vibrio* sp. (Prado *et al*., 2020; Andree *et al*., 2021; Künili *et al*., 2021), or a combination of both (Lattos *et al*., 2021), as potential contributing factors. Recent studies on the fan mussel suggest that a picornavirus from the Marnaviridae family, identified in the host's hemocytes, may induce MME by inhibiting the host's immune response, thereby increasing vulnerability to opportunistic infections (Carella *et al*., 2023; Carella *et al*., 2024). On the other hand, this study was not designed to screen for viral etiology, as this was not a consideration during the determination of the methodology; consequently, we lack data on this topic. In certain instances, the mortalities of *P. nobilis* were reported based solely on observational data (Öndes *et al*., 2020a; Özalp & Kersting, 2020), while in other investigations, various analytical methodologies were employed to ascertain the underlying causes.

The methodology employed predominantly includes histological techniques for detecting parasites in cystic form within tissues, along with molecular analyses for parasite identification. These approaches have been extensively utilized to investigate the underlying causes of mass mortalities in *P. nobilis* (Darriba, 2017; Catanese *et al*., 2018; Katsanevakis *et al*., 2019; López-Sanmartín *et al*., 2019; Panarese *et al*., 2019; Tiscar *et al*., 2019;

Fig. 7: Maximum-likelihood phylogenetic analysis of the small subunit ribosomal RNA (SSU rRNA) gene of *Haplosporidium* isolates based on the Tamura-Nei model (Tamura & Nei, 1993). The analysis involved 69 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 419 positions in the final dataset. Bootstrap values are shown at nodes.

Čižmek *et al*., 2020; Lattos *et al*., 2020, 2021; Grau *et al*., 2022). Furthermore, studies correlating these mortalities with the presence of pathogenic bacteria have incorporated bacteriological examinations (Prado *et al*., 2020; Scarpa *et al*., 2020) and electron microscopy techniques (Carella *et al*., 2019).

As the etiological factors of the co-infection impacting *P. nobilis* populations along the Mediterranean coasts continue to be elucidated, the research focus has shifted towards examining the responses and protective strategies exhibited by the surviving individuals. Accordingly, studies have not only identified the presence or absence of parasites through histological and molecular techniques but have also begun to assess oxidative damage and antioxidant responses in infected specimens (Box *et al*., 2020; Künili *et al*., 2021; Lattos *et al*., 2023). To the best of our knowledge, there remains a paucity of research in this domain. It is well-established that bivalves employ cellular immunity, particularly hemocytes, to eradicate infectious agents under pathological conditions. Hemocytes utilize reactive oxygen species (ROS) in pathogen combat, and any imbalance in this mechanism can lead to oxidative damage (Rahman *et al*., 2019; Lattos *et al*., 2023), thereby accelerating enzymatic and oxidative processes.

In the present investigation, the quantified levels of antioxidant enzymes, including SOD, CAT, and GST, exhibited their highest concentrations within the gill tissues of samples collected from the Düzler station. Despite this observation, the discerned variance in enzyme levels among tissue types did not reach statistical significance. Notably, the predominance of higher enzyme concentrations in gill tissues suggests a potentially prompt response, possibly attributed to the earlier exposure of these tissues to seawater, thus facilitating an earlier encounter with parasites and/or bacterial agents. Conversely, while the most significant quantities of MDA were detected in samples from the Erdek station, the differences noted across sampling stations did not achieve statistical significance. However, notable disparities in MDA levels were discerned among different tissue types, with elevated concentrations observed in the digestive gland. This elevation reinforces the proposition that the escalation in antioxidant levels might be insufficient to avert oxidative damage (Sureda *et al*., 2013a). Evaluations of *P. nobilis* oxidative responses to environmental pollutants generally advocate for an upward trajectory in antioxidant levels (Sureda *et al*., 2013a, 2013b; Capó *et al*., 2015; Natalotto *et al*., 2015). The current findings regarding antioxidant response align with existing literature, mirroring analogous outcomes obtained in investigations scrutinizing antioxidant enzyme levels in *P. nobilis* subsequent to parasitic/bacterial infection, construed as akin to pollutant exposure (Künili *et al*., 2021; Lattos *et al*., 2023). Nonetheless, Box *et al*. (2020) have reported a decline in antioxidant defense in infected *P. nobilis* specimens, intimating a potential vulnerability to infection and subsequent mortality resulting from immune system compromission.

