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ORIGINAL ARTICLE

Mass mortality in endangered fan mussels *Pinna nobilis* (Linnaeus 1758) caused by co-infection of *Haplosporidium pinnae* and multiple *Vibrio* infection in Çanakkale Strait, Turkey

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ABSTRACT

Purpose: *Pinna nobilis* (fan mussel) is one of the most important endemic bivalve molluscs in the Mediterranean and mass mortality events were observed in these mussels in recent years. In this study, we report mass mortalities caused by *Haplosporidium pinnae*, which has been spreading in the Mediterranean for 3 years, and reached the Çanakkale Strait, which is the entrance of the Marmara and the Black Sea.

Material and methods: Field observations during sampling and subsequent histopathological, biochemical, genetic, and microbiological analyses were carried out.

Results: These analyses showed that *H. pinnae* infection spread among the natural beds of *P. nobilis*, causing severe tissue damage and oxidative stress. Our phylogenetic analyses suggested that the parasite spread through the Mediterranean much faster than thought. The results showed that vibriosis originating from *Vibrio coralliilyticus*, *Vibrio tubiashii*, *Vibrio mediterranei*, and *Vibrio hispanicus*, acted together with *H. pinnae* in infected individuals and caused death.

Conclusion: It is highly probable that the spread of *H. pinnae* to the Sea of Marmara and the Black Sea may occur earlier than expected, and it was concluded that mass deaths were caused by co-infection with *H. pinnae* and a geographically specific marine pathogen that can infect *P. nobilis* populations.

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KEYWORDS
Pinna nobilis; *Haplosporidium pinnae*; *Vibrio*; histopathology; oxidative stress; mass mortality event

Introduction

Pinna nobilis (pen shell or fan mussel) is endemic and the largest bivalve species in the Mediterranean Sea. Their lifespan can vary between 27 and 45 years, and their height can reach up to 120 cm (Zavodnik *et al.* 1991, Galinou-Mitsoudi *et al.* 2006, García-March *et al.* 2011, Basso *et al.* 2015, Rouanet *et al.* 2015). *Pinna nobilis* lives at depths of 0.5–60 m on soft or sandy sea bottom covered with seagrass meadows. It is known that *P. nobilis* shells were sold as ornaments for many years in touristic areas in countries on the Mediterranean coast. It is also hunted because of the byssus threads, which are more valuable than the shell of *P. nobilis* and which the creature uses to fix itself to the seafloor. In the last 20–30 years, the population of *P. nobilis* decreased significantly due to reasons such as commercial and recreational hunting, decorating of the shells, trawling activities and boat mooring operations (Kurtay 2014).

Due to anthropogenic and industrial causes, the population of *P. nobilis* faces critical survival challenges (Alomar *et al.* 2015, Öndes *et al.* 2020a). But recently, scientific reports were published about their mass mortality in certain locations (Spain, Italy, France, Greece, Portugal, etc.) due to a highly contagious protozoan disease (Darriba 2017, Lattos *et al.* 2020a). The threat resulting in mass mortality of fan mussel populations was first reported in 2017 from Spain (Darriba 2017) with the causative agent a protozoan species named *Haplosporidium pinnae*. The term mass mortality event (MME) for *P. nobilis* was first used at that time and mortality started to be monitored. The frequent occurrence of the mussels' mass mortality in the different Mediterranean counties has raised severe ecological concern that needs to be addressed.

Works performed by several researchers during the periods of 2017–2020 in Spain (Catanese *et al.*, 2018, López-Sanmartín 2019), Italy (Panarese *et al.* 2019) and Greece (Katsanevakis 2019) revealed that the primary causative agent of the mass mortality of *P. nobilis* is *H. pinnae*. It is even thought the onset of *H. pinnae* infection in *P. nobilis* populations may even be the main cause of death. However,

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some recent studies suggested that co-infections along with *H. pinnae* may severely affect the course of mass mortality. Studies were carried out to show that *Mycobacterium* species, as well as *H. pinnae,* are responsible for mass deaths (Carella *et al.* 2019, Lattos *et al.* 2020a). The idea was strengthened when intensive bacterial strains, rather than the inherent flora of *P. nobilis* were reported in recent studies (Prado *et al.* 2020, Scarpa *et al.* 2020). Among these studies, bacterial strains from the gen I *Mycobacterium* and *Vibrio* were microorganisms most presumed to cause coinfection with *H. pinnae* leading to mass mortality events for *P. nobilis* (Lattos *et al.* 2020b).

In the present study, we evaluated the status and spread of mass death in *P. nobilis* populations in Çanakkale Strait, Turkey in terms of histopathological, antioxidative enzymes, microorganism levels and dominance assay. The study area is in a key position at the entry point to the Sea of Marmara and the Black Sea, the last extensions of the Mediterranean. The mass mortality in Turkish seas was reported by visual observations previously done by scuba divers (Öndes *et al.* 2020b, Özalp and Kersting 2020, Acarlı *et al.* 2021).

Previous literature reports about a load of pathogens in *P. nobilis* tissues (if any) and their findings using multibiomarkers indicated the need for the present study and examination of the efficiency of these biomarkers in determining the cause-effect relationship. To our knowledge, this study is the first report of *H. pinnae* infection in Turkish seas confirmed by molecular, histopathological, biochemical and microbiological analyses. In addition, this is the first scientific report suggesting that vibriosis is caused by *Vibrio harveyi* as well as other *Vibrio* species including *V. mediterranei*, *V. hispanicus*, *V. coralliilyticus* and *V. tubiashii* may be responsible for mass mortalities in the presence of *H. pinnae* infection.

Clinical significance

- *Pinna nobilis* is under protection and their populations face the threat of extinction.
- It was determined that *V. harveyi* could be related to the mass mortality in *P. nobilis*.
- Histopathological analyses showed the presence of *H. pinnae* in *P. nobilis*, especially within the mantle, connective tissue, digestive gland, and intestine.
- We determined that *H. pinnae* and *Vibrio* sp. infection caused an increase in the antioxidant defense of *P. nobilis*.

Material and methods

Study area and animal collection

Fan mussels ($n = 863$) were investigated using underwater visual observations from the different stations in Çanakkale Strait (1. Çoraklı k Cape, 2. Umurbey, 3. Çardak) between

Figure 1. Sampling areas of *P. nobilis* in Çanakkale Strait (1: Çoraklık cape, 2: Yapıldak, 3: Umurbey).

Table 1. Number of dead and alive fan mussel individuals and other study area informations.

Stations	Coordinates	Sampling date	Habitat type	Number of dead individuals	Number of alive individuals	Total
1	40°12′518″N 26°29′613″E	02.07.2020	S, P	185	58	243
2	40°14′312″N 26°32′482″E	02.07.2020	S, M, P	154	79	233
3	40°16′238″N 26°33′830″E	30.06.2020	P	193	194	387

S: sand; P: *Posidonia oceanica* meadows; M: muddy.

Table 2. Descriptive statistics of some morphological measurements of fan mussels ($n = 106$).

