Multipathogen infections and multifactorial pathogenesis involved in noble pen shell (*Pinna nobilis)* mass mortality events: background and current pathologic approaches

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Abstract

Disease outbreaks have been reported in recent years from several ecologically or commercially important invertebrate marine species all over the world. Mass mortality events (MMEs) have affected the noble pen shell (*Pinna nobilis*), causing its near extinction. Our knowledge of the dynamics of diseases affecting this species is still unclear. Early studies towards determination of the etiological agent responsible focused on a novel protozoan parasite, *Haplosporidium pinnae,* though further investigations suggested that concurrent polymicrobial infections could have been pivotal in some MMEs, even in the absence of *H. pinnae*. Indeed, moribund specimens collected during MMEs in Italy, Greece, and Spain demonstrated a systemic presence of a bacteria from within the *Mycobacterium simiae* complex and in some cases species similar to *Vibrio mediterranei*. Moreover, the diagnostic processes used for investigation of MMEs is still not standardized and requires the expertise of veterinary and para-veterinary pathologists, who could simultaneously evaluate a variety of factors, from clinical signs to environmental conditions. In this review we aim to report the data published until now and to discuss the urgent need of a consensus on the best research approaches to define MMEs in *P. nobilis*, in the context of the priorities required for their conservation. This approach should form the basis for establishing a broad foundation for future studies, aimed at preserving endangered populations of this native bivalves.

Keywords

Bivalves, diagnostics, *Haplosporidium*, *Mycobacterium*, *Vibrio,* polymicrobial infections

Epizootics caused by emerging infectious diseases often result in mass mortality events (MMEs), particularly in the case of newly introduced pathogens for which the host in question has had no previous exposure. These are becoming more frequent in shellfish over the last 30 years. Fish infectious diseases, their etiological agents and transmission mechanisms have been extensively studied, but much less is known about pathogens of shellfish and other benthic fauna, and particularly of diseases of bivalves.[36] The lack of information about the causes of disease in marine invertebrates is severely limiting our understanding of the aetiology and environmental factors contributing to MMEs.[18] In aquatic animals, complex interactions between heterogenous bacteria, viruses, and parasites are further complicated by an array of non-infectious environmental factors, resulting in polymicrobial infections with pathological outcomes that might be difficult to predict and control.[19,93] Novel cross-disciplinary approaches, involving simultaneous epidemiological and ecological studies at various levels of biological organization (molecular to population), are promising to provide a deeper understanding during host-pathogen interactions.[22,46]

The pen shell, also called fan mussel, *Pinna nobilis* is one of the largest bivalves of the Mediterranean Sea, inhabiting coastal areas and deeper areas in the range of 0.5–60 m. In the last decade, extensive mass mortalities of the species in many Mediterranean countries (Italy, Spain, Greece, Turkey, France) resulted in its inclusion in the Annex II Barcelona Convention (1992), Annex IV of the EU habitats directive (2007), and redefining the species as ‘critically endangered’ by the IUCN red list for threatened species.

75 The phenomenon began in 2016 and continues to devastate the populations of fan mussels to the
76 present day. MMEs can reduce a population in a short period of time, in some instances, due to specific
77 environmental conditions or weather events which can be the underlying and triggering causes [Mc Dowell]
78 These phenomena have been reported all over the world in different marine taxa [FEY]. In the
79 Mediterranean Sea, large-scale temperature anomalies, corresponding to increase in the frequency and
80 intensity of marine heatwaves (MHWs), have been reported since 1999 and later between 2015-2019,
81 for five consecutive years, and associated with MMEs of 4icrobenthic species belonging to different phyla
82 in Italy, France and Spain [1, 18, 22] The most affected species reported during these MMEs belong to the
83 coralligenous community that includincludedes gorgonians, echinoderms and sponges, which are
84 present along thousands of kilometres of coastline from the surface to 45 m. Reported data refer mostly
85 to temperature and non-infectious factors, but no pathologic data at individual/species level have ever
86 been reported for most of them [48].add
87 A clear understanding of the aetiology of the ongoing MMEs affecting the native *P. nobilis* populations in
88 the Mediterranean basin is necessary. Recent diagnostic investigations repeatedly confirmed the
89 simultaneous presence of several pathogens in the diseased bivalves. *Mycobacterium* sp. was identified
90 frequently, followed by *H. pinnae*, while *Vibrio mediterranei* and *Perkinsus* sp. were also detected in some
91 cases, suggesting that exposure to different pathogens could increase the complexity of disease
92 pathogenesis (Figure 1). The phylogenetic relationships of *Mycobacteria* spp. isolated from moribund
93 noble pen shells demonstrated a close relation to the *M. simiae* complex, which includes important
94 zoonotic agents responsible for the cause of emerging human and mammalian diseases.[48].[28,67]
95 Therefore, there is a strong need for implementing active surveillance programs focusing on new
96 emerging pathogens with zoonotic potential. This is especially relevant for the interface of the

human/marine habitat in the Mediterranean basin, currently experiencing a unique ecological transition represented by biological disturbance, climate change and modifications of the deep sea ecosystems. Altogether these factors can drive the emergence of new pathogens in unpredictable ways as already reported.[1,] NEW Improving the available diagnostic protocols for other species is mandatory for updating the surveillance capabilities for disease outbreaks and MMEs and to support healthy coastal ecosystems. Based on the available scientific evidence herein we discuss how the MMEs of *P. nobilis* are likely a consequence of a complex interplay between infections and non-infectious factors. In this review we stress the urgent need for future research to refrain from simplified disease hypotheses and take into consideration multiple additional elements that may have an impact on the physiology of the largest bivalve in the Mediterranean basin. At events of increased mortality in the marine environment, professionals investigate the episodes through qualitative clinical and pathological examinations, submit samples to laboratories for histopathological-assessment or diagnostics of infectious agents. However, the investigation into the pathogenesis of MMEs in marine environment has its limitations in revealing causality of disease without standardized diagnostic protocols.

Overview of the MMEs

The pathogens involved in *P. nobilis* MMEs and their interplay

Several pathogens have been found associated with the MMEs of the pen shell, and closely related with MHWs. The first report MMEs of *P. nobilis* described the presence of a Haplosporidian parasite in the

epithelium of digestive gland tubules in the autumn of 2016, although other pathogens have since been reported (Figure 1).[31]

Haplosporidians are endoparasites of invertebrates with a wide host range including bivalves, crustaceans, tunicates and polychaetes[21] .The phylum *Haplosporidia* has been associated with epizootic mortalities of farmed bivalves affected by the OIE-listed pathogens *Bonamia ostreae*/*exitiosa*, and by the MSX (Multinucleated Sphere Unknown) disease caused by *Haplosporidium nelsoni*. An outbreak due to *H. nelsoni* along the mid-Atlantic coast of the USA devastated local oyster populations and was responsible for significant economic losses.[37,50] In the case of the noble pen shell, the species in question is a member of the genus *Haplosporidium* and belongs to a large clade of species in the order Haplosporidia, and closely related to a *Haplosporidium* sp. infecting the cultured shrimp *Penaeus vannamei* in the Caribbean Sea and Indonesia. [72,92, 27] The parasite was reported in many areas, from Greece, Italy, Spain, Croatia and Turkey,[24,58,64,82] and considered as one of the major causative agents of the *P. nobilis* MMEs.

