



Wide-Geographic and Long-Term Analysis of the Role of Pathogens in the Decline of *Pinna nobilis* to Critically Endangered Species

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A mass mortality event (MME) affecting the fan mussel *Pinna nobilis* was first detected in Spain in autumn 2016 and spread north- and eastward through the Mediterranean Sea. Various pathogens have been blamed for contributing to the MME, with emphasis in *Haplosporidium pinnae*, *Mycobacterium* sp. and *Vibrio* spp. In this study, samples from 762 fan mussels (necropsies from 263 individuals, mantle biopsies from 499) of various health conditions, with wide geographic and age range, taken before and during the MME spread from various environments along Mediterranean Sea, were used to assess the role of pathogens in the MME. The number of samples processed by both histological and molecular methods was 83. The most important factor playing a main role on the onset of the mass mortality of *P. nobilis* throughout the Mediterranean Sea was the infection by *H. pinnae*. It was the only non-detected pathogen before the MME while, during MME spreading, its prevalence was higher in sick and dead individuals than in asymptomatic ones, in MME-affected areas than in non-affected sites, and it was not associated with host size, infecting both juveniles and adults. Conversely, infection with mycobacteria was independent from the period (before or during MME), from the affection of the area by MME and from the host health condition, and it was associated with host size. Gram (-) bacteria neither appeared associated with MME.

Keywords: *Pinna nobilis*, *Haplosporidium pinnae*, *Mycobacterium* sp., Gram negative bacteria, molecular biology, histology

INTRODUCTION

The fan mussel *Pinna nobilis* is a Mediterranean endemic, marine bivalve mollusc now critically endangered (IUCN, 2019). During the last decades, *P. nobilis* populations have progressively decreased due to anthropogenic impacts, mainly related to the destruction of their habitats, including illegal fishing, ornamental harvesting, and accidental killing by anchoring, bottom nets and trawlers (Basso et al., 2015; Vázquez-Luis et al., 2017). However, an unusual mass mortality event (MME) of this species was first detected in the SE Spanish coast in autumn 2016 (Vázquez-Luis et al., 2017) and fast spread north- and eastward, reaching France, Italy, Morocco, Tunisia, Malta, Greece, Croatia, Bosnia and Herzegovina, Cyprus and Turkey in 3 years (IUCN, 2018, 2020; Carella et al., 2019; Katsanevakis et al., 2019, 2021; Panarese et al., 2019; Čelebičić et al., 2020; Čižmek et al., 2020; Lattos et al., 2020a; Öndes et al., 2020; Özalp and Kersting, 2020; Scarpa et al., 2020; Zotou et al., 2020; Acarli et al., 2021; Çinar et al., 2021).

Knowing the cause of this MME is crucial to implement measures to avoid its extinction and to find recovery ways. However, its primary cause (or interrelated causes) remains an enigma until now. Two microorganisms have been proposed as the most plausible aetiological agents. From a chronological point of view, a member of Haplosporida, a protistan group enclosing parasites highly pathogenic for bivalves (Hartikainen et al., 2014; Arzul and Carnegie, 2015), was first detected associated to high fan mussel mortality in Calpe (Spain) (Darriba, 2017). Afterward, Fanelli et al. (2018) pointed out a *Haplosporidium* sp. as the aetiological agent in different Italian localities and Catanese et al. (2018) described that haplosporidan as the new species *Haplosporidium pinnae*, proposing it as the most likely cause of the MME. Later, Carella et al. (2019) reported *Mycobacterium* sp., close to a terrestrial-like non-tuberculous mycobacterium (NTM), with stronger association with the *P. nobilis* MME along the Tyrrhenian coastline (Italy) than *H. pinnae*, thus introducing a new possible aetiological agent into the aetiological scientific debate. At the same time, two publications again identified *H. pinnae* in the massive die-offs observed in the Aegean (Katsanevakis et al., 2019) and Ionian Seas (Panarese et al., 2019) but without making a differential diagnosis with *Mycobacterium* sp., the newly detected possible aetiological agent, as is also the case with the detection of *H. pinnae* in the Ligurian Sea (Betti et al., 2021). Since then, mixed infections of these two pathogens affecting fan mussels have been reported in Adriatic Sea (Čižmek et al., 2020; Šarić et al., 2020), in Aegean Sea (Lattos et al., 2020a) and, with further co-infection with other pathogens, in the Tyrrhenian Sea, Ebro Delta (NE Spain) and Aegean Sea (Carella et al., 2020; Lattos et al., 2020b, 2021; Scarpa et al., 2020). Additionally, the Gram (–) bacterium *Vibrio mediterranei*, previously described as an opportunistic emerging pathogen associated with mortalities in the wild (Rodríguez et al., 2017), has again been detected in natural and stabled fan mussels (Prado et al., 2020a; Andree et al., 2021; Lattos et al., 2021). Nowadays, co-infection with *H. pinnae* and bacteria of various *Vibrio* spp., has been proposed as the cause of the MME in the Çanakkale Strait (Turkey) (Künili et al., 2021). The more recent findings could suggest a more complex scenario than that originally

proposed in the first descriptions of the *P. nobilis* MMEs. Diseases with complex aetiology, influenced by various interacting drivers, frequently remain difficult to characterise due to reductionist approaches (De Lorgeril et al., 2018).

The International Union for the Conservation of Nature (IUCN, 2019) proposed a list of conservation recommendations with the objective of avoiding the plausible total extinction of the species. The main was “to investigate on the pathogens and vectors to conduct a deep study of the origin, epizootic dynamics and the complete life cycle of *H. pinnae*, as well as to clarify the pathogenicity and potential virulence of the reported mycobacteriosis.” However, the critically endangered status of this species involves many restrictions for experimentation, which complicates that research. To overcome these difficulties, contributions of multiple teams in various countries were assembled to configure a wide geographic, long term approach robust enough to support solid conclusions. This article reports the results of such a broad survey, based on histological and molecular diagnoses, including alive fan mussels collected before MME (2011–2014, from populations in a good state of conservation, but today extinct) and fan mussels in three classes of health condition (asymptomatic, sick and recently dead individuals) collected during the MME (2016–2020) in different areas, in order to: (1) assess the occurrence of potential pathogens (particularly *H. pinnae*, mycobacteria and Gram (–) bacteria) in fan mussels before the MME; (2) determine the occurrence of infectious agents in recently dead, sick and asymptomatic fan mussels during the different outbreaks of the ongoing MME; and (3) compare these two different scenarios, before and during MME, to unravel the relationships between the observed pathogenic microorganisms regarding the onset of the fan mussel MME.

MATERIALS AND METHODS

Sampling

Samples involving 762 fan mussels (whole individuals or biopsies) from 30 areas scattered through eight countries (Spain, France, Monaco, Italy, Croatia, Greece, Cyprus and Turkey) that had been collected by scuba diving were gathered (Table 1 and Figure 1). Due to the urgency of the situation, many researchers have contributed to the sampling collection, forming an interdisciplinary panel of researchers (all of them experts in the biology of *P. nobilis*). In the case of samples obtained during the various outbreaks of massive mortality detected, the fixed samples were immediately sent to the diagnostic laboratories, where they were processed for further study. All histology samples were processed at LIMIA (with the sole exception of some samples from Croatia-Zagreb University and Greece-HCMR Lab). Likewise, most of the samples for molecular analysis were also processed at LIMIA (with the exception of few samples from Greece). The processing protocols were the same in all laboratories. The processing time was always less than 1 month after sampling. All the samples before MME were analysed at LIMIA. The diagnosis of all the histological samples was always carried out in LIMIA, and was also the molecular diagnosis

TABLE 1 | Summary of sampling and diagnosis information, including sampling locations; sampling period; whether high mortality had occurred in the sampling location or not; sample size; number of fan mussels asymptomatic, sick and recently dead; age group (adults or juveniles); procedure of tissue removal (necropsy or biopsy); and the diagnostic results for *Haplosporidium pinnae*, mycobacteria and Gram (–) bacteria [No. positive (+) cases/No. diagnosed individuals].

<i>Pinna nobilis</i>													
Location	Country	Sampling period	High mortality	Environment	Sample size	Asymp./sick/dead	Age group (Adults/Juveniles)	Necropsy/Biopsy	Histology for <i>H. pinnae</i> (No. +/No. total)	PCR for <i>H. pinnae</i> (No. +/No. total)	Histology for Mycobacteria (No. +/No. total)	PCR for <i>Mycobacterium</i> sp. (No. +/No. total)	Histology for Gram (–) bacteria (No. +/No. total)
1. El Calón	Spain	Oct16	Yes	O	3	1/1/1	Adults	Necropsy	2/2	3/3	1/3	1/1	1/3
2. Mar Menor	Spain	Jul17	No	L	1	1/0/0	Adults	Necropsy	0/1	0/1	0/1	NA	1/1
2. Mar Menor	Spain	Dec19–Jan20	No	L	60	60/0/0	Adults	Biopsy	NA	8/60	NA	0/59	NA
3. Calpe	Spain	Oct16	Yes	O	4	1/3/0	Adults	Necropsy	3/4	3/4	NA	4/4	NA
3. Calpe	Spain	Nov17–Mar18	Yes	O	29	0/29/0	Juveniles	Necropsy	NA	21/29	NA	0/29	NA
4. Cabrera	Spain	Apr11–Mar12 (b. MME)	No	O	120	120/0/0	Adults	Necropsy	0/120	0/3	65/120	0/2	30/120
4. Cabrera	Spain	Jul–Aug14 (b. MME)	No	O	30	30/0/0	Adults	Biopsy	NA	0/30	NA	0/30	NA
4. Cabrera	Spain	Mar–May17	Yes	O	2	0/2/0	Adults	Necropsy	2/2	2/2	2/2	0/2	1/2
4. Cabrera	Spain	Jan18	Yes	O	1	1/0/0	Adults	Biopsy	NA	0/1	NA	1/1	NA
5. Majorca	Spain	Jun–Mar12 (b. MME)	No	O	17	17/0/0	Adults	Necropsy	0/17	0/12	11/17	6/12	7/17
5. Majorca	Spain	Nov16–Mar17	Yes	O	23	7/13/3	Adults	Necropsy	21/23	8/9	5/23	1/3	5/23
6. Minorca	Spain	Jun17	Yes	O	2	1/1/0	Adults	Necropsy	2/2	2/2	2/2	2/2	2/2
7. Delta de l'Ebre-Alfacs	Spain	Dec16, May18	No	L	9	9/0/0	Adults	Biopsy	NA	0/9	NA	0/9	NA
7. Delta de l'Ebre-Alfacs	Spain	Jul17, Jan18	No	L	5	2/1/2	Adults	Necropsy	0/5	0/5	0/5	NA	2/5
7. Delta de l'Ebre-Alfacs	Spain	May–Jun18	No	L	3	2/1/0	Juveniles	Necropsy	0/3	0/1	0/3	NA	3/3
7. Delta de l'Ebre-Alfacs	Spain	Jul–Dec18, Feb19	Yes	L	11	0/1/10	Adults	Necropsy	5/11	4/11	4/11	3/5	9/11
7. Delta de l'Ebre-Alfacs	Spain	Aug18–Mar19	Yes	L	21	20/0/1	Adults	Biopsy	NA	0/21	NA	0/21	NA
7. Delta de l'Ebre-Alfacs	Spain	Feb19	Yes	L	3	3/0/0	Juveniles	Necropsy	0/3	0/3	0/3	NA	0/3
8. Delta de l'Ebre-Fangar	Spain	Jan-18, Sep18	No	L	4	3/0/1	Adults	Necropsy	0/4	0/4	0/4	NA	1/4
9. Tossa de Mar	Spain	Nov17	Yes	O	4	0/3/1	Adults	Necropsy	4/4	4/4	2/4	1/2	2/4
10. Portlligat	Spain	Dec17	No	O	1	0/0/1	Adults	Necropsy	1/1	NA	1/1	NA	1/1
11. Port Vendres	France	Mar18–Nov19	No	H	31	31/0/0	Adults	Biopsy	NA	0/31	NA	0/31	NA