Morphological measurements	Min.	Max.	Mean ± S.D.
Height (cm)	11	47.5	28.99 ± 0.78
Width (cm)	55	18.5	12.70 ± 0.21
Thickness (cm)	1	5.5	3.46 ± 0.1
Weight (g)	15	1345	339.69 ± 26.21

June-July 2020 (Figure 1 and Table 1). For laboratory analyses (histopathology, oxidative stress parameters, microbiological and molecular biological tests), 22 healthy and infected individuals were collected and transported to the laboratory. A total of 106 fan mussels were examined morphologically (Table 2).

Microbiological analyses

The shells of specimens were washed and scraped with a sterile knife and cleaned with an alcohol swab. The shells were opened using a sterile knife from the umbo and the contents (consisting of gill, mantle and adductor muscle tissues) weighing 10 g were placed into 90 ml bacterial peptone (1%) and NaCl solution (2.75%). Then the samples were homogenized by using Stomacher 400 circulator (Seward, UK) for 4 min at 2000 rpm. The homogenates were then subjected to serial dilution with bacterial peptone water containing 2.75% NaCl. Isolation and enumeration of the bacterial strains were performed according to the plate count method as described in the USFDA bacteriological analytic manual (U.S Food and Drug Administration 1998). Thiosulphate Citrate Bile Salts Sucrose (TCBS) agar (Merck) were used for the growth of *Vibrio* sp., Endo and Violet Red Bile (VRB) agars for the growth of Enterobacteriaceae, and Marine Agar (Difco) was used for the growth of total viable bacteria.

Then the plates were incubated at 25 °C for 72 hours. After incubation, the number of colonies was counted and all morphologically varied strains were visually checked and predominant bacterial colonies (consisting of > 80% in petri dishes) were isolated from the plates for further identification by using 16S rRNA gene sequencing. For the growth of the selected predominant bacterial strains, Marine Agar was used and incubated at 30 °C for 24 hours.

Genomic DNA extraction

The bacterial isolates were collected from the culture medium and used directly, while the ethanol fixed tissue samples (about 100 mg wet weight) were first homogenized with a tissue homogenizer (Bullet Blender Storm – Next Advance Inc.) using glass beads (1.0 mm) for 5 min. Total DNA was then extracted from both samples using the PureLink Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's instructions and used for PCR.

PCR amplification and sequence analyses

For bacterial identification, partial 16S rRNA gene was amplified from the isolates using universal bacteria primers S-D-Bact-0008-a-S-20 (5′-AGAGTTTGATCCTGGCTCAG-3′) and S-*-Univ-0536-a-A-18 (5′-GWATTACCGCGGCKGCTG-3′) (Suau *et al.* 1999). Primer selection for detection of the pathogen in tissues was made according to the presumptive identification based on the examination of histological sections. Hence, a nested PCR was performed using haplosporidian-specific primer sets C5f-Hapl (5′-GTAGTCCCARCYATAAACBATGTC-3′)/sB1N (5′-GATCCHTCYGCAGGTTCACCTACG-3′) and V5f-Hapl (5′-GGACTCRGGGGGAAGTATGCT-3′)/sB2hap (5′-CCTTGTTACG ACTTBTYCTTCCTC-3′) (Hartikainen *et al.* 2014).

The PCR mixture included approximately 50 ng template DNA, 0.4 μM of each primer, PCR Master Mix (2X) (Thermo Scientific) and nuclease-free water (Thermo Scientific) and all amplifications were performed using a thermal cycler (BiometraTAdvanced – Analytik Jena AG). For the partial *16S rRNA* gene, the following parameters were used: initial denaturation at 95 °C for 3 min, followed by 30 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. For the nested PCR, the cycler was programmed as: initial denaturation at 95 °C for 3 min followed by 35 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 65 °C for 1 min, extension at 72 °C for 1 min) and a final extension step of 72 °C for 10 min. After amplification, PCR products were loaded on a 1.6% (wt/vol) agarose gel in TAE buffer containing ethidium bromide (0.5 μg/ml) and electrophoresis was performed at 90 V for 60 min. All products were visualized on a UV transilluminator and sizes of the products were estimated against GeneRuler 100 bp DNA Ladder (Thermo Scientific). All PCR products were purified and sequenced bidirectionally by Medsantek Ltd. (İstanbul, Turkey). For 16S rRNA gene products, amplification primers were used in sequencing; for sequencing, the nested PCR products and, 2nd round primers were used. Sequence editing and analyses were performed in Bioedit v7.0.0 (Hall 1999) using the ClustalX 2.1 (Larkin *et al.* 2007) and BLASTN 2.2.20 algorithm (Zhang *et al.* 2000). A ≥ 99% similarity criterion was used in gene sequences for the identification of the isolates at the species level (Clarridge 2004).

All gene sequences were deposited in the GenBank database.

Phylogenetic analyses

The nucleotide sequences obtained from this study were aligned with the matching sequences from GenBank using the ClustalW algorithm (Larkin *et al.* 2007) and phylogenetic analyses were performed by using the Maximum Likelihood method based on the Tamura–Nei model (Tamura and Nei 1993) with 1000 bootstrap replicates in MEGA7 software (Kumar *et al.* 2016).

Histopathological analyses

The mantle, connective tissue, intestine, and digestive gland of healthy and infected *P. nobilis* samples ($n = 18$) were fixed in Davidson's fixative for 24 h at room temperature. Afterwards, the tissues were dehydrated in progressive series of ethanol and embedded in paraffin. Tissues were cut to a 5–7 μm thickness with a Leica rotary microtome. The histopathological sections were stained with hematoxylin-eosin (Bancroft and Gamble 2008). Histopathological changes were evaluated, and micrographs were taken using a CX31 Olympus light microscope equipped with a digital camera by using DP2-BSW software.

Antioxidant enzyme analyses

The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes were investigated in the gill and digestive gland tissues of 6 infected and 12 non-infected *P. nobilis* samples. First, the homogenate was prepared with phosphate buffer (0.05 M, pH: 7.4, 1:5 w/w). The homogenate was centrifuged 2 times for 20 min at 4 °C and 10,000 *g* and supernatants were removed and stored at −45 °C. The amount of protein in each homogenate was determined by the method of Bradford (1976) and thus the enzyme activities were measured spectrophotometrically.

SOD enzyme activity was measured according to the method of Flöhe and Ötting (1984). The basic principle for this measurement is that the superoxide radical produced by the xanthine oxidase/hypoxanthine system causes colour formation by reducing Nitro Blue tetrazolium (NBT), and this colour change is the basic criterion in determining SOD activity. The reduction of the produced superoxide radical to NBT ends with the formation of blue-coloured formazan and this formation gives maximum absorbance at 550 nm.

CAT activity was measured according to Clairborne (1985), a method based on the decomposition of H_2O_2. The samples were measured at 240 nm for 90 s, with readings taken every 15 s. GPx activity was determined based on the method used by Wendel (1980). The measurement was made based on the principle that the produced GPx enzyme activity causes GR degradation and the absorbance decreases at this time. Measurements were recorded at 340 nm wavelength.