Haplospordian life cycle stages include two main phases: a uni-multinucleate plasmodium, and a sporulation phase producing resistant spores with a typical opening covered by an external lid.[26] The plasmodia stage divides by plasmotomy and can undergo sporulation. Spores are released into the environment after the death of the host, but they do not seem to be infective to the host in which they are produced, and may require a different host for completion of their life cycle.[26]

The morphological description of *H. pinnae* was firstly reported by Darriba et al.[31], and afterward by Catanese et al.[27]

Gross examination of affected animals can reveal in some case the presence of liquid cysts visible in tissues of the digestive apparatus, revealing an atrophic digestive epithelium underneath, related to the haplosporidium sporocysts dividing within the epithelial tubules [24].

The *H. pinnae* plasmodial phase is mainly uninucleate measuring 2-3 µm of length, with a central or slightly eccentric dense nucleus, present primarily in the connective tissue of the mantle and the digestive tissue. The plasmodia are generally observed within the hemocytes and in the connective tissue of the mantle and of the digestive gland (Figure 2a-c). Sporulation occurs in the epithelium of the digestive gland tubules, with transformed plasmodia having a lighter eosinophilic cytoplasm. Epithelial distention by sporocysts (30 µm) impinge the lumina of digestive tubules and when mature, sporocysts are released into the lumen (Figure 2d). In this phase, the host inflammatory response can appear mild or absent.[24,25,31] However, severe inflammation of the connective tissue of the digestive gland, with hypertrophy and hyperplasia of brown cells has been reported (Figure 2e-f).[27,64]

In terms of pathogen prevalence and intensity, the dynamics of haplosporidians in their hosts can be seasonal and dependent on environmental parameters like water currents.[21] In particular, as a pivotal step towards understanding mortality of *Pinna nobilis*, model simulations of particle drift dictated by regional surface currents have been performed[45]. Reported data showed that oceanic currents constitute a potential factor driving the expansion of these groups of parasites [23,45]. Unfortunately, the distribution of the disease pattern can't be conclusively ascribed solely to *H. pinnae* because much of the mortality reports were collected by "citizen scientists" who didn't perform any pathological analysis to assess what actual pathogens were present.[6,23]

Indeed, *H. pinnae* is not the only pathogen causing MMEs. Mortality of *P. nobilis* occurs also in the absence of *H. pinnae* and related to an infection by an incompletely classified *Mycobacterium*. The first report occurred in the two southern Italian regions in 2018, Campania and Sicily.[24] Later on, a diagnostic survey was conducted on moribund *P. nobilis* specimens retrieved from other Italian regions, such as Tuscany, Sardinia, and Apulia and then extended to Spain, in Catalonia. These studies showed that in the early phases of MMEs dying specimens presented a systemic disease associated with the infection by a *Mycobacterium* species, belonging to the *simiae* complex. Further molecular study of the *Mycobacterium* in *P. nobilis* used the genetic marker *hsp65* and the Internal Transcriber Spacer ribosomal DNA (ITS rDNA) for differentiating the strain. The study supported that the *Mycobacterium* infecting *P. nobilis* is close to *M. triplex*[25] that groups within the *M. simiae* complex, together with *M. sherrisi,* though phylogenetically distant to other mycobacteria reported from other mollusc species and other marine organisms. In fact, the *simiae* complex is composed of slow growing non-tuberculous mycobacteria (NTM), which were initially identified from *Rhesus* monkeys in 1965.[52,54] The complex has also been reported in humans in the Southern United States, Cuba, Palestine, Iran, Israel, Turkey, Japan, and more recently from Sri Lanka.[49,56] The species *Mycobacterium sherrisii* was recently described and characterized,[84] and now formally validated as a novel species.[53]

In most published cases, this *Mycobacterium* is reported to be an opportunistic pathogen, affecting immunocompromised individuals, such as HIV patients or those with pre-existing pulmonary diseases. Recent 16S rRNA gene data performed at the American University of Beirut Medical Centre, in Lebanon, revealed that *M. simiae complex* is the most common NTM isolated in human patients.[8]

In *P. nobilis* we still have scarce information on the pathogenetic mechanisms of this *Mycobacterium*.

Upon opening an infected pen shell, external clinical signs are non-specific, and include generalized tissue edema, characterized by a diffuse tissue swelling due to fluids that collect in the interstitial spaces of mantle and gill .[24] Microscopically, the bacteria seem to localize mainly within the eosinophilic granulocytes and in some cases in the Brown cells, as also shown by ultrastructure. They apparently escape from the phagosome, establishing in the cytoplasm of the host cell in a manner similar to other *Mycobacteria* (Figure 3).[24] Generally, 3 different patterns are described based on morphology of the inflammatory response and distribution, classified as focal, multifocal, and diffuse, involving granulocytes and brown cells. Diffuse inflammation is differentiated from focal inflammation when the affected area does not have multiple centers of hemocyte infiltration, and the immune cells are abundant and distributed broadly over a large section of tissue. During mycobacterial infection, moderate to severe multifocal inflammatory nodules are visible. Granulocytes can be admixed with aggregates of brown cells located in connective tissue of the digestive gland, and gonads (Figure 3a-c). Within the formed nodules composed of granulocytes, numerous intracytoplasmic acid-fast bacteria positive to Ziehl-Nelsen stain are visible (Figure 3b-d). Granulocytes, admixed with brown cells, could degenerate within the centre of the nodule in the connective tissue of the digestive gland and gonad. In reactive connective tissue proliferation of fibrous tissue is frequently observed, with infiltration by hyalinocytes and the presence of acid-fast bacteria within the brown cells (Figure 3 e-f).

Earlier studies before 2019, did not perform investigations to detect the presence of this *Mycobacterium*.[20,23,65] However, other research groups reported the same Mycobacterial species in specimens involved in MMEs, from many locations around the entire Mediterranean basin. Both *Mycobacterium* and *H. pinnae* have frequently been detected.[25,64,76,82]

Further phylogenetic analysis of the pathogens isolated from moribund animals showed that strains of *Mycobacterium* and *H. pinnae* had high similarity to samples previously reported from Greece, Spain, and Italy.[25,62,64,75] In Catalunya, *H. pinnae* was observed in 36% of the examined cases, always associated with *Mycobacterium* sp. [25] Histopathological studies in specimens from Thermaikos Gulf, Greece, showed the presence of *Mycobacterium* sp. with *H. pinnae*.[64] Interestingly, monitoring of a *P. nobilis* population from the Thermaikos Gulf, an estuary of extremely high importance for bivalve production, revealed the presence of both pathogens in a few specimens in higher quantity, without clinical symptoms of the disease.[64] The mass mortality of the population in the Thermaikos Gulf occurred during a prolonged period of raised seawater temperature in the Autumn, causing the collapse of all populations in shallow waters (4-10 m). In the Aegean Sea, the infection spread to all habitats by the late spring of the same year, limiting the distribution of surviving fan mussel populations to only the Kalloni Gulf Lesvos Island and in the Maliakos Gulf, Greece. Despite the temperature drop in the winter of 2019, mortality of the species continued, albeit at a lower rate than in the summer months of the previous year.[62] The histopathology of moribund animals showed greater lesion severity in specimens with concurrent infections with both *Mycobacterium* and *H. pinnae*.[82] Moderate to severe inflammatory lesions were linked to the unique presence of *Mycobacterium* sp., while absent or mild to moderate inflammatory foci were seen when *H. pinnae* was found alone. In the latter case, moderate inflammatory lesions were associated with the sporulation phases of the parasite. Lesions were absent, or of mild intensity, in the presence of plasmodia. This evidence suggests that the detection of different developmental stages of this parasite could have diagnostic and pathogenic relevance during *Haplosporidium* sp. infections. The simultaneous detection of both pathogens, and their presence within the inflammatory lesions, was observed in most of the examined sick/moribund animals. The absence of the *MME* in areas where these

pathogens were not detected,[82] supports the hypothesis that both *Mycobacterium* and *Haplosporidium* are cooperating in the progression of disease pathogenesis, thus synergistically leading to MMEs affecting *P. nobilis* in the Mediterranean Sea. Given the lack of clarity of *Haplosporidium* life cycles generally, there is also the possibility of additional biotic factors such as the presence or absence of alternate hosts that may contribute to the severity of MMEs. [38] The lack of such knowledge clouds some interpretations of the available data.