(Continued)

TABLE 1 | (Continued)

<i>Pinna nobilis</i>													
Location	Country	Sampling period	High mortality	Environment	Sample size	Asymp./sick/dead	Age group (Adults/Juveniles)	Necropsy/Biopsy	Histology for <i>H. pinnae</i> (No. +/No. total)	PCR for <i>H. pinnae</i> (No. +/No. total)	Histology for Mycobacteria (No. +/No. total)	PCR for <i>Mycobacterium</i> sp. (No. +/No. total)	Histology for Gram (–) bacteria (No. +/No. total)
11. Port Vendres	France	Mar18–Nov19	Yes	H	26	26/0/20	Adults	Biopsy	NA	9/26	NA	0/19	NA
12. Étang de Salses	France	May19–Jan20	Yes	L	144	142/2/0	Adults	Biopsy	NA	19/144	NA	0/144	NA
13. Port La Nouvelle	France	Dec19	Yes	H	1	1/0/0	Adults	Biopsy	NA	0/1	NA	0/1	NA
14. Agde	France	Jun19–Jan20	No	O	45	45/0/0	Adults	Biopsy	NA	1/45	NA	0/45	NA
15. Étang de Thau	France	Jun19–Jan20	No	L	53	53/0/0	Adults	Biopsy	NA	0/53	NA	0/53	NA
16. Sète	France	Mar18, Jan20	No	H	26	26/0/0	Adults	Biopsy	3/3*	0/26	0/3*	1/26	0/3*
17. Frontignan	France	Jul19–Jan20	No	H	20	20/0/0	Adults	Biopsy	NA	1/20	NA	0/20	NA
18. Port Saint Louis du R.	France	Jun–Nov19	No	O	14	14/0/0	Adults	Biopsy	NA	0/14	NA	0/14	NA
19. Ajaccio	France	Oct17	Yes	O	1	0/0/1	Adults	Necropsy	1/1	1/1	1/1	1/1	1/1
20. Tavolara	Italy	Oct18	Yes	O	6	6/0/0	Adults	Biopsy	NA	1/6	NA	NA	NA
21. Monaco	Monaco	Nov18	Yes	O	4	0/0/4	Adults	Necropsy	0/4	NA	3/4	3/3	3/4
21. Monaco	Monaco	Dec18	Yes	O	1	0/0/1	Adults	Biopsy	NA	1/1	NA	1/1	NA
22. Telašćica	Croatia	Feb19	Yes	O	3	3/0/0	Adults	Necropsy	0/3	0/3	3/3	0/3	NA
23. Elaphiti	Croatia	May19	Yes	O	3	0/3/0	Adults	Necropsy	3/3	3/3	2/3	2/3	1/3
24. Atica-Euboean G.	Greece	Aug18, Mar19	Yes	O	8	6/2/0	Adults	Necropsy	6/8	8/8	4/8	NA	NA
25. Halkidiki	Greece	Apr19	Yes	O	2	2/0/0	Adults	Biopsy	NA	2/2	NA	NA	NA
26. Crete-Bali	Greece	Mar19	Yes	O	1	0/1/0	Adults	Biopsy	NA	1/1	NA	NA	NA
27. Crete-Elounda	Greece	Feb19	Yes	O	2	2/0/0	Adults	Necropsy	0/2	2/2	2/2	NA	NA
28. Lesvos	Greece	Jan19	Yes	O	10	0/0/10	Adults	Necropsy	10/10	NA	10/10	NA	NA
29. Karaca Beach	Turkey	Jul18	No	O	3	2/1/0	Adults	Biopsy	NA	1/3	NA	NA	NA
30. Protaras	Cyprus	Mar18	No	O	5	5/0/0	Adults	Biopsy	NA	0/5	NA	0/5	NA
TOTAL					762	662/64/36	727/35	263/499	63/236	105/609	118/233	27/551	70/210
<i>Pinna rudis</i>													
3. Calpe	Spain	Mar19	Yes	O	3	3/0/0	Adults	Necropsy	0/3	0/3	0/3	NA	0/3
4. Cabrera	Spain	May17, Jan18	Yes	O	2	1/1/0	Adults	Necropsy	0/2	0/2	1/2	0/1	1/2
TOTAL					5	4/1/0	5/0	5/0	0/5	0/5	1/5	0/1	1/5

The samples collected in the period 2011–2014 (before the first detection of mass mortality) are marked with "(b. MME)" in the sampling period cells.

*The mantle fragments obtained by biopsy of three individuals from Sète were quite large and also processed by histology. NA: that diagnostic procedure was not performed in that group of fan mussels. Type of environment were also indicated: Harbour (H), Lagoon (L), and Open Sea (O).

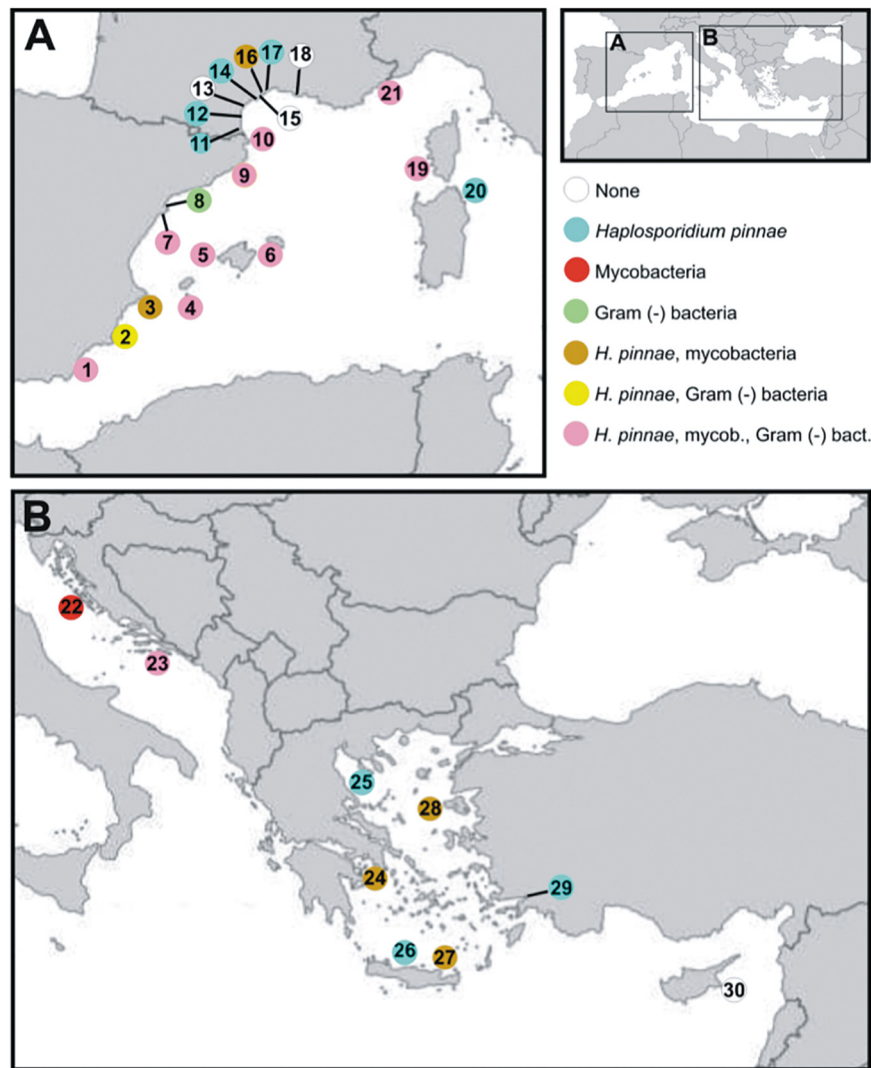


FIGURE 1 | Map of the Mediterranean Sea showing sampling locations. Next is the list of sampling locations with indication of the ecosystem type (H: harbour; L: coastal lagoon; S: open sea). **(A)** Spain: El Calón, S (1); Mar Menor, including Isla Barón, L, Isla Perdiguera, L, and Pinna Point, L (2); Calpe, including Calpe, S, and Villajoyosa, H (3); Cabrera, including Castell de Cabrera, S, and Santa María, S (4); Majorca, including Andratx, S and Magaluf, S (5); Minorca, including Son Saura, S, Boya Rey, Cala Gandulf, S, and Rates, S (6); Delta de l'Ebre-Alfacs, L (7); Delta de l'Ebre-Fangar, L (8); Tossa de Mar, S (9); Portlligat, S (10). France: Agde, including Agde, S, and Port Ambonne, H (11); Étang de Salses, including Port Leucate, H, Leucate Nord, L, Leucate Centre, L, Leucate Sud, L, and Lido Port-Leucate, H, (12); Étang de Thau, including Grau des Quilles, L, Marseillan Plage, L, and Meze, L (13); Port de Frontignan, H (14); Port La Nouvelle, H (15); Port Saint Louis du Rhone, L (16); Port Vendres, including Plage de Peyrefite, S, and Port Vendres, H (17); Port-Sète, H (18); Ajaccio, S (19). Italy: Tavolara, S (20). Monaco: Monaco, S (21). **(B)** Croatia: Telašćica, S (22); Elaphiti, S (23). Greece: Atica-Euboean Gulf, including Lavrio, S, Avilda, S, and Oropos, S (24); Halkidiki, S (25); Crete-Bali, S (26); Crete-Elounda, S (27); Lesvos, S (28). Turkey: Karaka Beach, S (29). Cyprus: Protaras, S (30). Colours of the circles correspond to the pathogens [*Haplosporidium pinnae*, mycobacteria or Gram (-) bacteria] detected in each location.

for the most part, with the exception of Greece. In LIMIA, the molecular results obtained in Greece were subsequently verified.

The sample set included fan mussels in three classes of health condition (asymptomatic, sick and recently dead individuals), three environments (coastal lagoons, harbours, and open sea) and wide size/age range, with both juvenile and adults. Asymptomatic specimens had typical healthy responses and normal mantle appearance when sampling. Sick fan mussels showed no response to stimuli, with pale and retracted mantle, and slow valve closure. Recently dead individuals were those in which decomposition

process had started but the presence of tissues that were still preserved and consistent allowed sampling. In the natural environment it is difficult to determine the time of death of these individuals. From observations of fan mussels maintained in captivity, recently dead individuals presumably corresponding to specimens that have died less than 36 h before (García-March personal communication).