Statistical analyses

The statistical analyses were carried out using SPSS 21.0 software. Normality of data and homogeneity of variances were tested using Kolmogorov Smirnov and Levene tests, respectively. The results between infected and non-infected samples were compared using One-Way ANOVA. The significant differences among concentrations were presented with different letters or figures. Significance level α was set to 0.05 for all analyses.

Results

Visual observations

In the Çanakkale Strait, in areas where the fan mussels are dense, healthy and infected individuals were identified at the same time and station (Figure 2).

In this study, the course of the disease originating from *H. pinnae* in *P. nobilis* individuals was observed in their natural beds (Figure 2(a)). Weak shell closing reflex when stimulated, the dull colouration of the inner part and poor general appearance were used as indicators in the selection of infected individuals (Darriba 2017, Catanese *et al.* 2018, Carella *et al.* 2019). The laboratory findings of the samples grouped as infected and healthy also supported the underwater observations and the method of distinguishing the infected specimens in the sampling field. According to the findings based on observations made underwater, it was clearly observed that among infected individuals of the same length, regardless of whether they were old or young, weak shell closing reflex was determined as common and was at similar levels; however, deterioration in colour, intense macro-organism involvement in or on the outer parts of the specimens, and increases in the level of mucus secretion were generally observed to be more severe among elderly infected specimens in classification according to recently decreased infected specimens (Figure 2(b–d)). The laboratory findings for specimens classified as infected in the sampling area, the bacterial levels, and the antioxidant enzyme activity were found to be significantly higher than the healthy specimens (Figures 3 and 9). Moreover, the devastating effects of the parasite in the internal organs of the infected sampled specimens were clearly distinguished by histopathological analyses that were also parallel to the field observations.

Microbiological analyses

Results of the microbiological analyses showed that total viable counts reached 10^4 and 10^6 cfu/g for healthy and infected specimens, respectively. Total viable bacterial colonies on TCBS agar, containing *Vibrio* spp. were determined as 10^3 and 10^4 cfu/g for healthy and infected specimens, respectively. *Enterobacteriaceae* species were limited at 10^1 cfu/g levels in all samples (Figure 3). While two dominant bacterial colonies were observed on the Marine Agar, three colonies were determined in TCBS agar. Among these dominant colonies, two strains from Marine Agar and TCBS agar were determined as *V. harveyi* (Figure 4).

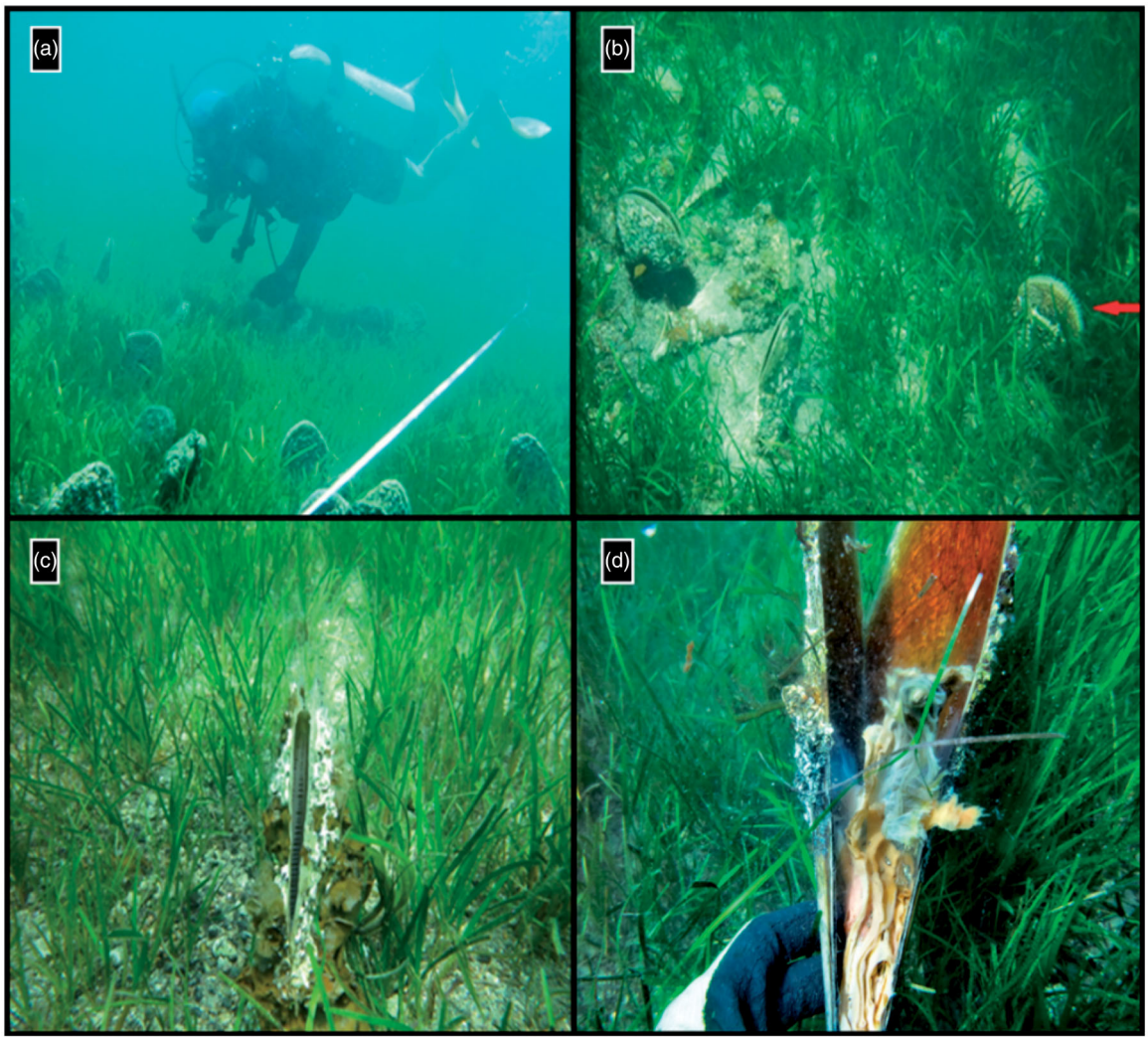

Figure 2. *P. nobilis* populations. (a) The habitat of *P. nobilis*, (b) healthy specimens (arrow) in infected population, (c) infected specimen, (d) recently expired specimen.