Further molecular diagnostic analyses on *P. nobilis*, *P. rudis*, and other bivalve species were performed in Sardinia (Italy),[83] showing that *H. pinn*ae was present in other bivalves at least 3 years before any first reported occurrence in *P. nobilis* associated with MMEs. Within the *P. nobilis* examined, positive *H. pinnae* PCR was reported in 27 (out of the 48) individuals analysed (56.3%). The protozoan was found in 71.4% of individuals with signs of disease such as weakness in closing the valves, but also in 44.4% of individuals without signs of disease. In a group of asymptomatic individuals, PCR testing for the presence of *H. pinnae* revealed a total of 12 positive and 15 negative specimens. PCRs targeting the Mycobacteria 16S rRNA were negative in 46 out of the 48 individuals tested. The only exceptions were retrieved from some individuals, such as PN19 from the north-western coast of Sardinia, and PN48 from the northern coast of Sardinia, which were found positive for *Mycobacterium* sp., showing 98% identity with the *Mycobacterium* sp. formerly described.[25]

Other typical bivalve pathogens such as bacteria belonging to *Vibrio* spp. and the dinoflagellate parasite *Perkinsus* sp. were detected in a few cases, suggesting that exposure to multiple pathogens could increase the complexity of disease pathogenesis [25, 65]. In Greece, 16S rRNA metagenomic sequencing was approached to assess the bacterial diversity within the digestive glands of diseased individuals.[65] Thirty moribund animals were collected in two different marine areas in the Aegean Sea. Sampling was carried

247 out between February and April of 2020. Along with the presence of pathogenic strains of *Vibrio*
248 *mediterranei*, multiple bacterial genera were detected including *Aliivibrio* spp., *Photobacterium* spp.,
249 *Pseudoalteromonas* spp., *Psychrilyobacter* spp. and *Mycoplasma* spp., with the latter found with a higher
250 abundance. Interestingly, in 8-10% of the cases animals displaying different lesions, were analyzed and
251 found to be negative for all the cited common pathogens. In early August of 2019, animals analysed in
252 Croatia, in an area where no mortality was detected (Seline), were negative for *Mycobacterium* and *H.*
253 *pinnae*, using both PCR and microscopic examination. However, histopathology revealed the presence of
254 extensive inflammatory nodules associated with brown cell hyperplasia in 40% of the samples (2/5
255 animals). Necrosis of the digestive gland was also recorded in a single individual, along with the presence
256 of intraepithelial Gram-negative bacteria in the digestive tract. In 60% of the animals (3/5), the presence
257 of unidentified ciliated protozoans on gills was also recorded.[82]
258 Negative molecular diagnostic (PCR) results were reported from Catalonia,[25] for 1 moribund, 1 dead
259 (though still with turgid flesh) and 3 animals in advanced state of autolytic processes, thus excluding the
260 presence of *Mycobacterium*, *Haplosporidium* and *V. mediterranei*.
261
262 *Vibrio mediterranei is associated with P. nobilis mortality under predisposing conditions*
263
264 The influence of pre-existing non-infectious factors on a bivalve's microbial communities, leading to the
265 establishment of disease conditions, is still not well understood. It is known that to cause disease,
266 pathogens must infect and invade the host's body, and subsequently dominate its microbial community.[2]
267 *Vibrio mediterranei* (synonym name of *V. shiloi* or *V. shilonii*) has been found in various marine
268 invertebrates.[81,85,90,91] The type strain (CECT 621T) was originally isolated from marine sediments,[77]

indicating that *V. mediterranei* is a cosmopolitan bacterial species.[3,10,41] Virulence genes were found in association with temperature/salinity-stressed animals, and include chitinases, proteases, and genes involved in an array of secretion and iron sequestration pathways.[10] The pathogenicity of *V. mediterranei* was first described in association with MMEs of the scleractinian coral *Oculina patagonica*, under the synonym name of *V. shiloi* or *V. shilonii*.[59–6181] Interestingly, in the case of coral bleaching by *V. shiloi*, in *Oculina patagonica*, the major effect of increasing temperature was reported to be the expression of virulence genes by the pathogen.[80] Elevated seawater temperature (a predisposing cause) increases the virulence of *V. shiloi,* synonym *Vibrio mediterranei* (a necessary cause), which enables the pathogen to adhere to a β-galactoside-containing receptor –produced by zooxanthellae – in the mucus on the surface of the coral host.[80] In the global decline of coral reef systems, the occurrence of multifactorial aetiology has been suggested, although still poorly understood.[32]

The first detection of *V. mediterranei* associated with a mass mortality in *P. nobilis* was reported during one of the early MMEs, registered in October 2016 along the coast of Alicante along the Southern Mediterranean coast of Spain. This multidisciplinary study used various methods including histopathology, bacteriology, virology, and parasitology, pathogen culture and PCR procedures.[79] A Saccharose positive bacterium was isolated in a TCBS medium from organs and tissues of two affected specimens and was absent in a third apparently healthy individual. The analysis of the 16s rRNA gene sequence obtained indicated the etiological agent was *V. mediterranei*. This information, together with experimental challenges of juvenile Manila clams (*Ruditapes philippinarum*) at 17ºC and 24ºC,[78,79] was reported and used as the basis for further testing the Koch postulates in juveniles of *P. nobilis*.[3] Similarly, in the Aegean Sea, the investigation of mortality events in *P. nobilis* populations conducted during the winter months along the Greek coastal zones, revealed the first detection of *V. mediterranei*, although

291 concurrently with another *Vibrio* sp. including a member of the *V. splendidus* clade.[62] In that study, apart
292 from the presence of *Vibrio* spp., *Mycobacterium* sp. was detected in all examined individuals together
293 with *H. pinnae,* which was present in 3 of 17 specimens studied.
294 Prado et al.[75] recorded over 90% cumulative mortality over 19-month period, peaking in summer and
295 early fall and coinciding with water temperatures above 25 °C. This temperature effect was also
296 observed in a challenge experiment in which *P. nobilis* individuals with mean shell length of 24 cm, were
297 injected with a strain of *V. mediterranei* (IRTA18-108) and held in open flow tanks for 23 days.[3] At the
298 start of the experiment, water temperature was 18°C. Mortalities started at 22°C on day 6 post-injection
299 and sharply increased after water temperature rose above 24ºC. Even though mortality rates were not
300 correlated with the bacterial doses injected, pathogenicity of the strain used for challenge was
301 confirmed in *P. nobilis* through PCR.
302 Field samples of *P. nobilis* collected in Alfacs Bay in the Ebro Delta were also found to be infected with *V.*
303 *mediterranei* in different tissues (particularly muscle and kidney), with 60% of the individuals having PCR
304 positive results in at least one tissue (adductor muscle, gonad, kidney, digestive gland, or branchia),
305 without displaying any disease signs. Apart from the challenge experiment described above, additional
306 individuals held in captive conditions suffered mortalities and necrosis of kidney and digestive tissue that
307 may be explained by consequences of generalized stress, and/or poor nutrition due to inappropriate
308 dietary maintenance leading to potentially reduced immune capacity and vulnerability to infection by *V.*
309 *mediterranei*. With individuals subjected to a more balanced diet, lower and later rates of mortality were
310 observed.[75] Yet, the pathogenicity of *V. mediterranei* in the context of other stressful events contributing
311 to debilitation of individuals, such as previous infections by other pathogens (e.g. *H. pinnae* or
312 *Mycobacterium* sp.), remains to be properly investigated. In a field study carried out in 2020 in the