Limited information has been reported about the life cycle of the genus *Haplosporidium*, and the available data have been obtained from cytological research conducted in the last decade (Tiscar *et al*., 2022). According to Darriba (2017), systemic infection by the haplosporidian parasite is observed in the connective tissue and di-

gestive gland tissues of mussels, where single-nucleated, early plasmodial, and plasmodial stages are reported. In our study, haplosporidian-like protozoans in different life stages were detected in the mantle and digestive gland sections examined. In the mantle sections of mussels collected from all stations in this study, single-nucleated and early plasmodial stages were observed, while plasmodial stage haplosporidian-like protozoans were observed in digestive gland sections. Studies have been conducted on the histological changes caused by protozoan infection in tissues (Catanese *et al*., 2018; Panarese *et al*., 2019; Box *et al*., 2020; Carella *et al*., 2020; Čižmek *et al*., 2020; Lattos *et al*., 2020; Künili *et al*., 2021; Lattos *et al*., 2021; Tiscar *et al*., 2022; Lattos *et al*., 2023). These studies reported that widespread hemocytic infiltrations, an increase in brown cell numbers, and damage to and necrosis of the digestive tubule epithelium are observed in mussel tissues due to *H. pinnae* infection. In the mantle sections examined in our study, widespread hemocytic infiltrations were observed and an increase in brown cell numbers was determined. Brown cells with phagocytic properties play an essential role in the immune system of mussels. These cells are also considered an indicator of the general health of mussels and their response to environmental stress factors.

Identifying the species and quantities of live microorganisms that can be cultured is crucial to understanding their potential impact on host organisms. In this study, the level of microorganisms found in *P. nobilis* was at least two logarithmic units higher than those detected in seawater samples from the same region (Fig. 5). The high number of microorganisms, detected by species, was mainly attributed to *Vibrio* species and *P. mandapamensis*, as shown in Fig. 6. The dominance of other microorganisms found in the natural sea flora and species not commonly found in marine environments, such as Enterobacteriaceae, was limited to a maximum of 7%. The presence of Enterobacteriaceae at less than 7% by species, as shown in Fig. 5, suggests that there was no significant land-based contamination in the seawater during sampling. This is because these bacteria are not typically supported in the marine environment, even if the geographical conditions are favorable for them (Künili & Ateş, 2021). However, the detection of species belonging to this family, which were relatively more abundant in *P. nobilis*, indicates that they can persist in living organisms or that contamination with these microorganisms continues intermittently, albeit to a lesser extent compared to seawater.

The detection of *Vibrio* species in the water samples from the study stations—and even more prominently, in the *P. nobilis* samples—indicates a distinct tendency for accumulation. This accumulation is likely influenced by environmental changes and the physiological characteristics of the host organism. *Vibrio* species are predominantly pathogenic to their hosts and can persist within them, particularly when the host is unable to expel these microorganisms, as has been observed in bivalve species (Arık Çolakoğlu *et al*., 2010; Arık Çolakoğlu *et al*., 2014). In *P. nobilis*, multiple *Vibrio* species, including *V. mediterranei, V. harveyi*, and *V. tubiashii*, have been reported as causative agents of mortalities (Prado *et al*., 2020; Künili *et al*., 2021). In particular, *V. owensii* and *V. harveyi* are the most dominant *Vibrio* species found in *P. nobilis* samples. They have been reported to induce chronic and acute diseases such as internal organ, tissue, and hepatopancreatic necrosis in fish, crustaceans, shellfish, and corals (Ushijima *et al*., 2012; Liu *et al*., 2018; Firmino *et al*., 2019; Zhang *et al*., 2020; Dai *et al*., 2022). In some cases, their presence is positively associated with mortalities of oysters and abalones, which is more severe when combined with the presence of other pathogens and changing environmental conditions that trigger immune responses in host marine organisms (Lee *et al*., 2023; Siboni *et al*., 2024). On the other hand, the genus *Photobacterium* of the Vibrionaceae family represents more than 40 species that are bioluminescent symbionts, halophilic, and halotolerant in character. They have a wide range of growth temperatures (5–25°C). They are capable of adapting to various environments, such as marine water, sediment, and the surfaces of various marine organisms, including their muscles and digestive systems, without solely relying on their hosts for survival (Urbanczyk *et al*., 2011; Moi *et al*., 2017; Tsoukalas *et al*., 2023). The presence of *P. mandapamensis* in marine organisms and environments was reported to be positively correlated with *Vibrio* species, especially those in the *Harveyi* clade, including *V. harveyi* and *V. alginolyticus*, which can constitute up to 40% of the total heterotrophic bacterial count (Chatragadda & Raju, 2020). This agrees with our results, which show that the predominant heterotrophic viable bacterial strains, as shown in Fig. 2, are mostly from the *Harveyi* clade and *V. owensii*.