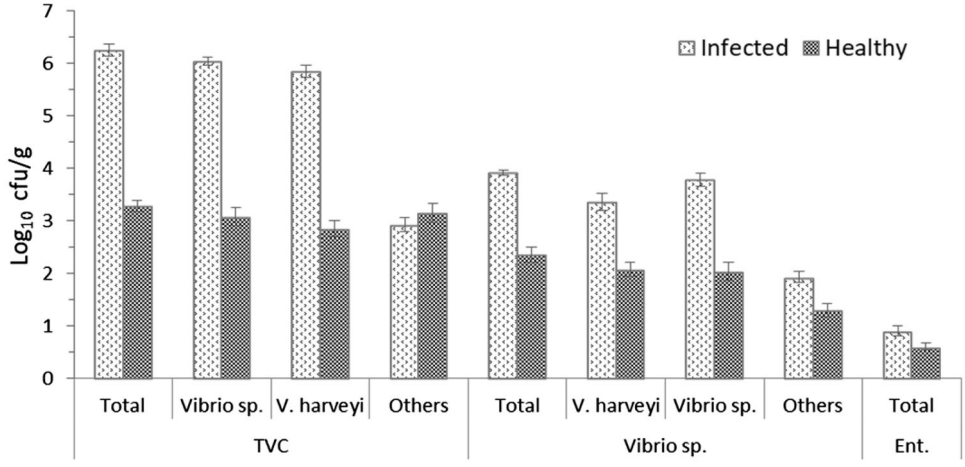

Figure 3. Dominances and total counts of viable bacteria in infected and healthy *P. nobilis* specimens.

The dominant bacterial strains on Marine Agar were identified as *Vibrio* sp. *and V. harveyi* with 82% of total viable colonies from infected and 20% from healthy *P. nobilis* specimens, respectively. The most dominant bacterial strain was identified at genus level since an equal similarity at >95% level was determined among two species named *V. coralliilyticus*, and *V. tubiashii* (Figure 5).

The second dominant bacterial strain was identified as *V. harveyi* accounting for 40% of total viable bacteria. The third dominant bacteria was also identified at the genus level as

Vibrio sp. with results showing equal similarity between two species, *V. mediterranei* and *V. hispanicus*. Remaining bacterial colonies were ignored as they were consisted of 11 different bacterial strains and accounted for a maximum of 19% of the total bacterial counts on all plates. These results showed that *Vibrio* species (*V. coralliilyticus*, *V. tubiashii*, *V. mediterranei* and *V. hispanicus*) and *V. harveyi* may be responsible for mortality in *P. nobilis* individuals when *H. pinnae* infection begin. The real reason for mass mortality was thought to be related to *V. mediterranei* and *V. harveyi* strains after immunity deficiencies occurring linked to the parasitic infection by *H. pinnae* (Austin and Zhang 2006, Carella *et al.* 2020, Scarpa *et al.* 2020, Zhang *et al.* 2020).

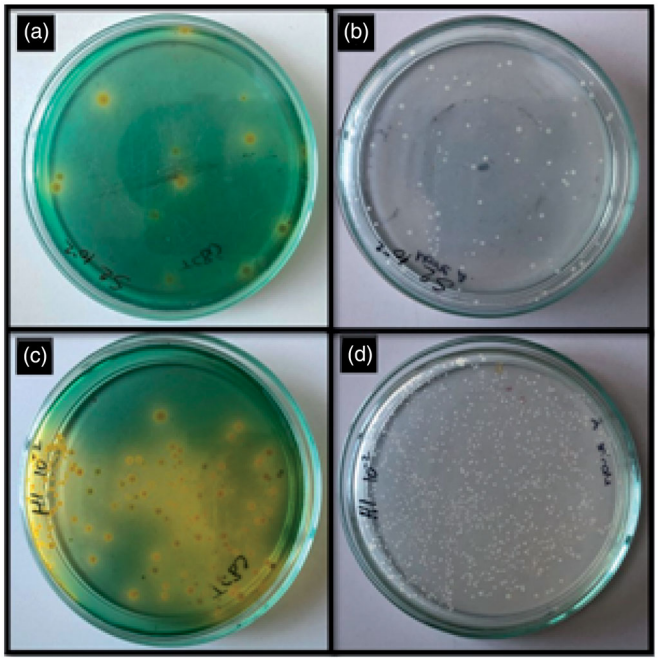

Figure 4. Bacterial strains and numbers in the plates of TCBS and Marine agars. The counts of *Vibrio* sp. at 10^{-2} cfu levels on TCBS agar. (a) The healthy specimens, (c) The infected specimens. The counts of total viable strains at 10^{-2} cfu levels on Marine agar. (b) The healthy specimens, (d) The infected specimens.

PCR and gene sequencing

According to 16S rRNA gene sequencing results, the predominant bacterial isolates obtained from *P. nobilis* individuals were identified as *V. harveyi* (acc. no. MW259975, MW259976, MW259978) and *Vibrio* sp. (acc. no. MW259974, MW259977). The *Haplosporidium*-specific primer set targeting the small subunit ribosomal RNA gene, produced a band around 659 bp in length, in nested PCR performed in this study. The sequence of this amplicon (acc. no MW255604) showed 100% similarity to sequences of *H. pinnae* entries in the GenBank Nucleotide collection (nt) database. Phylogenetic analyses showed that our isolate clustered together with the other *H. pinnae* isolates obtained from the Mediterranean region including Italy and Spain (Figure 6).

Histopathological analyses

The *Haplosporidium* protozoan was detected in all stations in the study. Histopathological studies showed the presence of *H. pinnae* protozoan in 6 out of 18 fan mussels, especially within the mantle, connective tissue, digestive gland, and intestine. Healthy *P. nobilis* specimens showed normal histological structure. Histopathological analyses revealed uninucleate and binucleate cells, plasmodial stages and sporocysts enclosing more or less mature spores of *H. pinnae* in the mantle, connective tissue, digestive gland and intestine of fan mussels (Figure 7). Significantly, heavily infected fan mussels showed a large vacuole with amorphous eosinophilic material (Figure 7(d)), abnormal haemocytic infiltration (Figure 8(a)), detachment of epithelial cell and *H. pinnae* spore deformations and necrosis (Figure 8(b,d)), and