313 Thermaikos Gulf on the remaining populations of *P. nobilis*, *Vibrio* species including *V. mediterranei*, were
314 detected alongside other *Vibrio* spp. in moribund individuals (unpublished data).
315
316 *Penshell immune and stress response associated with the MMEs*
317
318 The field of pathology is devoted to defining the causes of disease by describing the changes in cells,
319 tissues, and organs that are associated with disease and give rise to the presentation of signs and
320 symptoms in sick organisms.[33] Cells actively interact with their environment, constantly adjusting their
321 structure and function to changing demands and extracellular stressors.[40,87] As cells face physiologic
322 stresses or adverse conditions, they can undergo adaptation, achieving a new homeostatic state and
323 preserving viability and function by activating stress related genes and immune genes.[2,29]
324 Bivalves possess an innate immune system composed of cellular and humoral immune components
325 which are regulated by many immune-related genes.[9,88] Expression of some immune genes influences
326 genetic regulatory mechanisms, modifying cellular and organismal responses of ectotherms, such as
327 bivalves.[35] A first attempt to morphologically describe the pen shell immune cells, using cytology and
328 electron microscopy, was performed by Matozzo et al.[69] just before the spreading of the MMEs. Two
329 types of haemocytes were described: granulocytes and hyalinocytes; and granulocytes were further
330 subdivided either as basophilic, acidophil, and neutrophilic, capable of an active phagocytosis.
331 Haemocytes can produce superoxide anion and acid and alkaline phosphatases. Recently, during MMEs
332 Brunet et al.[17] reported a first insight into the genome of *P. nobilis* showing a large variety of genes
333 related to immunity, ranging from the pattern recognition receptors to effectors of the immune
334 response, and genes involved in apoptosis signalling, typically involved in the response of cellular damage

335 in bivalves.[94] Pattern Recognition Receptors such as Toll-like receptors, peptidoglycan recognition
336 receptors, glucan-binding proteins, lectins and laminins were highly represented in the *P. nobilis* genome
337 along with members of the Bcl-2 family, caspases and inhibitors and activators of apoptosis.
338 Peculiar immune cell types reported present in many bivalve species and highly represented in *P. nobilis*
339 are the so-called *brown cells*, abundant in the blood sinuses underlying the intestinal tract and renal
340 pericardial region filled with yellow-brown granules. They are fixed phagocytes common in the
341 connective tissue of bivalves. They aggregate in lesions and are found during *H. pinnae* and
342 *Mycobacterium* infection in *P. nobilis* but capable of active diapedesis. They may resemble higher
343 vertebrates Dendritic Cells , or Melanomacrophages Centres from birds and fish, and they are filled with
344 brown/yellow pigmented phagocytes that contain lipofuscin and melanin. In bivalves they contain
345 lysozyme, glutathione reductase and acid phosphatase[96] . When activated they pass from an immature
346 state into mature cells specialized for antigen capture with the initiation of lysosomal function.
347 Specimens of *P. nobilis* infected with either bacterial or parasitic pathogens generally display
348 hypertrophic and hyperplastic brown cells throughout the vesicular connective tissue in various states of
349 activity and degeneration (Figure 2-3).
350 Studies on stress response-related genes in the pen shell have identified members of the cytochrome
351 P450 gene family, heat shock proteins as well as sulfotransferases and glutathione-transferase genes.
352 Various triggering stimuli have been identified. Previous works showed that *P. nobilis* colonized by the
353 invasive algae *Lophocladia lallemandii* as well as individuals affected by anthropogenic activities have
354 increased levels of markers of oxidative stress.[15,89] *P. nobilis* affected by *H. pinnae* showed a reduction of
355 the antioxidant effectors catalase and superoxide dismutase, as compared to the healthy individuals,
356 while sick individuals also had higher levels of malondialdehyde, an indicator of lipid peroxidation.[14]

Other stress indicators like heat shock proteins and immune response pathways, apoptosis and autophagy were investigated in few affected animals in Greece by Lattos et al.[63] Analysis of both Hsp70/Hsp90 demonstrated that Individuals coinfected by *H. pinnae*, *Mycobacterium sp.* and *Vibrio sp.* species exhibited higher levels of the stress proteins, indicating an increased cellular stress response in comparison with the individuals infected only with *Mycobacterium* and *Vibrio*. Regarding specific immune genes, levels of the pro-inflammatory cytokines, Il-6 and TNF-*α*, did not show any significant differences between individuals infected only with *H. pinnae,* or the three pathogens together.

Considerations on false diagnoses or lack of knowledge of normal histology and histopathology

Histology remains a standard assessment tool for disease diagnosis in pathology, providing information on the state of the host tissues, the etiology of disease, the level of infection, and pathological alterations of affected tissues.

In the invertebrate pathologists' community, there is an increasing concern about the quality and veracity of histopathological findings published in peer-reviewed journals in the field. Animal pathology training programs encourage students to obtain rigorous, comprehensive education in histology, histopathology, and systemic pathology. The trained and experienced animal pathologist must know the studied animals in detail and have ability to distinguish normal histological variations from pathological processes in examined tissues. Historically, histopathology has mostly been used for the identification of bivalve parasites, with focus on the species of commercial interest such as clams, oysters and mussels, and to some extent as an endpoint in toxicological studies, sometimes using sentinel species such as zebra mussels. On the other hand, increased interest in publishing findings about invertebrate pathology has

not yet been met with an increase in availability of expert reviewers with specific training. Consequently, inaccurate interpretations of microscopic observations are being published in peer reviewed scientific publications.

The first histological misinterpretation about *P. nobilis* was reported by Katsanevakis et al.[35] where reported histopathologic findings of *H. pinnae* in the digestive gland are in fact photomicrographs of a female gonad follicle with evident regressing oocyte phase, surrounded by brown cells and scattered hemocytes. This error is apparently a combination of the authors' failure to recognize the normal tissue and the failure of the reviewers to reject the incorrect interpretations of the images and their associated findings. Recently Kunili et al.[58] also misinterpreted a normal tissue for *Haplosporidium*. Throughout the publication, the authors erroneously describe constitutive immune cells of a pen shell (brown cells) as "sporocysts enclosing more or less mature spores", and apparent nuclei of digestive epithelia are incorrectly reported as plasmodia or binucleate phase of the parasite. Furthermore, designated tissue types are hardly recognizable as such in the published low-quality images. These two examples of inaccurate and poorly presented histopathological data emphasize the need for augmentation of the knowledge on microanatomy and pathology of bivalve species (in particular those without commercial interest), encompassed by comparative pathologists.[7]

Non-infectious factors can play a role in the evolution and distribution of disease patterns in the field

Factors like temperature, humidity and soil nutrients and ocean chemistry in general can all have strong influences on spatial distributions of pathogens. Studies of relevant non-infectious factors, like water temperature and salinity have been reported.[68] These same non-infectious factors also affect animal