A total of 126 whole specimens and 469 mantle biopsies were collected during MME spreading (2016–2020); additionally, 137 alive whole specimens and 30 mantle biopsies corresponded

to the period 2011–2014, before the MME was first noticed. Samples in the period 2016–2020 were collected in the context of population surveillance or even awareness of first signs of abnormally high mortality in some of those populations by scientists, citizens and services for marine protected areas management, after the first detection of the MME in autumn 2016. Thus, high fan mussel mortality had affected some locations when sampling, while it had not occurred yet in others, which were sampled in the context of the alarming news from neighbouring areas. These samplings included collection of recently dead, sick and asymptomatic whole fan mussels, for a total of **126** specimens (more details in **Table 1**). The sacrifice of some asymptomatic and sick individuals was considered necessary to search for the causes of each mortality outbreak by analysing organs that cannot be sampled without killing the fan mussels. Most of those collected fan mussels were adults but 29 juvenile specimens (ca. 40 mm in dorso-ventral length) that had been settled on the anchorage ropes of finfish culture cages near Calpe and 6 more juveniles (ca. 180 mm in dorso-ventral length) collected from the “Banya” sandspit of Delta de l’Ebre-Alfacs were also included. Mantle biopsies were taken from other **469** adult specimens, without removing them from their habitat, during that period, using a specific procedure for *P. nobilis* preventing killing (Wesselmann et al., 2018). Additionally, samples that had been collected from 2011 to 2014 for other scientific purposes (characterisation of the gonad cycle and other physiological processes as well as search for potential pathogens) from Balearic Islands populations, extinct today, were included in this study to assess the potential pathogenic load of fan mussels before the MMEs, thus allowing comparison of the pathogenic load before and during MMEs. Namely, **120** live individuals were collected by scuba diving in the Cabrera National Park Marine Protected Area (CNP-MPA) and **17** live individuals from other fishing exploitation localities in Majorca Island in 2011–2012 (Deudero et al., 2017; Box et al., 2020); additionally, **30** mantle biopsies were taken from alive individuals located in CNP-MPA in 2014 (**Table 1**). A summary of sampling and diagnosis information is provided in **Table 1**. The number of samples processed by both histological and molecular methods was **83**.

Furthermore, five spiny fan mussels *Pinna rudis*, a species not affected by high mortality (Vázquez-Luis et al., 2017; Catanese et al., 2018), were collected from two areas affected by the *P. nobilis* MME for comparative analysis.

Due to *P. nobilis* being a strictly protected species (Annex II of Barcelona Convention and Annex IV of the European Council Directive 92/43/EEC), all samplings were carried out under permission of regional and national authorities (listed below in Ethics Declarations: Permissions). For this reason, during the MME, a limited number of recently dead, sick and asymptomatic individuals were collected from each location. However, in the authorised survey conducted before the MME (2011–2012), approximately 9–12 individuals were collected every month. This number represented a very low percentage of the fan mussel population in the CNP-MPA at that time and the natural population showed no signs of disease (Vázquez-Luis et al., 2014; Deudero et al., 2015).

Specimens collected before MME were measured (dorso-ventral and antero-posterior shell lengths) and weighed (total and fresh weight) in the laboratory. Other biometric parameters were obtained from stored tissues. During MME, measures were taken, when possible, while performing visual census surveys, although sometimes it was not done due to the inherent difficulties of field sampling by scuba diving. In the case of mantle biopsies from live individuals, only shell width was measured when possible. Individuals collected for diagnosis embraced a wide length range, from juveniles to large adults (from 2.4 to 71 cm in shell length). Additional information, such as depth, temperature and environment, was also recorded when possible. The sampling effort covered all the depths that scuba divers can easily reach (0.3–35 m) and the different types of bottoms this long-living bivalve inhabits. Environments were directly recorded by direct census or compiled from data reported in scientific articles (Darriba, 2017; Katsanevakis et al., 2019; Čížmek et al., 2020), also indicating if sampling was made in coastal open sea, coastal lagoons or harbours. Although it is not specifically a lagoon, Delta de l’Ebre estuarine (Alfacs and Fangar bays) was considered as such, due to its semi-enclosed water bodies with freshwater inputs, its geomorphology, environmental conditions and paralic sedimentation that make it different in relation to the open sea (Prado et al., 2021).

Sample Processing and Histological Analysis

The biopsies consisted of mantle pieces taken *in situ*, which were preserved in absolute ethanol for molecular diagnosis. Exceptionally, the mantle biopsies from three individuals located in Sète were large enough to be also processed by histology.

The collected whole fan mussels (necropsies), either asymptomatic, sick or recently dead, were dissected and soft tissues preserved in 10% phosphate-buffered formalin or in Davidson’s fixative for histological analysis. For molecular diagnosis, before MME, samples of soft tissues were stored in absolute ethanol or at -80°C while during MME the samples were preserved only in absolute ethanol (**Table 1**). However, the whole soft tissues of the 29 juvenile fan mussels collected from Calpe were preserved in absolute ethanol and processed for molecular diagnosis. Additionally, the Ray’s fluid thioglycolate medium (RFTM) assay (Ray, 1963) was performed with gill pieces from individuals collected in 2011–2014 to assess infection with *Perkinsus* spp. For histology, a cross section of the visceral mass at the level of the digestive gland was taken from the fixed soft tissues in the case of adults, while the whole fixed soft tissues were used in the case of juveniles. Digestive gland was chosen for diagnosis because it is the target organ for *H. pinnae* sporulation (Catanese et al., 2018) as well as one of the organs infected with *Mycobacterium* sp. when it occurs; due to anatomical continuity, organs in contact with the digestive gland, namely the gonad, were partially included in the tissue pieces processed by histology. Most samples were dehydrated in an increasing ethanol gradient and cleared with X-free, embedded in paraffin wax and sectioned at 3–4 μm thickness; other samples were included in Technovit 7100 (Kulzer, Germany) and sectioned at 1–2 μm . In the case

of the histological analyses of the samples prior to the MME (samples from 2011 to 2012) they were performed on new sections of the stored paraffin blocks. Sections from each block were stained with Mayer's haematoxylin and eosin (MHE). Some additional sections were stained with Mayer's haematoxylin-VOF (Gutiérrez, 1967), with which *H. pinnae* spores become highlighted (Catanese et al., 2018). Gram (Scharlab histo kit) and Ziehl-Neelsen (ZN; Luna, 1968) staining procedures were also performed to facilitate detection of bacteria and acid-fast bacteria (*Mycobacterium* sp.), respectively in most of the samples.

Molecular Analysis

Small pieces of the collected tissues were chopped with sterile scissors and used for DNA extraction; triplicate extractions were performed for each tissue, in order to minimise biases. Total DNA was extracted from mantle and/or digestive gland pieces (adults) or homogenised tissues (juveniles), using the NucleoSpin® Tissue DNA Isolation Kit (Macherey-Nagel), according to the manufacturer's instructions.

For the Greek samples, DNA was extracted by precipitation with isopropanol and ammonium acetate (5 M) following the manual of Sambrook et al. (1989). Each sample was washed with 800 μ l of sterile distilled water for 15 min, following centrifugation at $13,000 \times g$ for 2 min. The supernatant was removed and the wash was repeated. Afterward, each sample was washed with 600 μ l of lysis buffer (0.5 M Tris, 0.1 M EDTA, 2% SDS, pH 8.8) for 15 min, following centrifugation at $13,000 \times g$ for 2 min and removal of the supernatant. The washes with the lysis buffer were repeated twice. The pellet was mixed with 600 μ l of lysis buffer and 6 μ l of proteinase K (20 mg/ml) and incubated at 55°C overnight.

In the final step of the DNA extraction protocol, i.e., the elution of the DNA pellet, replicate samples were pooled and their concentration was measured in a NanoDrop 1000 spectrophotometer.

The nucleic acids were subjected to PCR amplification employing the primer pairs HPNF3/HPNR3 (Catanese et al., 2018; López-Sanmartín et al., 2019) for *H. pinnae*, and mycgen-f/mycgen-r for *Mycobacterium* spp. (Böddinghaus et al., 1990; Carella et al., 2019), using the same PCR conditions applied by the authors who described them. All the obtained PCR products were purified using a mi-Gel Extraction Kit (Metabion, Germany) and bi-directionally sequenced using an ABI 3130 Genetic Analyser (Applied Biosystems, Carlsbad, CA, United States); the sequences were analysed using the BLAST algorithm at the NCBI server¹ for species identification. PCR products obtained from samples collected before the onset of MME were sequenced to confirm and/or to identify the species detected by histology. However, the fact of obtaining PCR amplifications after years of sampling indicates good quality and acceptable storage of the samples.

Ray's Fluid Thioglycolate Medium Assays

A piece of one gill lamella of each fan mussel collected in 2011–2012 (see Deudero et al., 2017) from CNP-MPA and Majorca, was incubated in Ray's fluid thioglycolate medium

(RFTM, Ray, 1963), supplemented with chloramphenicol and nystatin, for 1 week at room temperature in the dark, to detect hyphospores of *Perkinsus* spp. Afterward, tissues were crushed on a slide, stained with Lugol's iodine solution, covered and observed under light microscopy to estimate the severity of *Perkinsus* infection using the modified Ray's scale (Andrews and Hewatt, 1957) with six severity degrees, from 0 (no parasite detected) to 5 (very heavy infection).

Statistical Analysis

Due to the variable nature of the available samples (biopsies or necropsies), some could only be analysed by PCR, others by histology and others by both. In the absence of a gold standard to evaluate the sensitivity and specificity of each diagnostic method, the agreement between the histological and molecular diagnostic methods for both *H. pinnae* and *Mycobacterium* sp. was evaluated using Cohen's kappa coefficient (κ) (Cohen, 1960) and interpreted according to Landis and Koch (1977). The following associations were tested using contingency tables and the chi square (X^2) test: (1) association between the period regarding the MME (either before the first detection or during the MME spreading) and the occurrence of *H. pinnae*, mycobacteria or Gram (–) bacteria, respectively, in the fan mussels; (2) between the health condition of the fan mussels (either asymptomatic, sick or recently dead individuals) and the occurrence of the mentioned pathogens; (3) between the affection of the sampling area by MME (either yes or not) and the occurrence of the mentioned pathogens; (4) between the occurrence of the mentioned pathogens and host size (10 cm length) classes; (5) between the occurrence of *H. pinnae* and the environment type (either coastal lagoon, harbour or open sea) and (6) paired associations between the mentioned pathogens. Significance was considered at $P \leq 0.05$.

RESULTS

Histological and Molecular Diagnosis

Histological examination showed infection with *H. pinnae* in 63 of the 236 analysed fan mussels; no detection corresponded to individuals collected before the MME. Stages of the parasite sporulation process were detected in eight asymptomatic and eight sick individuals (Table 1 and Figure 2). Considering the 60 cases of detection of *H. pinnae* in histological sections that included digestive gland (the only organ where sporulation occurs), sporulation stages were detected in 16/60 samples (27%) of the infected individuals. Regarding diagnosis by PCR, *H. pinnae* was detected in 105/609 (17%) of the analysed fan mussels; again, no positive case was detected in individuals collected before the MME (Table 1).