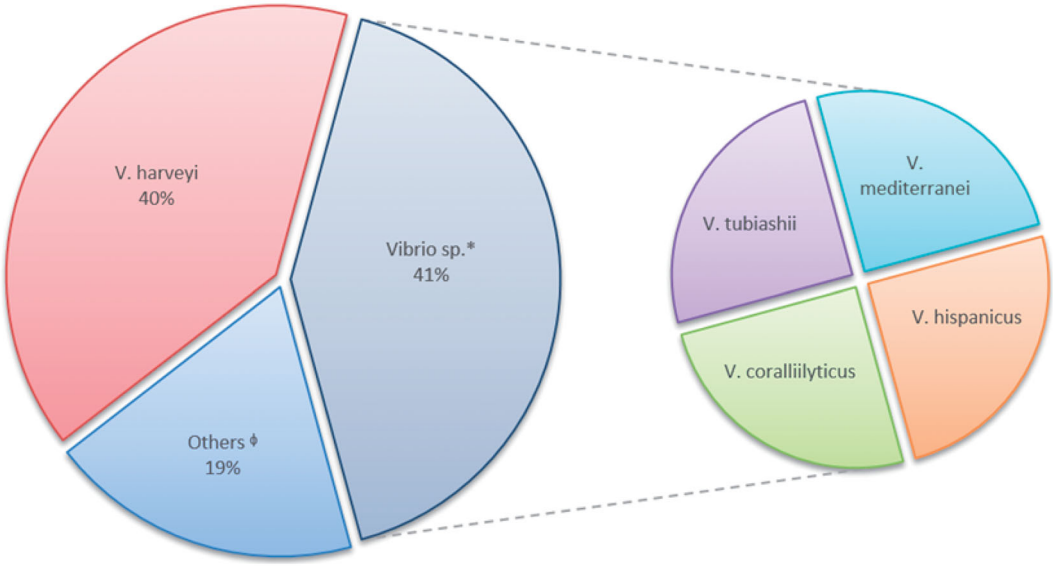

Figure 5. Dominant bacterial strains in infected *P. nobilis*. **Vibrio* sp. species was determined equally similar according to the genetic analyses. ϕThe number of other bacterial strains were 11 and they were ignored due to their total presence not more than 19%.

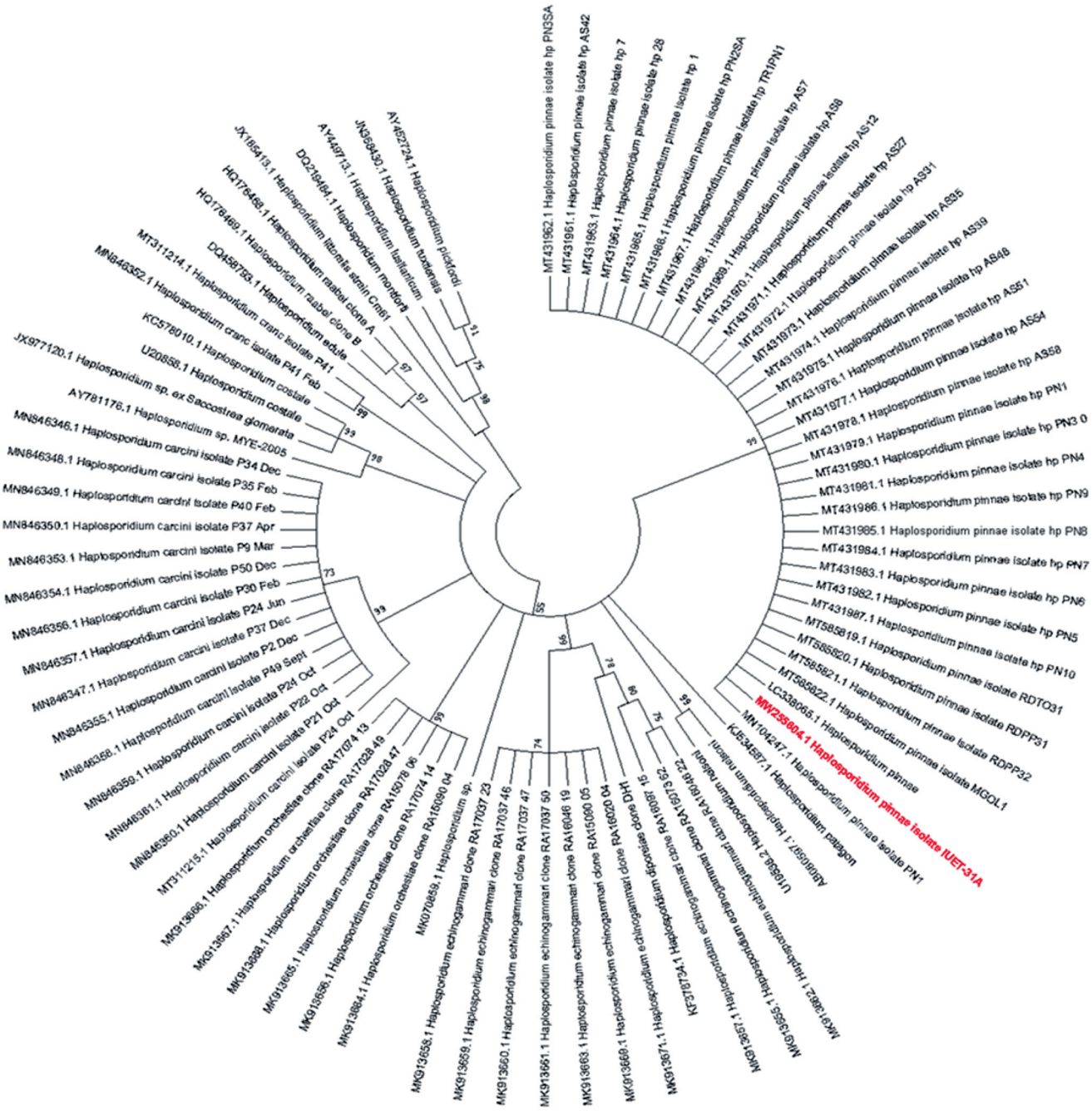

Figure 6. Maximum-likelihood phylogenetic analyses of small subunit ribosomal RNA (SSU rRNA) gene of *Haplosporidium* isolates. The analyses involved 84 nucleotide sequences and all positions containing gaps and missing data were eliminated. Bootstrap values are shown at nodes.

increased counts of brownish cytoplasm cells in the intestine (Figure 8(c)).

Antioxidant enzyme analyses

SOD enzyme activity values measured in gill tissues of *P. nobilis* samples collected in the study were 46.2 ± 4.9 U mg^{-1} in healthy individuals, while it was measured as 64.1 ± 10.4 U mg^{-1} in infected individuals. This difference seen in the gill tissue in terms of SOD activity was statistically significant ($F = 25.4$, $p < 0.05$). Similarly, SOD activity values measured in digestive gland tissues of the same individuals were 49.1 ± 14.4 U mg^{-1} in healthy individuals, while it was measured as 67.5 ± 6.8 U mg^{-1} in infected individuals. This difference in SOD activity between healthy and infected individuals was statistically significant ($F = 8.6$, $p < 0.05$) (Figure 9(a)). The CAT activities were measured in gill tissues as 181.5 ± 30.5 U mg^{-1} in healthy individuals. The measured values increased in infected individuals and were 244.6 ± 15.8 U mg^{-1}. This increase was statistically significant ($F = 22.2$, $p < 0.05$). CAT values measured in the digestive gland are also quite different in terms of healthy and infected individuals. The values were 211.1 ± 23.8 U mg^{-1} in healthy individuals and 240.7 ± 23.5 U mg^{-1} in infected individuals. Again, this difference was also statistically significant