401 physiology, such as spawning and the subsequent recruitment of juveniles that are affected by water
402 temperature. An absence of recruitment has been noted previously among the Ebro Delta populations,[5]
403 and investigations using *P. nobilis* -specific qPCR detected very low levels of *P. nobilis* eggs/larvae in
404 seawater samples collected in this region during Aug-Sept. 2016.
405 Currently surviving *P. nobilis* populations are found only in enclosed bays, like in the Sea of Marmara and
406 in a few scattered lagoons. With such geographical limitations in distribution, recruitment bottlenecks
407 can be a driver of reduced genetic diversity that ultimately may enhance the severity of MMEs regardless
408 of the cause. The last known remaining population from Greece, is located near the estuaries of the
409 Spercheios River, Phthiotis, central Greece, in a habitat characterized as having lower salinity levels than
410 at the other populations in the same Gulf. This population was the last one that survived during the rising
411 temperature regime in the spring of 2020 (unpublished data). Mortalities occurred in the populations of
412 Maliakos, Phthiotis, central Greece, during the winter 2020, however not at the scale considered to be
413 an MME. Temperature of these sampling sites ranged between 10°C and 15°C. Also, regarding the Greek
414 coastline, despite the numerous diving efforts in different sites, no living individuals were detected in the
415 Ionian Sea in 2020, except for the population originating from Laganas Bay in Zakynthos Island, Ionian
416 Sea.[97] Cabanellas-Reboredo et al.[23] collected data on pen shell MMEs and environmental conditions
417 assembled by scientists and citizens across the Spanish Mediterranean coast, south of France, and some
418 more isolated locations in the Tyrrhenian and Adriatic Sea, Crete and Chypre and found that disease
419 expression was closely related to temperatures above 13.5 °C and to a salinity range between 36.5-39.7
420 %. Although no pathological evaluation was conducted to determine the exact cause of mortality, the
421 results indicated a clear influence of salinity and temperature in outbreak patterns. This suggests that
422 the interaction between these factors at the local scale could influence the outcome of MMEs. Similarly,

in the outer part of Alfacs Bay (South Ebro Delta), mass mortality events in 2018 and 2019 did not occur until the months of July and August, when temperatures rose above 28 °C (considerably higher than 13.5 °C) and coincided with salinity increases above 35 g/L (36.5 to 38.5 range).[76] In this case of Alfacs Bay, it is important to note that mortality rates (100% near the mouth, 43% in middle regions, and 13% in inner regions) were significantly associated with the summer salinity gradient across the bay (averages of 37.4 to 35.7) caused by freshwater agricultural discharges during the spring-summer season. *H. pinnae* was detected in individuals from all study zones of Afacs Bay, whereas *Mycobacterium* was only found in the region near the mouth of the bay, featuring the highest salinity. Interestingly, neither *H. pinnae* nor Mycobacterium were found in the small population of Fangar Bay (North Ebro Delta) subjected to summer salinity ranges of ca. 30.5 to 33.5 g/L.[76] Also, importantly, a small contingent of young surviving individuals (3 years of age) was found in the region of Alfacs Bay subjected to MMEs where both *H. pinnae* and *Mycobacterium* were present.[76]

Given that the prevalence of infection with Mycobacterium and other Gram (-) bacteria increases with host size, these patterns suggest that both pathogens are to some degree involved in the overall mortality rates observed in the field. In addition to those from the Ebro Delta, surviving pen shell populations have also been found in other confined or semi-confined environments featuring higher or lower salinities as compared with the open sea.[39,42,44,73,86] All these observations taken together, stress the importance of environmental monitoring to assess the mortality risks to populations and to consider possible palliative management actions such as controlled release of freshwater, where possible, to balance possible increases in salinity above 36.5 g/L during the summer period.

The need for standardized diagnostic protocols for noble pen shell MMEs

Diagnostic procedures have a key role in disease control and infection prevention in the case of captive animals: medical care, risk evaluation, management, and mitigation, as well as development of government policies in a framework of One Health, all rely on diagnostic tools to guide further decisions.[11,12,16,51,] .[98] However, diagnosing any infectious disease in aquatic animals requires more than just the result of a laboratory-performed diagnostic tests to identify (known) pathogens. The diagnostic process usually requires the expertise of a veterinarian and/or trained para-veterinary pathologists, who could simultaneously evaluate all known factors, from clinical signs to environmental conditions, that could be associated with the presence of a pathogen to support their diagnosis as happens in human medicine; this would be the approximation of collecting the patient's history through written and oral records, which is given primary importance in human medicine. Moreover, this process is prone to errors, and undermine the ability to identify the primary causative agent with certainty. The availability of specific and sensitive diagnostic tests is also of high importance to assist veterinary services in providing correct diagnosis.[99]

In the case of bivalves, it is challenging to determine the health status considering that there is an absence of observable clinical signs until very late stages of infection. Preliminary information on the health status of *P. nobilis* populations is usually based on field observations of animal behavior describing animals generally as *healthy* or *sick* /*moribund*. For both adults and juveniles, assigning a bivalve to one of these categories is based on presence/absence of signs of adductor muscle weakening, such as gaping, and retraction of the mantle from the edge of the shell, to define it as sick.[27,31,74] Moreover, during field examinations of bivalves, the valve closure speed is usually estimated by applying a gentle or more

stronger touch, or by pushing the water in the direction of the valve opening; slower closing response indicating a sick or moribund animal. However, studies performed on bivalves in Croatia and Italy showed that the valve closing speed may be unreliable evidence (Carella pers. communication), thus, the determination of their health status based only on this observation could be incorrect. In fact, bivalves considered healthy in the field for the quick closing speed, at a direct or "hands-on" physical examination may show evident clinical signs such as generalized tissue oedema, cysts or areas or discoloration along with infection with pathogens when once subjected to microscopic evaluation. This can be true even with the presence of clear evident gross lesions (i.e. digestive gland cysts associated with the presence of *H. pinnae* in apparently healthy animals) (Figure 4). Variability in clinical signs can often be confusing and misleading due to the lack of knowledge of physiology and pathology of the studied host animals, with consequent difficulty in correct interpretations of clinical signs/animal behavior.[95] Such absence of a reliable clinical interpretation can be frustrating, leading to misinformed and possibly wrong decisions. Indeed, a prerequisite for formulating correct etiopathogenetic hypotheses and for the development of treatment or conservation strategies, is that they must be based on a correct assessment of a bivalve's health status.[43]

Gaps in the diagnostic processes applied during investigations of mass mortalities in *Pinna nobilis* are clearly emerging from the increasing number of reports: there is no comprehensive and methodologically standardized description of the morphology, stage and grade of the macro-and microscopic lesions associated with the presence of specific pathogens. Therefore, it is not possible to link a pathogen to a specific response, lesion, or molecular pattern.

Possibly due to the endangered status of this species, most studies have utilized only mantle biopsies and molecular diagnostics to define the animal's health status, which is itself a very limiting approach

that can lead to "over-interpretation" in pathological diagnosis.[20] Taking into consideration that a) multiple pathogens are potentially associated with MMEs, and b) these pathogens show systemic distribution patterns in several organs and tissues during different phases of disease progression, it is obvious that a simplified mantle biopsy/PCR approach cannot work as the optimal diagnostic strategy. Moreover, molecular diagnostics alone do not give enough information to differentiate the infection and the associated disease. Different developmental stages of *H. pinnae* can elicit very different tissue reactions and lesions; information which cannot be derived from molecular analyses that are qualitative on the presence/absence of the pathogen, and possibly quantitative, in the case of qPCR.[25] Furthermore, the absence of detailed histopathology descriptions from tissue lesions linked to the pathogens leads to confusing results.

A proposal for an integrated microscopy and molecular diagnostic protocol

Various techniques and an integrated approach needs to be used to collect, identify, and monitor host populations for pathogens and related lesions, along with the associated environmental data. For animals still alive in the field, mantle biopsy is usually collected to define if an animal is infected by *H. pinnae* and coupled with associated animal behavior. This type of sampling can't be used alone and should be coupled to the collection of other biological material, like hemolymph used for smear preps for microscopy and DNA-based diagnostic approaches, also for a parallel testing for other pathogens.