Regarding bacterial infections in the samples taken before MME, ZN staining revealed mycobacteria in the connective tissue of the digestive gland and surrounding area (Figures 3A,B) in 76/137 (55%) of the analysed individuals, while Gram staining showed infection with Gram (–) bacteria in 37/137 (27%) (Table 2); coinfection with mycobacteria and Gram (–) bacteria was detected in 24% of the individuals. Positive detection of

¹<http://blast.ncbi.nlm.nih.gov/Blast.cgi>

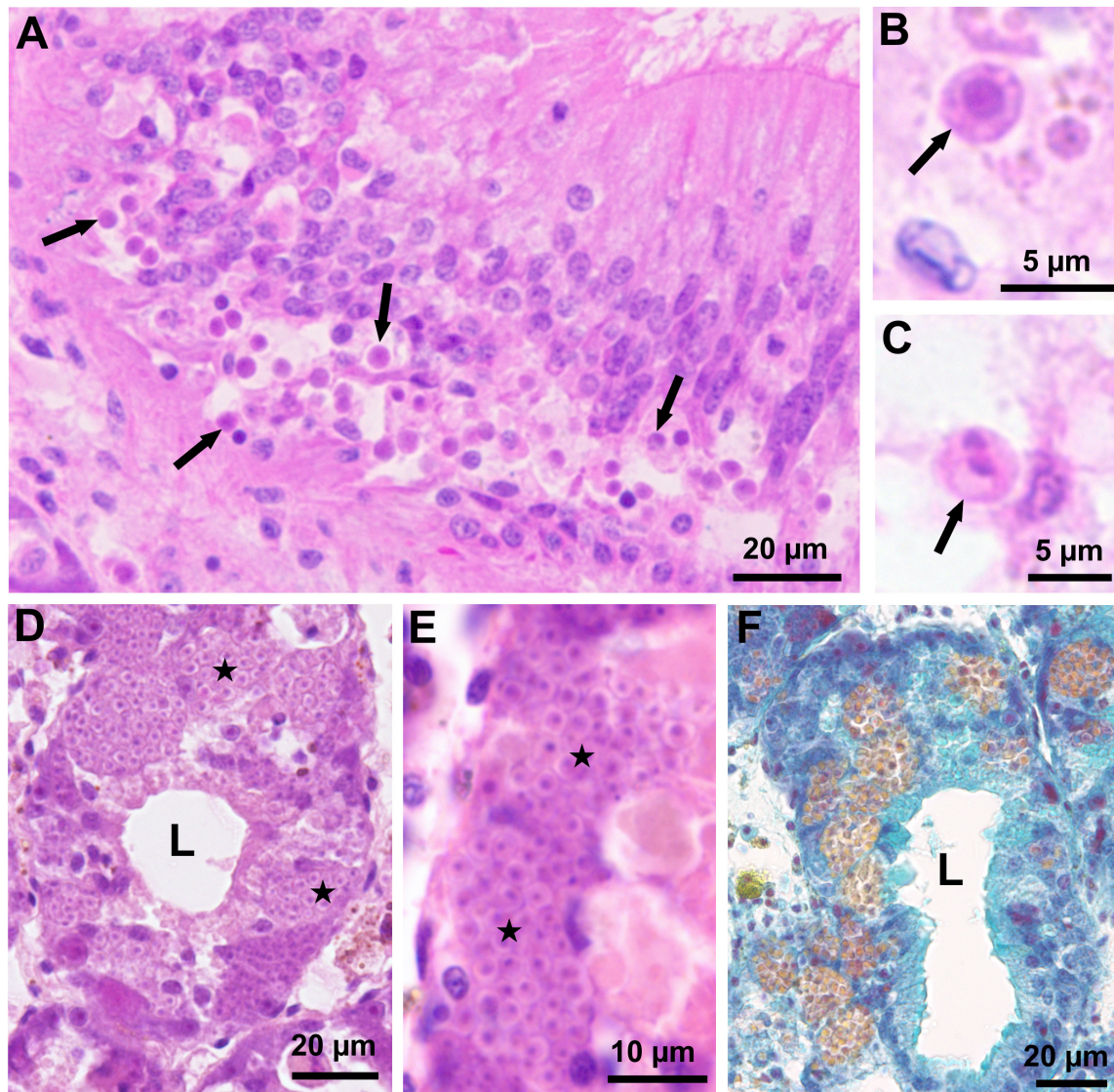


FIGURE 2 | Light micrographs of histological sections through the visceral mass of fan mussels *Pinna nobilis* infected with *Haplosporidium pinnae*. **(A)** Section through the intestine showing numerous uninucleate cells of *H. pinnae* (arrows) associated with vacuolisation of the host intestinal epithelium (MHE; Lesvos, January 2019). **(B)** High magnification showing a uninucleate cell of *H. pinnae* (arrow) in the host connective tissue (MHE; Lesvos, January 2019). **(C)** High magnification showing a binucleate cell of *H. pinnae* (arrow) in the host connective tissue (MHE; Lesvos, January 2019). **(D)** Section through the host digestive gland showing masses of *H. pinnae* spores (stars) in the epithelium of a digestive tubule (MHE; Majorca, November 2016). **(E)** High magnification showing masses of *H. pinnae* spores (stars) in the epithelium of a digestive tubule (MHE; Majorca, November 2016). **(F)** Section through the host digestive gland, stained with VOF, showing sporulation stages of *H. pinnae* in the epithelium of digestive tubules; mature spores are easily distinguished due to their yellow colour (Majorca, November 2016). L, lumen of digestive tubules.

Mycobacterium sp. occurred in 6/44 (14%) of the PCR-analysed individuals **before** MME (**Table 2**). **During** MME, 42/96 (44%) of the analysed individuals were positive to mycobacteria with the ZN staining and 33/73 (45%) to Gram (–) bacteria (**Table 2**); mixed infections were also detected. Total positive detection of *Mycobacterium* sp. occurred in 21/507 (4%) of the PCR-analysed individuals (**Table 2**).

The tested DNA sequences through BLAST search showed 100% similarity with that of *H. pinnae* (accession number: LC338065; LR131124) and those of *Mycobacterium*

sp. (accession numbers: X88906, MH569645, MH569646, MT642064, LR135613), respectively.

A total of **83** fan mussels were diagnosed both with histological and molecular procedures for *H. pinnae*, with agreement between them in 72/83 (87%) individuals (**Table 3**); that was “substantial” agreement ($\kappa = 0.734$). Regarding diagnosis of mycobacteria, 42 fan mussels were diagnosed both with histology and PCR, with agreement only in 28/42 (67%) individuals (**Table 3**); that was “fair” agreement ($\kappa = 0.211$). Remarkably, 20/34 (59%) of the positive cases for mycobacteria by histology were negative

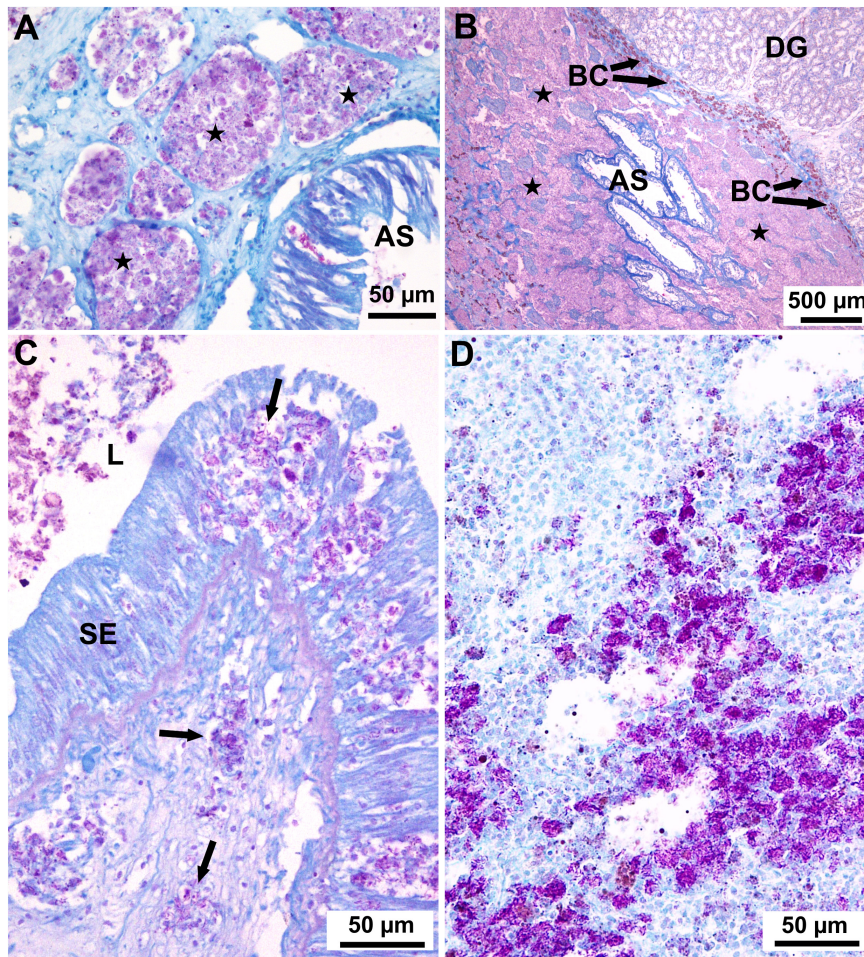


FIGURE 3 | Light micrographs of histological sections through the visceral mass of fan mussels *Pinna nobilis* stained with Ziehl-Neelsen to show infection with mycobacteria. **(A)** Aggregations of haemocytoid nodules filled with mycobacteria (purple deposits) forming nodules (stars) in the connective tissue surrounding the anterior stomach (AS; Cabrera, January 2012). **(B)** Low magnification showing massive aggregation (stars) of haemocytoid nodules throughout the connective tissue surrounding the anterior stomach (AS); BC: brown cells; DG: digestive gland (Cabrera, November 2011). **(C)** Section through the stomach showing free haemocytoid nodules (not enclosed in nodules, arrows) filled with mycobacteria (purple deposits), both in the stomach epithelium (SE) and in the subjacent connective tissue; L, lumen of the stomach (Majorca, March 2017). **(D)** Section through the connective tissue showing masses of mycobacteria (purple deposits) spreading outside haemocytoid nodules (Minorca, June 2017).

TABLE 2 | Percentages of cases of infection with *Haplosporidium pinnae*, mycobacteria and Gram-negative bacteria detected before (2011–2014) and during (2016–2020) the MME, using histology, PCR assays or any of these procedures.

Period	<i>Haplosporidium pinnae</i>			Mycobacteria			Gram (–) bacteria
	Histology	PCR	Any	Histology	PCR	Any	Histology
Before MME	0% (137/0/0)	0% (45/0/0)	0% (167/0/0)	55% (137/0/0)	14% (44/0/0)	48% (167/0/0)	27% (137/0/0)
During MME	64% (35/31/33)	19% (485/61/18)	23% (495/64/36)	44% (35/27/34)	4% (451/46/10)	9% (477/62/36)	45% (22/26/25)

The total number of asymptomatic, sick and recently dead individuals analysed with each diagnostic procedure for each pathogen are shown in brackets, in this order, separated by bars.

by PCR while every positive case for *Mycobacterium* sp. by PCR was also positive by histology. The high percentage of putative PCR-false negatives for *Mycobacterium* showed that the molecular procedure implemented in this study was not always appropriate to diagnose these bacteria, likely due to the fact that the mycobacteria infections were frequently restricted

to digestive gland and surrounding connective tissue, while it occurred less frequently in the organs involved in the biopsy PCR assays (mantle).

Infection with *Perkinsus* sp. was not detected in histological sections either before or during MME. The RFTM screening of perkinsosis performed before the MME revealed a moderate

TABLE 3 | Contingency tables for comparison between diagnostic procedures, histology and PCR assays, for *Haplosporidium pinnae* (A) and mycobacteria (B).

A			
Histology for <i>Haplosporidium pinnae</i>	PCR for <i>Haplosporidium pinnae</i>		Total
	No. positive cases	No. negative cases	
No. positive cases	34	6	40
No. negative cases	5	38	43
Total	39	44	83

B			
Histology for <i>Mycobacterium</i> sp.	PCR for <i>Mycobacterium</i> sp.		Total
	No. positive cases	No. negative cases	
No. positive cases	20	20	40
No. negative cases	0	8	8
Total	20	28	48

TABLE 4 | Results of RTFM assays for perkinsosis.