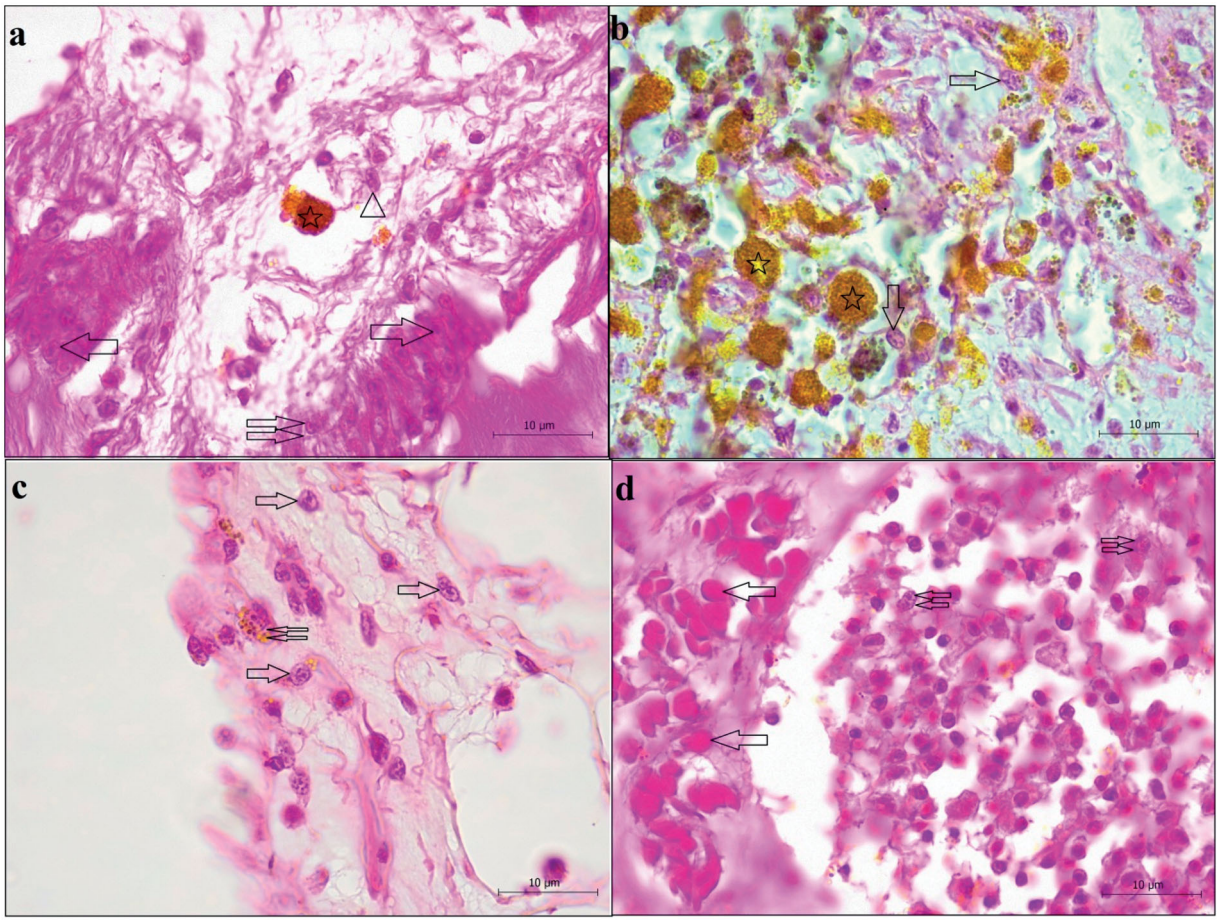

Figure 7. Histological sections through the different part of *P. nobilis* infected with *H. pinnae*. (a) Histopathological section of a connective tissue around the intestine; uninucleate cell of the parasite (arrows), binucleate cell of the parasite (double arrow), plasmoidal stages (triangle), sporocyts enclosing more or less mature spores (star), H&E. (b) Histopathological section of a connective tissue around the digestive gland; plasmoidal stages (arrows), sporocyts enclosing more or less mature spores (star), H&E. (c) Histopathological section of a mantle; plasmoidal stages (arrows), sporocyts enclosing more or less mature spores (double arrow), H&E. (d) Histopathological section of connective tissue around the digestive gland; large vacuole with amorphous eosinophilic material (arrows), plasmoidal stages (double arrows), H&E.

($F = 6.3$, $p < 0.05$) (Figure 9(b)). GPx activity measurements were 10.8 ± 2.8 U mg^{-1} in the gill tissue of healthy individuals and 14.0 ± 3.8 U mg^{-1} in infected individuals. The difference between these values was not statistically significant ($F = 4.1$, $p > 0.05$). Looking at the values of GPx in the digestive gland tissue, values were; 9.8 ± 1 U mg^{-1} in healthy individuals and 11.8 ± 1.5 U mg^{-1} in infected individuals and the difference was statistically significant ($F = 12.3$, $p < 0.05$) (Figure 9(c)).

Discussion

Mass mortalities of the *P. nobilis* species began to be observed along the Mediterranean coasts in 2016 (Darriba 2017, Vázquez-Luis *et al.* 2017). In addition to cases in the Mediterranean, mass deaths were recorded in individuals in the Aegean Sea over time (Catanese *et al.* 2018, Katsanevakis 2019). When these mortalities began to be investigated, the findings indicated that they were caused by *H. pinnae* (Catanese *et al.* 2018, Panarese *et al.* 2019, Tiscar *et al.* 2019), but recent studies suggest that deaths in individuals occur due to co-infection of the protozoa with bacteria such as *Mycobacterium* sp. or *Vibrio* sp. (Carella *et al.* 2019, 2020, Scarpa *et al.* 2020). Studies based on the observation of recent mass deaths of *P. nobilis* in the Aegean Sea and Çanakkale Strait (Öndes *et al.* 2020b, Özalp and Kersting 2020, Acarlı *et al.* 2021) raised the question of whether these deaths were caused by *H. pinnae* alone or related to other bacterial infections besides this pathogen.

Several studies emphasize that the mortality of *P. nobilis* could be related to *Mycobacterium* (Carella *et al.* 2019, Carella *et al.* 2020, Lattos *et al.*, 2020b) and *V. mediterranei* (Prado *et al.* 2020, Andree *et al.* 2021). To our knowledge, *V. harveyi* was not reported previously as a causative agent in mass mortality events of *P. nobilis*. In the present study, it was determined that *V. harveyi* could be related to mass mortality in *P. nobilis*. *V. harveyi* is a marine, gram-negative, well-known bacterial species causing mortalities in marine organisms. This species was identified as the aetiological agent of diseases in a wide variety of marine animals, both vertebrates and invertebrates including aquaculture fish species, sharks, abalones, Penaeid shrimps, lobster, sea cucumbers, and sea horses (Austin and Zhang 2006). The presence of *V. harveyi* in tissues infected with *H. pinnae* obtained in this study suggests the possibility of this bacterium causing