In the field, mantle biopsy is generally performed maintaining the valves opened with the use a wooden stick (diameter = 0.5 cm), put in proximity of the hinge ligament. A tissue fragment of approximately 0.5 cm^2 is taken using a sterilized bite forceps and fixed in absolute alcohol for subsequent DNA diagnostic.

This method ensures no damage for the sampled animal, performed by many researchers. A reliable technique for sampling hemolymph is needed to complement and expand this current base protocol for non-lethal assessment. In bivalves, hemolymph collection is done at the adductor muscle anterior or posterior, depending on the species, and less frequently the ventricle of the heart. Similarly, to the blood tests in humans and livestock, it has been demonstrated to be a suitable tissue to evaluate the state of infection in marine invertebrates.

Hemolymph can be taken from live animals in the field without sacrificing them. This practice needs experienced manipulation, since a needle of 150 to 300 mm is used, inserted in the posterior adductor muscle, accessible from the upper part of the valve (Carella pers. communication). The adductor muscles, visible through gaping valves, allow needle insertion. Successful collection from a 50 cm animal can yield approximately 1.5 ml of fluid.

When moribund animals are collected instead, a complete panel of microscopic evaluation composed of hemolymph smear and histopathology of all the tissues, with routine and special staining, should be performed (**Table 1**). After the field examination, animals can be collected directly from their natural habitats and processed as soon as possible (max. 3-4 hours), transported in refrigerated containers. Specimens can survive much better in a chilled environment at 5-15°C. Species that tend to gape should be placed in damp towels or seaweed, then bagged and chilled. Care should be taken during collection to prevent damage and stress to specimens, which may affect histological interpretations. Animals are measured from the tip of the right valve near the hinge to the longest point on the bill, and their size (cm) is recorded. Few of the published articles on mussel mortality events describe gross lesions. The general appearance and overall body condition of each animal should be assessed as part of the routine examination or the investigation of mussel declines. External examination must consider shell

532 abnormalities, presence of fouling organisms, parasites, gross abnormalities, predators, and
533 physiologically related conditions as performed for other bivalves. Before animal opening, withdrawal of
534 hemolymph can be performed. Hemolymph can be collected via a 23-gauge needle from the posterior
535 adductor muscle of mussels. Collected hemolymph can be stored for cytology or for molecular diagnostic
536 assays. The mussels are opened through severance of the adductor muscle and then examined for color,
537 condition (fat, medium, or watery), macroparasites, and shell and tissue abnormalities. The body is
538 removed from the shell by severing the adductor muscle as close to the left valve as possible.

539 All the tissues (digestive tissues, gonad, kidney, gills, muscles) should be fixed in fresh fixative solution
540 (buffered Formalin or Davidson's) in the right amount of volume ratio (1:10). Poor fixation can make
541 tissues useless for histological assessment. Formalin and Davidson's are good general fixatives for
542 bivalves because they have good penetration, prepare the tissue for histological stains, and give superior
543 staining results with hematoxylin and eosin stains and other staining techniques. Some tissues require
544 special care and handling, like the bivalve's digestive gland that degrades very rapidly and need to be
545 immediately fixed after sampling as it is highly sensitive to weak fixation displaying artifacts.

546 Development of DNA-based diagnostic methods currently offer a broad panel of probes and tests but for
547 the pathogens related to *P. nobilis* MMEs the effort is still scarce. These methods have the theoretical
548 advantages of high sensitivity and high specificity and possible rapid screening for the presence of a
549 targeted pathogen. These methods should be coupled with highly sensitive qPCR for multiple targets,
550 ideally multiplexed using TaqMan or similar fluorochrome probes. Molecular tools are valuable for
551 establishing the presence of a pathogen, but they do require care for their interpretation. This is crucial
552 because many laboratories rely heavily on polymerase chain reaction (PCR) methods, which provide
553 information as to the presence or absence of a pathogen, although in some assays the primers are not

554 designed or tested adequately to diagnose a pathogen correctly. Primers sets for a PCR assay that are
555 overly-specific may not detect novel emerging strains, thus making all aspects of the pathological
556 examination equally relevant.

557 A routine diagnostic approach should at least include a classification of the extent and severity and nature
558 of the disease (i.e. pathological process, or lesions), based on a standardized quantitative evaluation of
559 tissue lesions at the time of the diagnosis.

560 The lesion severity should be classified using a standardized histological score, based on semi-
561 quantitative estimates of amount of tissue involved (in %), and on the type of lesions seen (inflammation,
562 degeneration, necrosis, etc.) with consistent lesion nomenclature / definitions in relation to the pathogen
563 detection.[82] Such an approach was developed before for salmonid whirling disease,[4] by assigning an
564 arbitrary numerical scale to identifiable discrete stages of disease development. Although, such disease
565 grading needs to be optimised for each etiological agent and related lesion, thus it will require careful
566 preparation and validation when multiple concomitant aetiologies are suspected.

567 At light microscopy, the lesions can be graded, and the extent of involvement recorded and related to
568 *Mycobacterium* and *H. pinnae.* The importance of consistent grading has been reviewed, and many
569 grading schemes have been reported for bivalve pathology and in one case in *P. nobilis.*

570 The sections need to be analysed blinded to molecular diagnostic results to minimise bias. In the case for
571 *Mycobacterium* and *H. pinnae*, the scorings are similar for both the pathogens and must consider the
572 phase of development, only for *H. pinnae*, and the site of infection. Special stains can help in visualizing
573 them (**Table 1**). As reported by [82] for *H. pinnae* we can give a score 1, for a mild infection, when we
574 observe the presence of few plasmodia in mantle or digestive tissue; mild to moderate infection (score
575 2): the parasite is present in the digestive gland and within digestive tubules in the pre-sporulation phase

within digestive epithelium (until 30% of the digestive tubules in a histological section are involved); score 3, marked infection, when the parasite is present within digestive tubule epithelium (more than 30% of the digestive tubules filled).

For *Mycobacterium*, Ziehl-Neelsen slides are prepared, using similar criteria; mild infection (score 1): few immune cells filled with Ziehl-Neelsen + bacteria in the mantle and digestive tissue capsules; mild to moderate infection (score 2): aggregates of immune cells filled with Ziehl-Neelsen (+) bacteria spreading in the connective tissue of the mantle as well as in the digestive gland, infiltrating tubules and hemolymph vessels; marked infection (score 3): big aggregates of Ziehl-Neelsen (+) bacteria spreading in all the tissues within nodules of hemocytes (digestive gland, mantle and gonad).

This approach, accompanied by a detailed description of the lesions per case, can give us a better and more complete overview of the pathogenesis leading to the mortality.

In parallel, standardized molecular diagnosis should also consider the "quantity" of any identified pathogen(s), to better estimate their relative importance when a polymicrobial infection is suspected. Taken together, it has become evident that one of main reasons for our limited progress in understanding the epidemiology and pathobiology of MMEs affecting the noble pen shell is the lack of standardized diagnostic protocols that could be sensitively applied when multiple pathogens are occurring.

In **Table 1** is presented a list of all the necessary analyses for the evaluation of *P. nobilis* health status and possible causes related to the mortality.