Location	Date	Sample size	No. of infected individuals	Prevalence	Mean severity
Magalluf	03/06/2011	5	0	0%	0
Cabrera	23/05/2011	10	0	0%	0
Andratx	21/06/2011	6	3	50%	1
Cabrera	27/06/2011	10	3	33%	0.2
Cabrera	20/07/2011	10	8	80%	1.1
Cabrera	29/08/2011	11	6	60%	0.7
Cabrera	21/09/2011	10	10	100%	1.7
Andratx	11/10/2011	6	6	100%	2
Cabrera	31/10/2011	9	5	55%	0.4
Cabrera	25/11/2011	11	0	0%	0
Cabrera	10/01/2012	10	0	0%	0
Cabrera	24/01/2012	10	3	30%	0.3
Cabrera	17/02/2012	10	3	30%	0.3
Cabrera	15/03/2012	10	0	0%	0
Whole		128	47	38%	0.55

prevalence (47/128, 38%) and low severity degree (mean: 0.55) of *Perkinsus* sp. (Table 4).

In the case of *P. rudis*, *H. pinnae* was not detected, while mycobacteria were observed in one asymptomatic individual (Table 1). Gram (–) bacteria were detected in the only sick individual of the five examined.

Histological Remarks of *Haplosporidium pinnae* Infection

Uninucleate and binucleate cells of the parasite and, less frequently plasmodia with up to six nuclei, occurred in the connective tissue throughout the visceral mass, mantle and gills, as well as in the stomach and intestine epithelia (Figures 2A–C). The infection was frequently associated with heavy haemocytic

infiltration of the connective tissue and the affected epithelia. Additionally, stages of parasite sporulation process, including uninucleate and binucleated cells, multinucleate plasmodia, and sporocysts enclosing sporoblasts or more or less mature spores, were detected in the epithelium of digestive tubules (Figures 2D–F) of eight asymptomatic and eight sick individuals from El Calón (1 fan mussel), Calpe (3), Majorca (8), and Attica (4). Parasite sporulation caused severe damage of the digestive tubule epithelium.

Histopathological Remarks of Bacterial Infections

In the samples taken before MME, multiple nodular aggregates of haemocytes were often observed scattered in the connective tissue circumscribing the visceral mass and the anterior stomach,

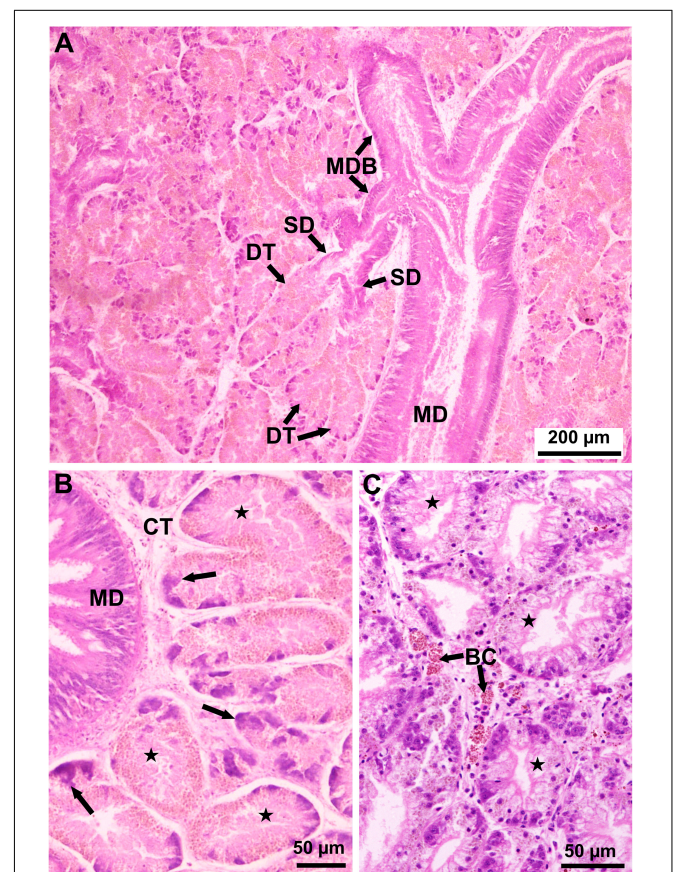


FIGURE 4 | Light micrographs of histological sections stained with MHE showing the normal (healthy) structure of the *Pinna nobilis* digestive gland. (A) Lower magnification showing a main duct (MD) leading to main duct branches (MDB) from which primary ducts (PD) arise leading to secondary ducts (SD) ending in blind digestive tubules (DT) (Cabrera, May 2011). (B) Higher magnification showing digestive gland tubules (stars) in absorptive phase surrounding a stomach branch (SB); the digestive tubules show highly reduced lumen and tall epithelium, consisting of acidophilic absorptive cells and basophilic young, differentiating cells (arrows); CT, connective tissue (Cabrera, May 2011). (C) Higher magnification showing digestive gland tubules (stars) in holding phase, in which digestive tubules show larger lumen; some brown cells (BC) occur in the connective tissue (Cabrera, March 2012).

sometimes forming aggregate-rich regions encapsulated by a haemocytic and brown cells response (Figures 3A,B). In some instances, its larger size became a bacterial abscess. Acid-fast positive bacteria were seen within the affected haemocytes and brown cells in the core of the lesion. Less frequently, Gram negative bacteria were also observed. Both nodules and abscesses appeared to effectively sequester the bacteria, since haemocytes enclosing bacteria were not seen outside aggregates. However, mycobacterial infection showed some differences in the samples collected during MME. Mycobacteria-filled haemocytes located in nodular aggregates, were also observed. They were also detected in the connective tissue surrounding visceral mass and anterior stomach but also in the mantle and in the connective tissue surrounding gonadal follicles. In addition, mycobacteria-filled isolated haemocytes were frequently seen spreading throughout the connective tissue of the visceral mass and mantle as well as in the digestive epithelia (Figure 3C). Occasionally, free mycobacteria were seen (Figure 3D).

For comparison, micrographs showing the normal structure of the fan mussel digestive gland, corresponding to a healthy condition, are provided in Figure 4.

Pathogen Occurrence in Samples Collected Before and During Mass Mortality Event

Remarkably, *H. pinnae* was not detected in any of the 167 fan mussels analysed before the MME, while it occurred in 63/99 (64%) of fan mussels examined by histology collected during MME (Table 2 and Figure 5A). Conversely, mycobacteria were detected both before and during MME (Table 2 and Figure 5A). Considering the histological diagnoses, differences in distribution into negative and positive cases between samples taken before and during MME were significant for *H. pinnae* and Gram (–) bacteria but not for mycobacteria. The ratios of individuals in the health condition classes were different between diagnostic procedures (Table 2), which contributed to differences in the percentages of positive cases between procedures.

Association Between Pathogen Occurrence and Health Condition

The distribution into negative and positive histological detections of *H. pinnae* was significantly different between the three classes of health condition, with the lowest percentage of infection in the asymptomatic individuals and the highest in the sick ones (Figure 5B). No significant difference was found between histological mycobacteria detections of the three health conditions, while Gram (–) infections showed significant differences between the recently dead condition and the other ones (Figure 5B). Considering the conditions in which a putative aetiological agent of the MME should be more likely found, namely in sick individuals from high mortality affected areas, *H. pinnae* was detected in 26/31 (88%) of the sick fan mussels from MME-affected areas that were analysed with histology, mycobacteria in 13/27 (48%), and Gram (–) bacteria in 9/26 (35%) (Figure 5B). Regarding the 26 sick fan mussels from MME-affected areas that were found infected with *H. pinnae*

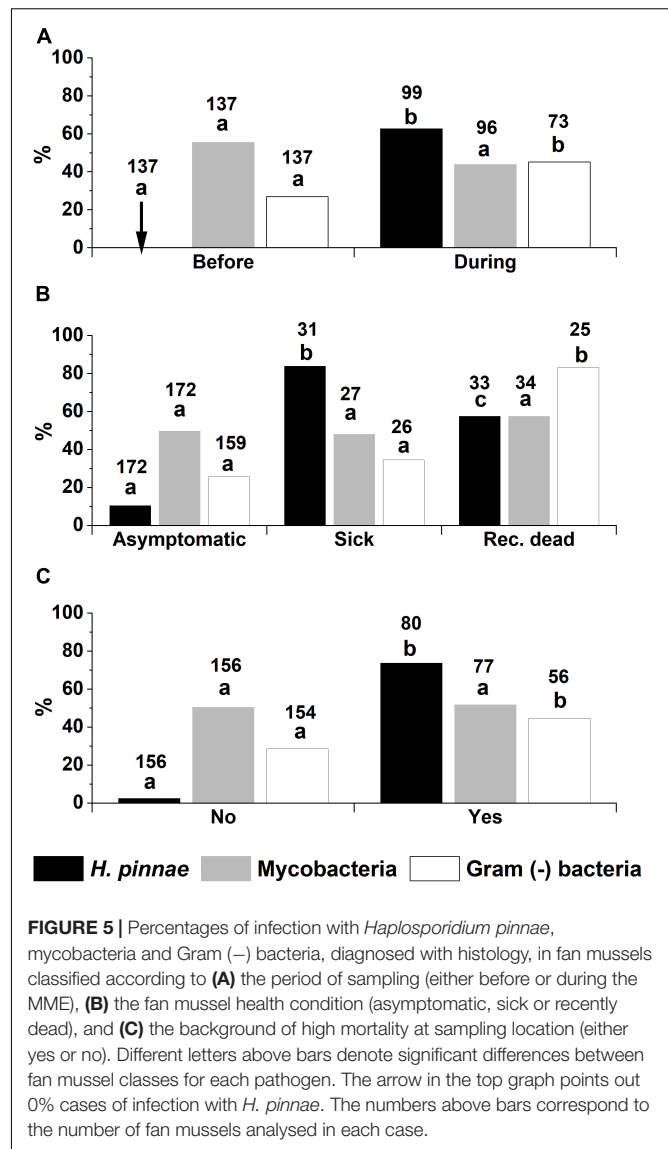


FIGURE 5 | Percentages of infection with *Haplosporidium pinnae*, mycobacteria and Gram (–) bacteria, diagnosed with histology, in fan mussels classified according to (A) the period of sampling (either before or during the MME), (B) the fan mussel health condition (asymptomatic, sick or recently dead), and (C) the background of high mortality at sampling location (either yes or no). Different letters above bars denote significant differences between fan mussel classes for each pathogen. The arrow in the top graph points out 0% cases of infection with *H. pinnae*. The numbers above bars correspond to the number of fan mussels analysed in each case.

by histology, 11/26 (42%) of them were simultaneously infected with mycobacteria and 5/26 (19%) of them were also infected with Gram (–) bacteria. During MME, *Mycobacterium sp.* but no *Haplosporidium* was detected in only four recently dead individuals.

Relationship Between Pathogen Occurrence and the Local Affection by Mass Mortality Event

As it has been explained before (see the section “Materials and Methods”), not all the locations were affected by a high mortality at the moment of sampling. The frequency of histological detection of *H. pinnae* was significantly higher in the locations that had been affected by high mortality than in the sites that had not been affected when sampling (Figure 5C). That was not the case for histological detection of mycobacteria, which showed similar distribution of positive and negative cases in the high

mortality-affected and in the non-affected locations. Detection of Gram (–) bacteria was higher in high mortality-affected sites (Figure 5C).

Juvenile Condition and Occurrence of Pathogens

A total of 35 juvenile fan mussels were analysed, 29/35 of which were sick individuals taken from Calpe and 1/35 sick plus 5/35 asymptomatic specimens collected from Delta de l'Ebre-Alfacs. The 21/29 sick juveniles from Calpe were positive for *H. pinnae* by PCR (Table 1). However, every juvenile (29/29) from that location was PCR-negative for *Mycobacterium* sp. (Table 1), even though the use of the whole soft tissues would facilitate the detection of this bacterium by PCR. Regarding the six juveniles from Delta de l'Ebre-Alfacs, everyone was negative (6/6) for *H. pinnae* (histology and PCR) and for mycobacteria (histology) but 3/6 showed Gram (–) bacteria in histological sections.