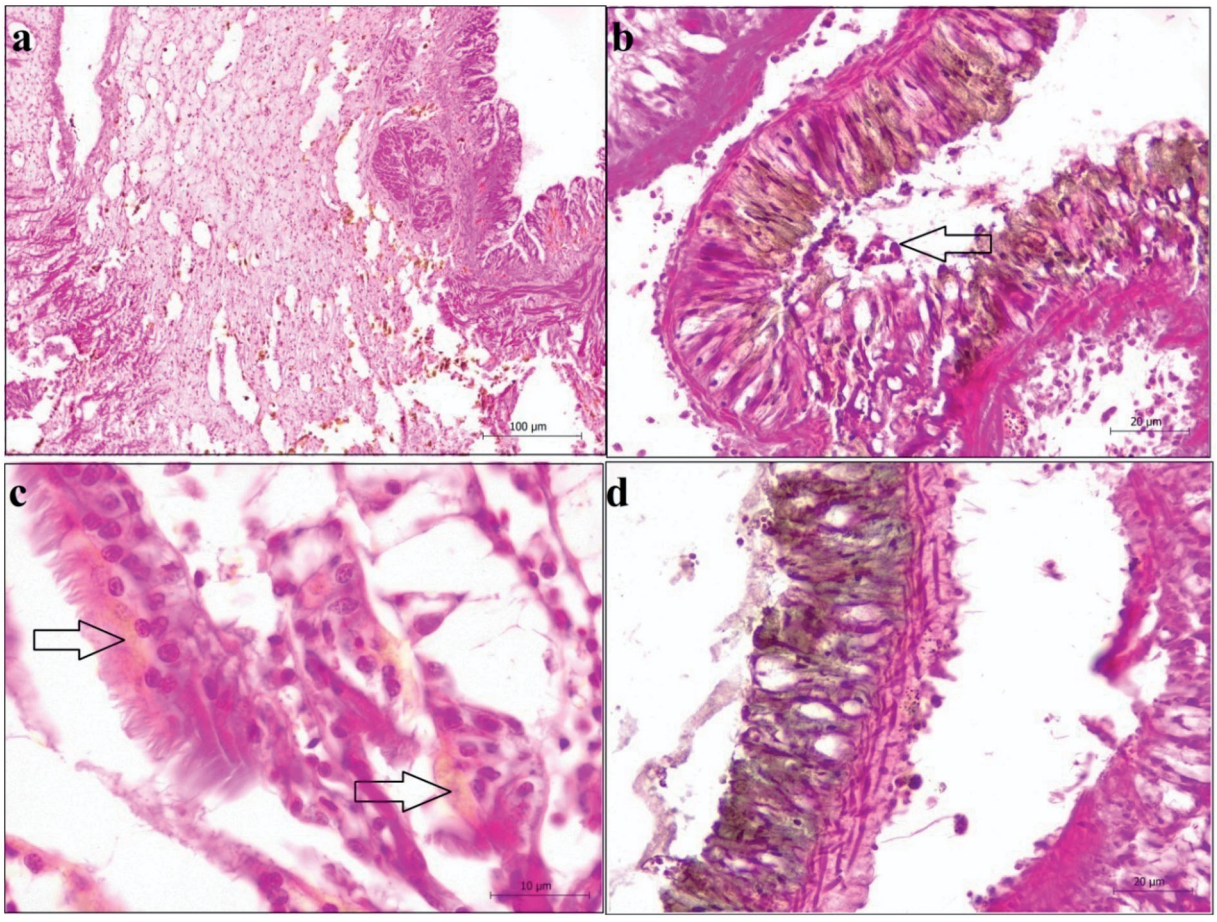

Figure 8. Histopathological findings of the different part of *P. nobilis* infected with *H. pinnae*. (a) *P. nobilis* specimen showing haemocytic infiltrations of the connective tissue around the digestive gland and intestine, H&E. (b) Parasitic stages of *H. pinnae* (arrow) in the intestinal lumen, epithelial deformations and necros, H&E. (c) The cells with brown cytoplasm (arrow) in the intestine, H&E. (d) Detachment of epithelial cell and *H. pinnae* spores in the intestine, H&E.

opportunistic infection in *P. nobilis* along with the presence of the determined *Vibrio* sp. species.

Polymerase chain reaction (PCR), one of the molecular biological techniques, was used as an indispensable tool in both life sciences and human/veterinary pathology studies since the day it was invented. This powerful tool was also used for pathogen screening/confirmation purposes in *P. nobilis* infections (Catanese *et al.* 2018, Katsanevakis 2019, Panarese *et al.* 2019). Even in cases where the parasite could not be observed in histopathological sections, the pathogen could be detected in tissues with this tool (Catanese *et al.* 2018). In this study, the parasite observed in histopathological sections was confirmed as *H. pinnae* by nested PCR applied to digestive gland tissues and subsequent sequencing. When compared with Genbank records, the *H. pinnae* sequence obtained in this study showed high similarity with other samples isolated from the Mediterranean region including isolate PN1 (100%, acc. no. MN104247) and isolate hp_AS42 (99.78%, acc. no.MT431961) from Italy and an isolate from Western Mediterranean Sea/Spain (99.54%, acc. No. LC338065).

The histopathological results of this study showed that each known life stage (uni nucleated, bi nucleated, plasmodial, sporocysts, spores) of *H. pinnae* was found in the same infected fan mussels. The excessive frequency of protozoa occurred in the connective tissue, haemolymph sinuses, and the gut epithelium, where the uninucleate and the less frequent sporocysts and plasmodial stages again were associated with systemic infection (Catanese *et al.* 2018). Our histological analyses findings are in agreement with reports by Catanese *et al.* (2018), we observed severe infection of the fan mussels in the connective tissue and digestive glands (Figures 7 and 8). Histopathological findings are important for the assessment of the health status of the organisms. Histopathological studies revealed that the presence of *H. pinnae* protozoan was related to important histological lesions in the infected specimens of *P. nobilis* (Darriba 2017, Catanese *et al.* 2018, Katsanevakis 2019, Panarese *et al.* 2019, Tiscar *et al.* 2019, Box *et al.* 2020a, Carella *et al.* 2020, Lattos *et al.* 2020a). In our study, histological examination of fan mussels showed that severe infection of *H. pinnae* was observed especially in the mantle, connective tissue, intestine, and digestive gland. These findings are in agreement with reports by Darriba (2017), Catanese *et al.* (2018), Panarese *et al.* (2019), and Katsanevakis (2019). The observed lesions were especially present in the epithelium of the digestive gland and intestine. Also, we observed increases in brownish cell and haemocytic infiltration and focal necrosis. Our findings are in agreement with a previous study which was reported by Panarese *et al.* (2019).