In the table is given an overview of the diagnostic approaches with all the analyses needed to be performed in dead/or sacrificed animals, or in tissue biopsies from live animals: histopathology, cytology,

597 and DNA based methods for both *H. pinnae* and *M. sherrisi,* using both qualitative and quantitative PCR
598 correlated with sequencing.
599 In field studies, when animals are to be kept alive, the alternative diagnostic procedure until now has
600 been to perform mainly mantle biopsy, but previous studies showed that the mantle is not suitable for
601 *Mycobacteria* detection, so only *H. pinnae* can be revealed using this method. The alternative, currently
602 in course of validation, is to collect and use hemolymph with a non-lethal method.
603
604 Recent research perspectives are mainly focused on the optimization of the already described techniques
605 to gain sensitivity and specificity with faster and easier application, and that allow a positive diagnosis in
606 even early stages of infection. Molecular tools can detect DNA sequences of the pathogen, which does
607 not imply that the pathogen is visible in the host cell, although with use of *in situ* hybridization techniques
608 its reliability can be determined.
609 In **Table 2** we present the analysis performed and the pathogens detected in different cases in many
610 Mediterranean countries.
611
612
613 **Future directions**
614 Reported investigations of MMEs and other research efforts addressing the spread of *P. nobilis* disease
615 have been using rather diverse methodological approaches, primarily due to lack of standardized
616 diagnostic tools, unknown/complex disease aetiology, largely missing information on basic biology of
617 healthy animals, and lack of communication between scientific groups. Furthermore, diagnostic
618 techniques with inherent limitations were used, including clinical signs based on the closure of the valves,

mantle biopsies, and reliance mostly on molecular information. Such an approach interferes with comprehensive analysis and interpretation of the rapidly accumulating data about mass mortalities. Further problems in understanding disease arise due to limited availability of knowledge about *P. nobilis* physiology, anatomy, histology, nutrition, and microbiology. Taken together, our efforts to understand this disease are currently undermined by limitations in both quality and quantity of empirical knowledge.

Apparently, we are facing a crisis of complexity, in which many factors and relations intertwine. Mortalities do not appear to be related to a single pathogen, and it is also possible that a primary agent common to all mortalities remains elusive and is yet to be described. It is therefore necessary to clarify the role of each suspected pathogen and their association to inflammatory lesions of each of the affected organs in observed mortalities, their relationships with environmental variables, routes of entry and dispersion (vectors), and multiple other epidemiological characteristics of this disease. As a way forward to further research, the following points should be considered:

Solve the diagnostic issue

We have incomplete knowledge of the problem, due in part to variable approaches to analyse the collected samples and the differences among focus areas of the studies that were carried out to date. It is therefore necessary to standardize the pathogen detection protocols, supported with the further development of reliable detection methodology. A manual of such an approach should be created in the context of *P. nobilis* MMEs involving all the experts of the sector. Moreover, we advise that collection of hemolymph from the adductor sinus can be safe for sampled *P. nobilis* and should be explored as a relatively non-invasive, and potentially useful, approach to the evaluation of mussel health.

641 Researchers studying MMEs of *P. nobilis* should be trained within a Ring test. "Ring" tests are
642 competence tests coordinated among multiple collaborating laboratories including enough replicates
643 needed for statistical analysis of assay reproducibility (between laboratories) and repeatability (within a
644 laboratory) and facilitates process improvement.

645 Finally, the analysis of eDNA raises interesting perspectives to better detect, characterise and monitor
646 known pathogens. These techniques will not replace, but rather complement diagnostic tools currently
647 used including general methods such as histopathology, which helps to ensure detection of emerging
648 diseases.

649 Once such protocol is generally accepted by the scientific community, the next step will be to use it in a
650 re-analysis of available samples from previous works, including quantitative molecular search for
651 multiple suspected pathogens.

652 Establishing targeted surveillance programs with the participation of multiple stakeholders (i.e.
653 governments, non-governmental agencies, academic partners, veterinary contributors, and the general
654 public) is of high importance, especially due to the pan-Mediterranean distribution of the MMEs and the
655 official IUCN status as a critically endangered species. Developing highly specific and sensitive non-lethal
656 diagnostic methods, combined with standardized investigation protocols will be crucial in supporting
657 national veterinary diagnostic laboratories, or other diagnostic facilities in fulfilling the requirements of
658 the EU habitats directive and Barcelona Convention (Anex II). Furthermore, surveillance programs may
659 assist in the discovery and characterization of resistant individuals or populations, allowing further
660 studies on resistance mechanisms and the establishment of a hatchery for production of *P. nobilis* for
661 reintroduction/repopulation of previously affected regions. Improved communication and increased

662 awareness of the problem should be a priority to all involved research groups and stakeholders. Timely
663 sharing of research data and surveillance information between stakeholders will accelerate our
664 understanding of the disease epidemiology and will certainly help in conservation efforts. For example,
665 opening a shared data repository with online access to interested parties, or setting up a forum/list of
666 research groups, government and non-government agencies or other entities to facilitate rapid exchange
667 of information related to emerging/ongoing outbreaks. It is also foreseeable that several public/private
668 partnerships can be founded at various levels (from local coastal communities to multi-national
669 consortia) with a task, among others, to encourage and support communication, dissemination of
670 information and conservation actions based on solid scientific evidence.
671
672 *Database of full genome sequences and genomic approach to pathogenesis*
673
674 The simplification of genomic methods for acquisition of whole genomes, transcriptomes and
675 metagenomes offers an opportunity to identify virulence factors, develop better molecular detection
676 methods, gain knowledge of the molecular mechanisms at work of both the pathogen and the host
677 immune response, and understand pathogens and hosts phylogeny. In this sense, it is recommendable
678 to build a unique and dedicated database that includes genomes from *Mycobacterium, Vibrio* strains and
679 *Haplosporidium* isolates collected from various mortality episodes and geographical locations. Future
680 effort should also be put into development of isolation and culture of the pathogens most often involved
681 in the MMEs to define their virulence and pathogenicity for the pen shell. The lack of bivalve molluscan
682 cell lines has greatly limited the possibility of the study of experimental transmission of pathogens and

683 to better define the host-pathogen interaction. Moreover, classic serological methods are not suitable
684 for diagnostic purposes since mollusks do not produce antibodies.

685

686 *Definition of optimal environmental variables for P. nobilis to better understand the disease pathogenesis*
687 *and to find areas for animal refugia*

688

689 Ambient conditions can be a source of increasing stress on host populations involved in MMEs, and cause
690 changes in pathogen virulence and infectivity, but the exact role or mechanisms of the non-infectious
691 factors is currently unclear. To understand the epidemiological connections between various locations
692 and differences in the appearance of the disease, it is necessary to collect further information about
693 environmental conditions preceding and during *MMEs*. Enlightening the association between the abiotic
694 factors of the local environment and the occurrence of *MMEs* is critical to identify potential refugia that
695 could be used for relocation and introduction of (previously confirmed) healthy specimens. Furthermore,
696 additional studies are needed to investigate the effects of environmental pollution and of the
697 contaminant concentrations present in these animals. Considering a typical habitat of fan mussels (eg,
698 coastal embayments both natural and man-made: bays, gulfs, etc.), increased urban activities and
699 industrial discharge may incrementally increase pollutant concentrations in areas with slow water
700 exchange, causing higher stress and lowering resistance to any and all pathogens.

701 Predisposing host factors allowing engagement of opportunistic pathogens need further investigations.
702 Current information about major potential pathogens suspected in *MME*s supports the idea of their
703 being opportunistic in nature, rather than exclusively pathogens. Therefore, a remarkable lack of

mortalities in other bivalve species that share habitat with *P. nobilis* suggests there may be an important intrinsic component of the host acting as a species-specific predisposing factor, making *P. nobilis* vulnerable to opportunistic infections. While there is only limited information about normal physiology and ecology of *P. nobilis*, a possible differential factor could be the high incidence of micro-lacerations in *P. nobilis* mantle or gills caused by commensal crustaceans like *Nepinnotheres pinnotheres* and *Pontonia pinnophylax*, commonly found living inside almost all *P. nobilis* specimens, but not in other bivalve species sharing the habitat. The extreme size of *P. nobilis* sets it apart from other bivalves and enables the cavity between the valves to provide shelter for these supposedly commensal crustaceans. Such micro-lacerations may act as entry points for opportunistic pathogens, specifically predisposing *P. nobilis* to development of diseases [42].