Relationship of Pathogen Occurrence With Host Size

The percentage of fan mussels found infected with *H. pinnae* by histology was not associated with size (dorso-ventral length; Figure 6). However, a significant association was found between the percentage of fan mussels found infected with mycobacteria by histology and the host size; the same applied for infection with Gram (–) bacteria. The higher the size class, the higher the percentage of infection, both for mycobacteria and Gram (–) bacteria (Figure 6).

Association Between Pathogens

The paired tests to evaluate the association between pathogens, based on histological diagnosis, showed that the occurrence of *H. pinnae* was independent of that of mycobacteria and Gram (–) bacteria, while the occurrence of mycobacteria was associated with that of Gram (–) bacteria (Table 5).

Relationship Between the Occurrence of *Haplosporidium pinnae* and the Environment

The highest percentage of individuals infected with *H. pinnae* corresponded to those living in open sea, while the lowest percentage corresponded to those from coastal lagoons and those from harbours in intermediate position (Figure 7). The differences in the distribution of fan mussels into *H. pinnae*-infected and non-infected categories between environments were significant.

Geographic Distribution of the Pathogens

Haplosporidium pinnae was detected in 24/30 locations while it was not in 6/30 sites (Table 1 and Figure 1). In 4/6 of the locations where *H. pinnae* was not detected, abnormally high mortality had not been recorded, while in the other 2/6, Port La Nouvelle and Telašćica, which had been affected by MME, the analysed fan mussels were asymptomatic. Regarding the 24 locations in which the parasite was detected, MME had affected 20/24 and the other 4/24 sites (Mar Menor, Agde, Sète, and Frontignan).

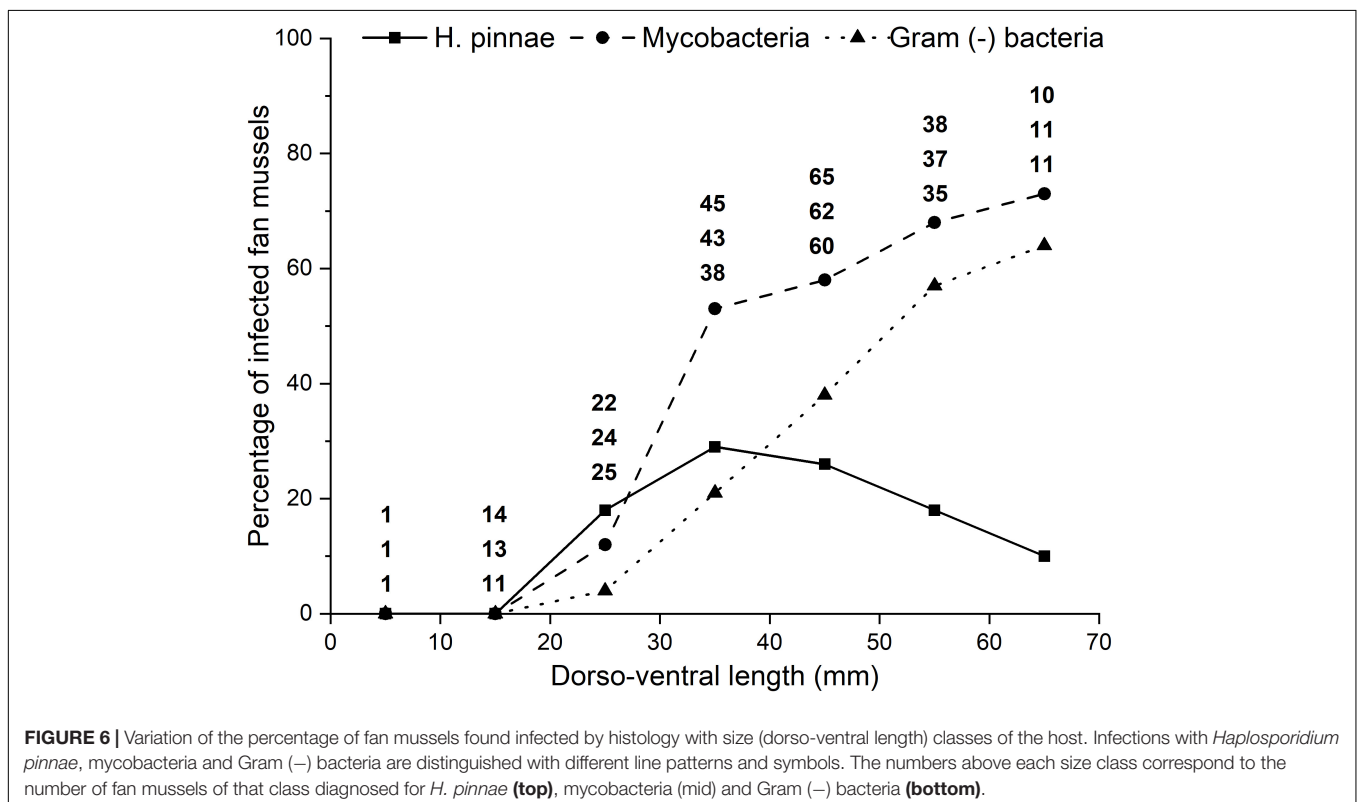


TABLE 5 | Contingency tables and statistical test to evaluate the association between the occurrence of *Haplosporidium pinnae* and that of mycobacteria (A), *H. pinnae* and Gram (–) bacteria (B), and mycobacteria and Gram (–) bacteria (C).

A			
Mycobacteria	<i>Haplosporidium pinnae</i>		Total
	No. positive cases	No. negative cases	
No. positive cases	29	88	117
No. negative cases	31	86	117
Total	60	174	234
$\chi^2 = 0.09$; DF = 1; $P = 0.76$			

B			
Gram (–) bacteria	<i>Haplosporidium pinnae</i>		Total
	No. positive cases	No. negative cases	
No. positive cases	16	53	69
No. negative cases	29	111	140
Total	45	164	209
$\chi^2 = 0.17$; DF = 1; $P = 0.68$			

C			
Gram (–) bacteria	Mycobacteria		Total
	No. positive cases	No. negative cases	
No. positive cases	50	20	70
No. negative cases	49	91	140
Total	99	111	210
$\chi^2 = 24.85$; DF = 1; $P < 0.001$			

The values correspond to histological diagnosis.

that had not been affected by MME when sampling, were close to affected locations. The occurrence of mycobacteria was tested, either by histology or PCR, in 26 locations; positive cases were recorded in 16/26, 14 that had been affected by MME and 2 that had not (Table 1 and Figure 1). Regarding the 10 locations in which mycobacteria were not detected, diagnoses were based on mantle biopsies analysed with PCR (Table 1), a procedure likely yielding high proportion of false negatives, as mentioned above. Considering the 16 locations for which histological diagnosis for mycobacteria was performed, mycobacteria were detected in 14/16, all them except in Mar Menor (only one specimen), and Delta de l'Ebre Fangar (four individuals) (Table 1). Gram (–) bacteria were analysed in samples from 13 locations, 9/13 had been affected by MME and 4/13 had not; infections were found in 12/13 sites except in Sète (only three mantle biopsies) (Table 1).

DISCUSSION

Samples of fan mussels taken through the Mediterranean coast by various teams of different countries were used to gather a wide-geographic, long term data set with which assess the role of previously blamed and suspicious pathogens in the panzootic MME. This approach allowed compiling highly diverse information, including samples taken before and during MME; asymptomatic, sick and recently dead animals; wide geographic

and fan mussel size (from juvenile to old adults) ranges; and different environments (coastal lagoon, harbour, open sea). The involvement of various sampling/research teams and the different protection degree of *P. nobilis*, depending on country and sampling period (before and during mortality outbreaks), explained the heterogeneity of diagnostic techniques and sample types (either necropsy or biopsy), but that was compensated with the unprecedentedly high number of analysed fan mussels and highly diverse scenarios.

Concerning the health status of the analysed individuals, accurately describing what is considered as “recently dead” specimens in the wild is important in the context of the current controversy in the scientific community on the cause of the *P. nobilis* MME. In our study, the individuals considered “recently dead” were dead animals in which the decomposition, lytic post-mortem processes had begun although the presence of still consistent soft tissues allowed taking soft tissue pieces and their histological analysis. Previous reports on the mass mortality of *P. nobilis* did not explain the meaning of “recently dead” individuals. Remarkably, the detection of multiple pathogens only in individuals classified as “recently dead” may be due to the decomposition process, i.e., post-mortem proliferation of opportunistic or saprophagous organisms (particularly bacteria) in degraded specimens, which were in autolytic phase at sampling (Dent et al., 2004; Tsokos, 2005). If decomposition and tissue lysis is advanced, bacterial proliferation is obvious while the detection of other pathogens (such as *H. pinnae*) that occurred before the post-mortem lysis could be very difficult or impossible, causing interpretational problems (Tsokos, 2005). In this sense, in marine environments, as the putrefaction proceeds, Gram-negative, non-pigmented rods constitute a major component of the flora (Masami and Hiroshi, 1959). However, parasites do not survive in dead molluscs because tissue putrefaction probably leads to an acidic degradation of the cyst or oocyst wall (Gómez-Couso et al., 2003).

Regarding heterogeneity of diagnostic techniques and procedures, histology and PCR showed substantial agreement for detection of *H. pinnae*. However, the PCR procedure to detect *Mycobacterium* sp. seemed to yield a high number of false negatives, likely due to the solely use of mantle biopsies for PCR assays, because the mycobacteria infections were frequently restricted to the digestive gland and the surrounding connective tissue. According to this and considering that Gram (–) bacteria were only evaluated with histology (using Gram staining), all the analyses involving comparisons between different pathogens were based on histological diagnoses. Nevertheless, positive PCR results were useful at least to suggest (pending of confirmation) the occurrence of mycobacteria and to get the DNA sequence for its correct identification in various geographic locations for which PCR-based diagnoses were the only available diagnostic. The use of histological techniques in studies looking for aetiology is important to confirm true infections because just a positive PCR result does not involve true infection on its own (Burrison, 2008).