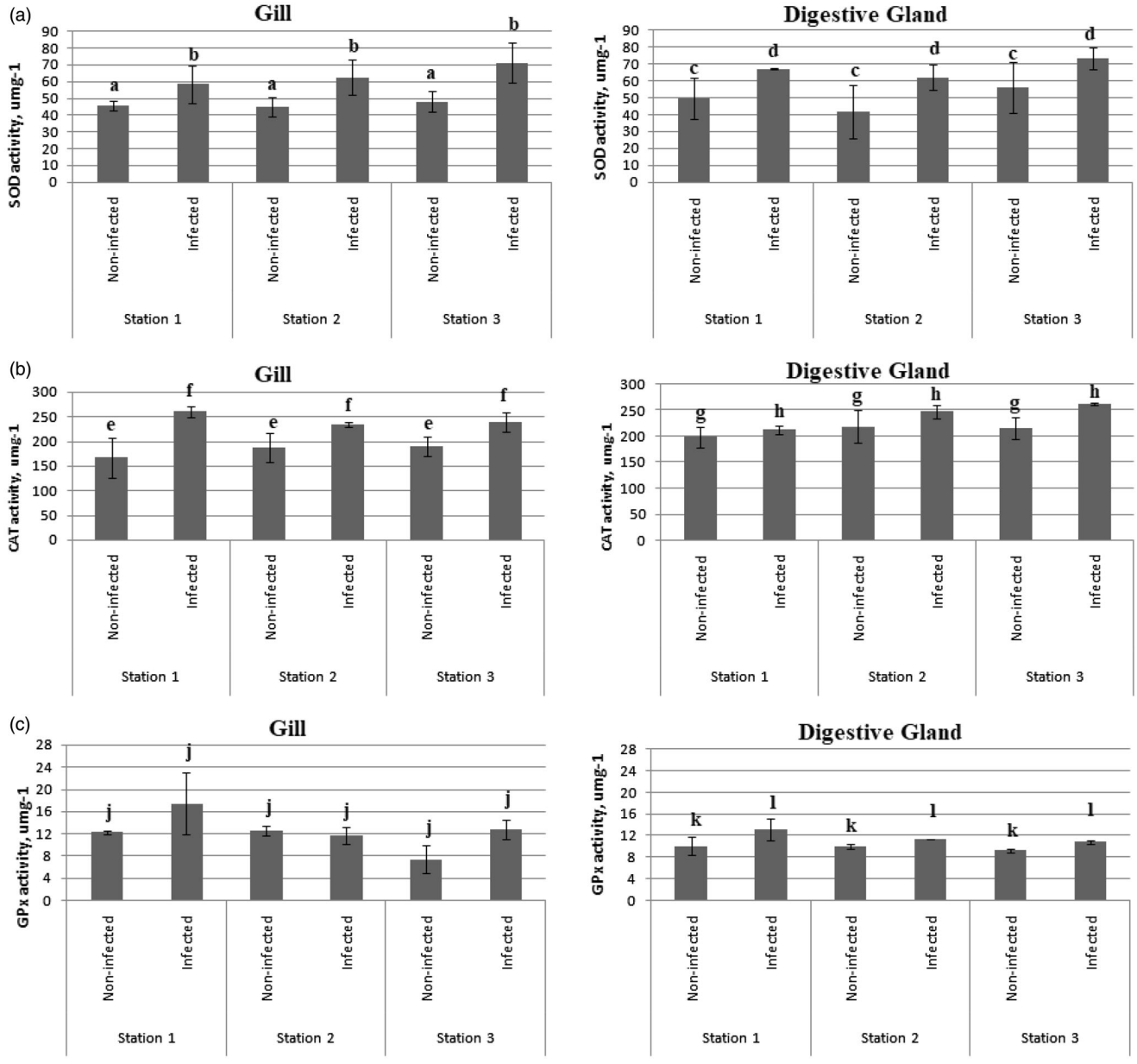

Figure 9. (a) SOD, (b) CAT and (c) GPx activities in gill and digestive gland tissues of healthy and infected *P. nobilis* samples. Significant differences ($p \leq 0.05$) among concentrations are represented with different letters.

Antioxidant responses were also evaluated in this study, in which the causes of mass deaths thought to be caused by both pathogenic and bacterial infections were investigated. In the processes in which the infection spreads through this type of transmission, it is expected that there will be an increase in ROS production to eliminate pathogens and oxidative stress and thus antioxidant defense activation are triggered if they are not eliminated sufficiently (Capó *et al.* 2015, Box *et al.* 2020a). The antioxidant responses of *P. nobilis* species as a result of various environmental pollutants or anthropogenic processes were demonstrated in various studies. The general belief in these studies is that antioxidant responses are also increased due to oxidative stress (Sureda *et al.* 2013a, 2013b, Natalotto *et al.* 2015, Capó et al. 2015). In addition, the antioxidant status of *P. nobilis* individuals surrounded by invading *Lophocladia lallemandii* colony and non-invasive individuals were compared, and significant increases were detected in both digestive gland and gill tissue of individuals after the invasion (Box *et al.* 2009). Only one study investigating the antioxidant enzyme level of *P. nobilis* as a result of *H. pinnae* parasite infection was found (Box *et al.* 2020a). In this study, a decrease in antioxidant defense was detected and this decrease could lead to the inability to resist infection and thus high mortality. In the same study, it was mentioned that a bivalve species (*Mimachlamys varia*) infected with *Perkinsus mediterraneus*

parasite had a progressive increase in antioxidant level (Box *et al.* 2020b), and it was reported that they obtained the opposite results in their own studies. In this study, we determined that *H. pinnae* and *Vibrio* sp. infection caused an increase in the antioxidant defense of *P. nobilis*, and this increase strengthens the possibility that it is due to ROS production to eliminate both parasitic and bacterial infection.

Conclusion

Based on the results, it could be concluded that the *H. pinnae* protozoan was one of the primary causative organisms responsible for the mass mortality event of *P. nobilis* in Canakkale Strait, Turkey. The histological studies have demonstrated various developing stages of the protozoan in the different vital tissues (connective tissues, digestive glands, and intestine) of these mussels. The microbiological analyses also confirm *V. harveyi* and *Vibrio* sp. bacteria in these tissues along with *H. pinnae* infection. Further, enhanced antioxidative enzymes (SOD, CAT, and GPx) activity levels in the gills and digestive glands of mussels in Çanakkale Strait, Turkey, represent that the organism is facing oxidative stress due to the coinfections. Hence, with the help of multibiomarkers present study showed that these two pathogens could invade various other vital tissues of mussels, leading to their devastating health impact. Furthermore, it is suggested that investigation related to the impact of bacteria coinfection with protozoan on the economically essential mussels should be carried out in other parts of countries located on the Mediterranean coast so that the government authorities could manage their rehabilitation in time.

Authors' contributions

İEK was involved in the microbiological analyses, writing of manuscript, editing, and finalization. SEG was involved in the antioxidant enzyme analyses, writing of manuscript, editing, and finalization. AA was involved in the visual observations. ET was involved in the molecular analyses, writing of the manuscript; editing. FÇ was involved in the work plan of this study. MG was involved in the histopathological analyses, writing of manuscript editing, and finalization. UA was involved in the visual observations, writing of the manuscript, statistical design and analysis, study administration. All authors read and approved the final manuscript.

Ethical approval

The samples were collected with permits from the Ministry of Agriculture and Forestry of the Turkish Republic (28.04.2020/39871594-600-E.2000061639).

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No potential conflict of interest was reported by the author(s).

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