In addition, *Mycobacterium* sp. was detected in all samples from the Düzler station, and 50% of samples from Erdek. The antioxidant activity related to host immunological defensive systems was relatively higher (Fig. 2) in *Mycobacterium* sp. positive samples. Moreover, observational evaluation of the samples from these stations, according to their sample numbers, also supports their weak health appearance, even though the measured physicochemical properties of seawater, a major stress factor for aquatic organisms among the stations remain similar (Table 2). *Mycobacterium* sp. has been reported as one of the important pathogens for *P. nobilis* along with *H. pinnae* in previous studies as a complex event involving multiple potential pathogens and environmental factors that contribute together (Carella *et al*., 2019; Lattos *et al*., 2020; Šarić *et al*., 2020; Carella *et al*., 2023).

In this study, we determined that the co-occurrence of other varied pathogens, including haplosporidian parasites and bacterial strains, may be major factors in the weak but not dying state of *P. nobilis*. The presence of the *Haplosporidium* sp. parasites along with *Mycobacterium* sp. in *P. nobilis* populations may not be deadly in the SoM. This may be related to the presence of genetic variation of the parasite, indicating it may have mutated to avoid causing death, thus prolonging its presence in the host even in the case of the presence of other pathogens, including strains belonging to the genera of *Mycobacterium* and *Vibrio*. In this study, SSU rRNA gene-based

screening/diagnostic techniques, which many researchers have widely used, were used to determine the cause of MME in fan mussels, especially for *H. pinnae* (Catanese *et al*., 2018; Katsanevakis *et al*., 2019; Grau *et al*., 2022). A study by Moro-Martínez *et al*. (2023) on *H. pinnae* isolates from the Mediterranean found that the SSU rRNA gene region only had a single haplotype. On the other hand, as one of the most noteworthy findings in our study, we observed that the SSU rRNA gene sequence of the *Haplosporidium* sp. we detected in the tissues differed greatly from *H. pinnae*. Considering all these together, the isolates we obtained from the SoM may belong to a distinct variant of *H. pinnae* or possibly to another haplosporidian rather than *H. pinnae*.

Conclusion

This study provides a comprehensive analysis of the first occurrence of *Haplosporidium* sp. in *P. nobilis* populations in the SoM. Unlike the Mediterranean basin, where MMEs are primarily attributed to the aggressive pathogenicity of *H. pinnae*, our findings, including underwater population observations among various stations, indicate that the presence of *Haplosporidium* sp. along with *Mycobacterium* sp. and elevated levels of *Vibrio* species do not induce MME in the SoM. However, individuals still exhibited weak health status according to their immune responses, as determined via biochemical, histopathological, and microbiological analyses.

Along with clinical, morphological, and advanced laboratory analyses, changes in marine environmental parameters other than those monitored in this study should also be considered in more in-depth measurements as climate change continues. Also, it should not be ignored that the special structure of the Sea of Marmara may have a protective effect for P. nobilis against mass deaths. This study is the first to report from a pathogenic perspective that there is no condition attributable to MME in the fan mussel populations in SoM. As of January 2025, the population in the monitored stations remains stable, with no signs of MMEs observed following the sampling period. This situation was proven via advanced laboratory analyses and underwater clinical symptoms, showing the populations are alive despite their weak health status, most likely due to the presence of *Haplosporidium* sp., *Mycobacterium* sp., and elevated microorganism levels of *Vibrio* sp. and *Photobacterium*.

In light of the genetic differences within the same clade that they represent, future research should focus on making more profound diagnostic tools and better understanding methods for the life cycle of *Haplosporidium* sp. The *Haplosporidium* identified in this study may indicate a non-pathogenic or less virulent for the fan mussel populations. This study shows that instead of causing death, these variations result in a weakened health state for *P. nobilis*. To gain a comprehensive understanding of the unique circumstances in the SoM and draw a definitive conclusion, it is necessary to employ advanced imaging techniques such as electron microscopy, as well as gather more detailed information on the causes and spread of the disease. In addition to analyzing the SSU rRNA gene, it is crucial to sequence multiple housekeeping genes or even the whole genome of both the parasite and the host. Investigating the factors causing the SoM to experience a different outcome than the Mediterranean basin's and preventing it from becoming a mass mortality event could have a significant impact on the survival of the endangered fan mussel, which plays a crucial role in Mediterranean ecology.

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