Histopathological features of the infections of fan mussels with *H. pinnae* and those with mycobacteria observed in this study are consistent with descriptions provided by Catanese et al. (2018)

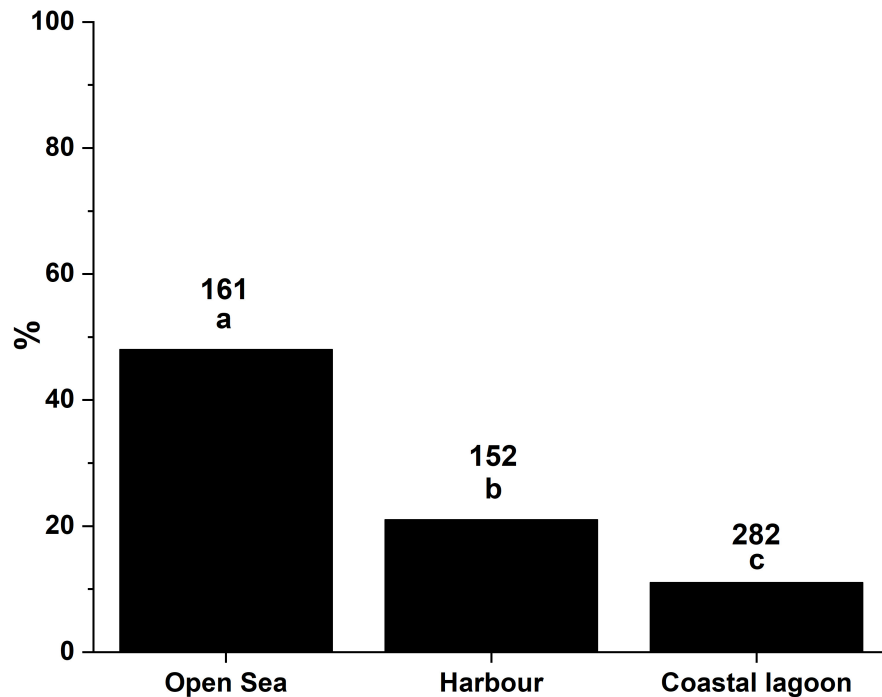


FIGURE 7 | Percentages of infection with *Haplosporidium pinnae* corresponding to different ecosystems. Different letters above bars denote significant differences between environments. The numbers above bars correspond to the number of fan mussels diagnosed in each case.

and Carella et al. (2019, 2020), respectively. The connective tissue of the visceral mass was the main location for mycobacteria; nevertheless, the detection of some positive PCR-results for *Mycobacterium* sp. from mantle biopsies as well as the reports of mycobacteria infections occurring in mantle (Carella et al., 2019, 2020) and gills (Lattos et al., 2020a) evince a broader histological range, especially in advanced infections in which mycobacteria occur free in the host tissues in addition to those sequestered in haemocytic nodules.

Although some authors suggest the multifactorial causality of the *P. nobilis* mortality, the results of this work supported that the onset of the MME is strongly associated with the detection of *H. pinnae*, which exhibits a preeminent role with respect to the others pathological agents considered in the study. Although it is true that the causal relationship with the disease can only be unequivocally established with controlled laboratory experiments, the strong relationship between the presence of *H. pinnae* with the MME is unquestionable, as shown in our results. This strong relationship, however, is not exhibited by other putative pathogenic agents identified as possible aetiological agents. It was the only analysed pathogen that was not detected in the period before MME was noticed, while it was widely detected during MME. Infection with *H. pinnae* was significantly associated with health condition, with significantly higher probability of finding the parasite in sick and dead animals than in asymptomatic ones; the lower histological detection of *H. pinnae* in recently dead individuals than in sick ones could be due to the difficulty of detection of the parasite in post-mortem delayed tissues (Gómez-Couso et al., 2003; Alaeddini et al., 2010;

Allen-Hall and McNevin, 2013). The frequency of histological detection of *H. pinnae* was significantly higher in areas that had been affected by high mortality. Assuming that the most likely conditions to detect a putative aetiological agent of the MME would be in sick fan mussels from affected areas, *H. pinnae* complied with that contingency and did it more frequently without mycobacteria or Gram (–) bacteria co-infection. Considering that the MME has affected fan mussels of all ages (Vázquez-Luis et al., 2017; Zotou et al., 2020), from juveniles to old adults, the detection of *H. pinnae* in many juveniles, more frequently in the sick ones, from Calpe, an area heavily affected by high mortality early in the MME, together with the lack of association between the occurrence of this parasite and the host size (age), contributes to support the strong association between *H. pinnae* and the MME. The lack of detection of this parasite in the six juvenile fan mussels from Delta de l'Ebre-Alfacs could be due to the fact that three of them were taken before abnormal high mortality got the area and that the other three individuals, taken once high mortality had been observed, were asymptomatic. Finally, this parasite was not detected in any specimen of *P. rudis*, which has not been affected by MME even in the areas where *P. nobilis* became extinct.

On the contrary, evidence supporting association of MME with mycobacteria was not found. Infection with mycobacteria was present with a high prevalence, in the healthy, and reproductively capable fan mussel population of the Balearic Islands, regularly monitored before the onset of the MMEs (Hendriks et al., 2012; Vázquez-Luis et al., 2014; Alomar et al., 2015; Deudero et al., 2015, 2017; Box et al., 2020). These

mycobacteria were identified as *Mycobacterium* sp., very closely related (with 100% sequence similarity) to those detected by Carella et al. (2019) during MME (Box et al., 2020). The percentage of individuals infected with mycobacteria in the period before detection of MME was not significantly different from that during the MME spreading. The probability of finding infection with mycobacteria did not significantly vary depending on health condition. The percentage of infection with mycobacteria in the high mortality-affected areas was not significantly different from that of the non-affected sites. Mycobacteria were not detected in half of the sick individuals from high mortality-affected areas. The lack of detection of mycobacteria in juveniles and the significant association of the occurrence of mycobacteria with host size (age) would support a dominant chronic condition of the infection with mycobacteria rather than acute infection leading to host death. Histological observation revealed mycobacterial chronic nodular lesions, resembling those described by Carella et al. (2019). The digestive route of infection could be the most plausible, due to the localisation of lesions at the stomach, digestive gland and surrounding area. These observations support the hypothesis of the opportunistic character of mycobacteria, acting as a fan mussel coloniser, being sequestered in nodules, close to the “entrance door.” Moreover, mycobacteria were also detected in an asymptomatic, *P. rudis*, suggesting an opportunistic character in the Pinnidae family. Mycobacteria have been mainly found in marine bivalves when asymptomatic populations are studied focussing on their role as carriers involved in human disease (Aubry et al., 2002; Kotlowski et al., 2004; Hashish et al., 2018). Interestingly, mycobacteria were detected during a study of microbiome assessment of the predominant bacterial populations in asymptomatic *Pinna nobilis* individuals from a healthy population of the Eastern Adriatic Sea before MME arrival (Pavlinec et al., 2020). The detection of *Mycobacteria* in a few recently dead individuals, without detecting *H. Pinnae*, could be explained by their greater resistance capacity to environmental factors, withstanding the autolysis process and inducing their minor cellular fragmentation in the sample (Lindahl, 1993).

Gram (–) bacteria neither appeared associated with MME. They were detected both before the first detection of MME and during the period of MMEs. The higher probability of finding them in recently dead individuals than in sick and asymptomatic fan mussels suggests their post-mortem proliferation rather than being the cause of death. If so, the higher percentage of infection with Gram (–) bacteria in areas with high mortality background than in MME non-affected areas would be due to the fact that the proportion of recently dead individuals collected from the former areas was higher than that from the latter sites.

Gram (–) bacteria were only detected in one third of the sick individuals from MME-affected areas. As in the case of mycobacteria, the fact that the prevalence of infection with Gram (–) bacteria increased with host size (age) while MME equally affected fan mussels of all sizes (ages), debilitates a potential role of Gram (–) bacteria in fan mussel MME. These bacteria were also detected in a sick *P. rudis*. The opportunistic condition of a Gram (–) bacterium, *V. mediterranei*, was suggested to explain high mortality in stabled fan mussels; the bacteria

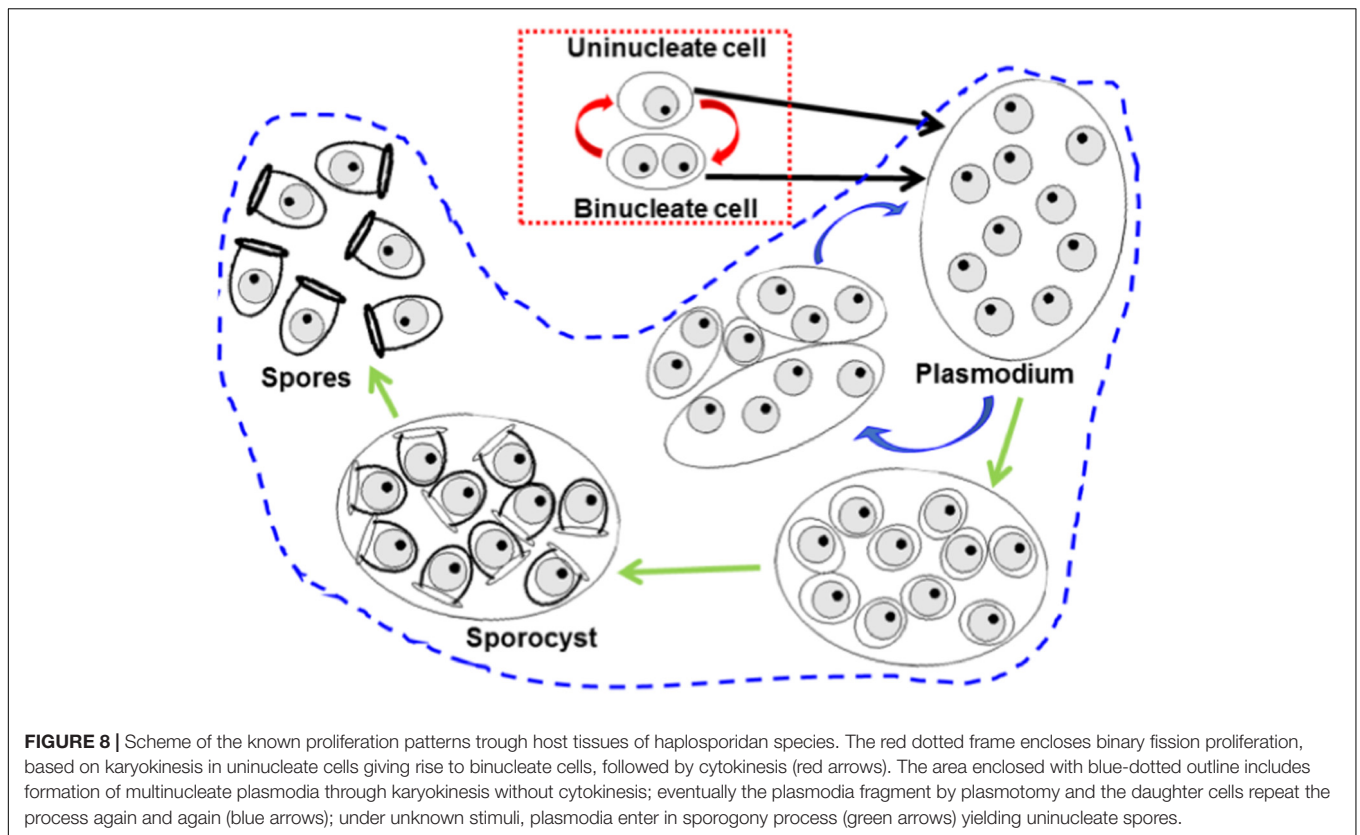
would have taken advantage of the host stress due to captivity and other possible factors to proliferate through host tissues (Prado et al., 2020a).

The occurrence of *H. pinnae* in the tissues of fan mussels was independent of those of mycobacteria and Gram (–) bacteria, while these two types of bacteria were significantly associated, which could be consistent with an opportunistic character of both types of bacterial infections. Accordingly, the pathophysiological response in sick animals is much greater in co-infections with *H. pinnae* in comparison with co-infections without presence of haplosporidan parasite (Lattos et al., 2021).

No case of infection with protistans *Perkinsus* spp. was detected with histology. Cases of infection with these parasites were only detected with the RFTM procedure, which was used in before the first evidence of MME. Diagnosis of *Perkinsus* spp. with RFTM is more sensitive than with histology (Bushek et al., 1994; Rodríguez and Navas, 1995; Villalba et al., 2005). Therefore, cases of light perkinsosis could have been unnoticed with histology during the MME spreading but, if systemic (moderate and heavy) infections had occurred, they would have been detected even with histology. The occurrence of cases of perkinsosis before the MME, as light infections, and the lack of detection of infections with *Perkinsus* sp. by histology through the study showed that perkinsosis is not an emerging disease for *P. nobilis* and it did not reach moderate or heavy intensity in *P. nobilis*, which allowed discarding association of perkinsosis with MME. No other serious pathogens with potential to cause high mortality were detected in the study.

The aetiology of the fan mussel MME was addressed previously. Since *H. pinnae* was proposed as its most likely cause (Vázquez-Luis et al., 2017; Catanese et al., 2018), subsequent surveys performed in areas affected by high mortality of fan mussels emphasised the search for this parasite and, as it was found, it was blamed for the high mortality in the respective areas (Fanelli et al., 2018; Katsanevakis et al., 2019; Panarese et al., 2019). The detection of more cases of infection with *Mycobacterium* sp., associated with heavier histopathological damage, than with *H. pinnae* in sick fan mussels from Campania and Sicily (South Italy) led to Carella et al. (2019) to state that *Mycobacterium* sp. was associated with the fan mussel high mortality in those areas but *H. pinnae* was not. However, Carella et al. (2020), in a broader (involving more fan mussels with wider geographic range) study, proposed that *Mycobacterium* sp., *Vibrio* spp., *H. pinnae* and *Perkinsus* sp. cooperate to disease pathogenesis, among which *Mycobacterium* sp. and *H. pinnae* seemed to be the most relevant. Subsequent surveys in other Mediterranean regions highlighted the occurrence of both *Mycobacterium* sp. and *H. pinnae* in fan mussels from MME-affected areas (Lattos et al., 2020a; Scarpa et al., 2020; Čizmek et al., 2020; Šarić et al., 2020).

The lack of detection of *Mycobacterium* sp. and *H. pinnae* in some sick fan mussels from affected areas was highlighted to argue that other factor/s could be the cause of (or contribute to) the MME (Scarpa et al., 2020; Šarić et al., 2020). The critically endangered condition of *P. nobilis* implies exceptional difficulties to perform *ad hoc* experiments addressing the aetiology of the MME. Previous studies on the aetiology of the fan mussel MME



were based on scarce number of specimens, from 4 (Darriba, 2017) to 46 (Scarpa et al., 2020). Our study has been the broadest attempt, in terms of time period, geographic range and sample size, addressing the aetiology of the MME; the results supported that *H. pinnae* was the pathological agent of those considered in the study with a preeminent role in the onset of the MMEs; even the argument used in other studies to weaken or discard the consideration of *H. pinnae* as the aetiological agent of the MME, namely its absence in sick fan mussels from MME-affected areas, would not apply to our results because 26 out of the 27 sick individuals collected from affected areas that were diagnosed with histology showed infection with *H. pinnae*. Nevertheless, assuming the strong association between *H. pinnae* and the MME found in this study, it cannot be considered as a conclusive evidence of a cause-effect relationship.

Scarpa et al. (2020) stated that the Mediterranean mussel *Mytilus galloprovincialis* and the carpet shell clam *Ruditapes decussatus* are also hosts of *H. pinnae*, and thus *P. nobilis* is not the exclusive bivalve host of this parasite, all it based on PCR-positive diagnosis for *H. pinnae* obtained from tissues of three clams and one mussel. However, only PCR amplification cannot be considered as a valid evidence of a true infection and, thus, of a parasite-host relationship; further validation is needed with an established technique, typically histology or *in situ* hybridisation, as thoroughly explained by Burreson (2008). The fact that the three clam samples that gave PCR-positive results for *H. pinnae* had been collected in 2014 would involve that the parasite occurred at that time in The Mediterranean Sea (at least in the

eastern coast of Sardinia). If *H. pinnae* really occurred at that time, did it infect its currently well-known host *P. nobilis* long time before the first evidence of MME, in 2016? According to our results, *H. pinnae* was not detected in samples of 167 fan mussels collected in the period 2011–2014 from various locations in the Balearic Islands while it was widely detected since 2016 onward in the same locations, where the fan mussel is nowadays almost extinct. Assessing whether *H. pinnae* occurred or not in fan mussels before 2016 in other areas would be useful if recorded samples were available.

Considering that all the known proliferation patterns of haplosporidans have been found in *H. pinnae* (see information below), likely this species can be directly transmitted among fan mussels and endure free in the environment keeping infective potential, all that contributing to facilitate proliferation through fan mussel populations. According to our results, stages of the *H. pinnae* sporulation process were only detected in 18% of the infected fan mussels. The stages of the sporulation process (multinucleate plasmodia, sporocysts and spores) are more easily detected than the smaller uni- and binucleate stages during histological examination, thus special care is needed to detect the parasite when sporulation does not occur. The detection of uni- and binucleate stages is particularly difficult in decomposed tissues of dead fan mussels.

Results showed a wide geographic distribution of *H. pinnae*. The earliest *H. pinnae* detections corresponded to SE Spain (El Calón and Calpe) and Majorca Island (autumn 2016), being found later (2017) northward in Minorca island, NE

Spain (Tossa de Mar and Portlligat) and eastward in Corsica, coinciding with the transmission models of a waterborne parasite dictated by surface currents (Cabanelas-Reboredo et al., 2019), which completely fits with the reduced SIR model validated with available data of *Haplosporidium pinnae* transmission in controlled water tanks (Giménez-Romero et al., 2021). The parasite was detected through 2018 and 2019 in more eastern Mediterranean locations as sampling effort coincided with MME spread. Both mycobacteria and Gram (–) bacteria were also widespread in *P. nobilis* populations but, very likely, the lack of their detection in some areas would be due to the lack of using histological diagnostic techniques with ZN and Gram staining, respectively.

Remarkably, differences in the prevalence of *H. pinnae* between fan mussels living in open sea, in harbours and in coastal lagoons were significant. Accurately determining whether particular environmental conditions make difficult or impede parasite proliferation could be relevant to recovery purposes, especially if some coastal lagoons remain as sanctuaries of *P. nobilis*. In fact, environmental factors (water temperature, salinity, presence of pollutants, trophic conditions) have significant effects on the pathogens themselves, on the host and on the host-pathogen relationship (Barbosa Solomieu et al., 2015). Notably, the dynamics of haplosporidan parasites is modulated by environmental parameters, especially temperature and salinity (Burreson and Ford, 2004; Arzul and Carnegie, 2015). Specific tolerance of *H. pinnae* to temperature and salinity has been suggested (temperatures above 13.5°C and salinity range between 36.5–39.7 psu; Cabanelas-Reboredo et al., 2019; García-March et al., 2020; Prado et al., 2021; Nebot-Colomer et al., 2021). Accordingly, salinity range outside the common Mediterranean salinity range (36.7–39.5 psu; Vidal-Vijande et al., 2011) is a shared feature of these coastal lagoons: on the one hand, the Mar Menor, exhibiting high salinity, up to 47 psu (Pérez-Ruzafa et al., 2005; Giménez-Casalduero et al., 2020); on the other hand, the other Spanish and French coastal lagoons, strongly affected by freshwater supplies (Prado et al., 2014, 2020b). Recent data demonstrated that until now the lagoons of the Gulf of Lion are still free from parasite (almost 90% of *P. nobilis* population; Peyran et al., 2021). Interestingly, mortality of fan mussels has been shown to be associated with salinity gradient in one of the coastal lagoons involved in the study, Delta de l'Ebre-Alfacs, influenced by freshwater discharges; the lower the salinity the lower the cumulative mortality (Prado et al., 2021). However, many coastal lagoons (as well as harbours) share higher anthropogenic pressures implying higher stress for fan mussels living there (García-March et al., 2020; Giménez-Casalduero et al., 2020; Prado et al., 2021), which could influence in their redox balance, cause cellular damage, and depress their immune system (Rivera-Ingraham et al., 2013; Capó et al., 2015; Espinosa and Rivera-Ingraham, 2016). In this sense, before the MME (deeply associated with *H. pinnae* according to our results), anthropogenic stressors rather than environmental or other variables explained most of the variability in the spatial distribution of the density of *P. nobilis* in the Balearic Islands (Deudero et al., 2015). Indeed, environmental adverse conditions such as pollutants or infections can induce oxidative stress in

marine bivalves. However, when analysing the differential effects of *H. pinnae* and *Mycobacterium* sp. on *P. nobilis* antioxidant response, significant effects on biomarkers were only observed in the presence of *H. pinnae* (Box et al., 2020). Unfortunately, we do not have data in *P. nobilis* to relate the effect of anthropic pressure on biomarkers before the presence of the parasite, which prevents us from elucidating whether the anthropic pressure may also have an adverse effect on *P. nobilis* inhabiting coastal lagoons, the last relicts for the survival of the species.

Special attention deserves the known stages of the *H. pinnae* life cycle, which have implications in the potential for disease spreading and parasite permanence in the ecosystem. The life cycle of haplosporidan parasites is a complex issue. Binary fission is the only known proliferation process (red dotted frame in **Figure 8**) of some haplosporidan species infecting oysters, such as *Bonamia ostreae* and *Bonamia exitiosa*, in which uninucleate and binucleate cells can leave the host with potential to infect new hosts, thus making possible direct parasite transmission among individuals of the type host species (Sas et al., 2020); additionally other potential transmission vehicles or reservoirs have been identified, including oyster larvae (Arzul et al., 2011) and other invertebrate species (Lynch et al., 2007, 2010; Costello et al., 2020). In other haplosporidan species, such as *Haplosporidium nelsoni*, the only known stages are plasmodia and those of sporogonia (area enclosed with blue dashed outline in **Figure 8**); for these haplosporidan species, their transmission way is unknown; direct transmission has never been experimentally confirmed and the need of a host has been suggested (Burreson and Ford, 2004; Arzul and Carnegie, 2015); the spores are likely enduring stages whose infective potential last longer than that of naked uninucleate and binucleate stages. Among haplosporidans infecting molluscs, the species in which the occurrence of all the mentioned proliferation patterns have been demonstrated through the host are scarce; that is the case of *Bonamia perspora* (Carnegie et al., 2006) and *H. pinnae* (Catanese et al., 2018), whose sporulation processes take place in different tissues: connective tissue and epithelium of digestive gland tubules, respectively.

Knowing the aetiology of the fan mussel MME is essential to avoid the complete extinction of this species and to design measures to recover their populations. The results supported a strong association of *H. pinnae* with the MME and allowed discarding an essential role of mycobacteria and Gram (–) bacteria.

PERMISSIONS

Samplings were performed with the necessary permits of the competent authorities (national and/or local). The corresponding permissions were detailed below: GOIB-ENB-AC-01/31/2011, SPE-CMAP-RE127 (7/11/2011), CE-5614-05-24-2013, CMAP-DGENB-40/2016, GC-DTS-SF/1088-2017, GC-DTS-02-11-2017, GC-DTS-17-11-2017, DIV/BDM/AUSPP/45/2017, DIV/BDM/AUSPP/47/2017, GMEE175828/2195 (11/14/2018), KLASA:UP/I-612-07/18-48/134, URBROJ: 517-05-1-1-18-4, prefectural decree 2018-s-24, prefectural decree 2018-s-28,

SGPM/BDM/AUTSPP/57/2018, PTOP-2019-404, GC-8428-05-23-2019, MAF-FA67852565-140.03.03-E.2935960 (09/26/2019).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

AUTHOR CONTRIBUTIONS

AG and GC conceived the study. AG, GC, BH, JV, PK, CP, and GS analysed the samples. AG, GC, AV, and JN analysed the data. AG, AV, and GC wrote the manuscript with input from all authors and revised the final manuscript. The rest of the authors participated in the field work. All the authors reviewed the manuscript improving it substantially. All documents were edited and approved by all authors.

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