Conclusions

This work offers an objective review of the current status of knowledge about possible causes of recent *MME*s of the noble pen shells in the Mediterranean basin. We agree that the disease etiology is complex, involving multiple pathogens causing co-infections, and strongly related to environmental conditions that are specifically predisposing *P. nobilis* to poor disease outcomes. We strongly advocate that a standardized *MMEs* investigation protocol should be jointly developed and adopted by all involved in studies of epidemiology and pathology of these MMEs. Furthermore, we ask all stakeholders that full consideration should be given to an open data-sharing approach, including access to archived samples, gathering molecular genetic information, and other physical or electronic data from both past and ongoing investigations. This will require a timely response on the part of all Mediterranean coastal

nations and will require focused funding to achieve. Only by working together will we be able to keep ahead of the devastating consequences of this poly-microbial pathogenic and multifactorial syndrome affecting the noble pen shell in the Mediterranean Sea and possibly having a chance to stop their disappearing from their native habitats.

References

1. Albano PG, Steger J, Bošnjak M, et al. Native biodiversity collapse in the eastern Mediterranean. 2021; *Proceeding of Royal Society* **288**(1942).
2. Allam B, Raftos D. Immune responses to infectious diseases in bivalves. 2015;**131**:121–136.
3. Andree KB, Carrasco N, Carella F, Furones D, Prado P. *Vibrio mediterranei*, a potential emerging pathogen of marine fauna: investigation of pathogenicity using a bacterial challenge in Pinna nobilis and development of a species-specific PCR. 2021; *J Appl Microbiol*. **130**(2):617–631.
4. Andree KB, Hedrick RP, MacConnell E. A review of the approaches to detect *Myxobolus cerebralis*, the cause of salmonid whirling disease. Vol. 2002, *American Fisheries Society Symposium*. American Fisheries Society 2002:197–211.
5. Andree KB, Trigos S, Vicente N, Carrasco N, Carella F, Prado P. Identification of potential recruitment bottlenecks in larval stages of the giant fan mussel *Pinna nobilis* using specific quantitative PCR. *Hydrobiologia* 2018;**818**(1):235–247.
6. Arzul I, Carnegie RB. New perspective on the haplosporidian parasites of molluscs. 2015; J Invert Path **131**:32–42.
7. Au DWT. The application of histo-cytopathological biomarkers in marine pollution monitoring: A review. Vol. 48, *Marine Pollution Bulletin*. 2004:817–834.
8. Bachofner JA, Ikenberg K, Schulthess B, Nemeth J. Disseminated *Mycobacterium simiae* and *Mycobacterium* avium infection causing an immune reconstitution inflammatory syndrome in a female patient with HIV infection. 2021; *BMJ Case Rep* **14**(5).
9. Balouet, G., Poder M. A consideration of the cellular reactions in bivalve molluscs, with emphasis on haemocytic diseases. 1985:381–385.
10. Banin E, Israely T, Kushmaro A, Loya Y, Orr E, Rosenberg E. Penetration of the coral-bleaching bacterium *Vibrio shiloi* into *Oculina patagonica*. 2000; *Appl Environ Microbiol*. **66**(7):3031–3036.
11. Binder S, Sanderson LM. The role of the epidemiologist in natural disasters. 1987;**16**(9*): Ann Emerg Med*. 1081–1084.

12. Birkhead GS, Maylahnand CM. State and Local Public Health Surveillance. In: Teutsch SM, Churchill RE, eds. *Principles and Practices of Public Health Surveillance*. Oxford University Press 2000:

13. Boddinghaus B, Rogall T, Flohr T, Blocker H, Bottger EC. Detection and identification of mycobacteria by amplification of rRNA. 1990; *J Clin Microbiol* **28**(8):1751–1759.

14. Box A, Capó X, Tejada S, et al. Reduced Antioxidant Response of the Fan Mussel *Pinna nobilis* Related to the Presence of *Haplosporidium pinnae*. 2020; *Pathogen***9**(11):1–14.

15. Box A, Sureda A, Deudero S. Antioxidant response of the bivalve *Pinna nobilis* colonised by invasive red macroalgae *Lophocladia lallemandii*. 2009; *Comp Biochem Physiol C Toxicol Pharmacol* **149**(4):456–460.

16. Buehler JW, Hopkins RS, Overhage JM, Sosin DM, Tong V. Framework for evaluating public health surveillance systems for early detection of outbreaks: recommendations from the CDC Working Group. 2004; *MMWR* **53**(RR-5):1–11.

17. Bunet R, Prévot JM, Vicente N, et al. First insight into the whole genome shotgun sequence of the endangered noble pen shell *Pinna nobilis*: a giant bivalve undergoing a mass mortality event. 2021; *Journal of Molluscan Studies*. **87**(1).

18. Burge CA, Hershberger PK. Climate change can drive marine diseases. In: Behringer DC, Silliman BR, Lafferty KD, eds. *Marine Disease Ecology*. Oxford University Press 2020:83–94.

19. Burge CA, Shore-Maggio A, Rivlin ND. Ecology of Emerging Infectious Diseases of Invertebrates. In: *Ecology of Invertebrate Diseases*. John Wiley & Sons, Ltd 2017:587–625.

20. Burreson EM. Misuse of PCR assay for diagnosis of mollusc protistan infections. 2008*; Dis Aquat Organ* **80**(1):81–83.

21. Burreson EM, Ford SE. A review of recent information on the Haplosporidia, with special reference to *Haplosporidium nelsoni* (MSX disease). 2004; *Aquat. Living Resour*. **17**(4):499–517.

22. Byers JE. Marine Parasites and Disease in the Era of Global Climate Change. 2021; *Annual Reviews*; **13**(1):397–420.

23. Cabanellas-Reboredo M, Vázquez-Luis M, Mourre B, et al. Tracking a mass mortality outbreak of pen shell *Pinna nobilis* populations: A collaborative effort of scientists and citizens. 2019; *Scientific Reports* **9**(1):1–11.

24. Carella F, Aceto S, Pollaro F, et al. A mycobacterial disease is associated with the silent mass mortality of the pen shell *Pinna nobilis* along the Tyrrhenian coastline of Italy. 2019; *Scientific Reports*; **9**(1):1–12.

25. Carella F, Elisabetta A, Simone F, et al. In the Wake of the Ongoing Mass Mortality Events: Co-occurrence of *Mycobacterium*, *Haplosporidium* and Other Pathogens in *Pinna nobilis* Collected in Italy and Spain (Mediterranean Sea). 2020; *Frontiers in Marine Science* **7**:48.

26. Carnegie RB, Barber BJ, Culloty SC, Figueras AJ, Distel DL. Development of a PCR assay for detection of the oyster pathogen *Bonamia ostreae* and support for its inclusion in the Haplosporidia. 2000; *Dis Aquat Organ* **42**(3):199–206.

27. Catanese G, Grau A, Valencia JM, et al. *Haplosporidium pinnae* sp. nov., a haplosporidan parasite associated with mass mortalities of the fan mussel, *Pinna nobilis*, in the Western Mediterranean Sea. 2018;*JIP* **157**:9–24.

28. Chen X, Liao B, Cheng L, et al. The microbial coinfection in COVID-19. Vol. 104, *Applied Microbiology and Biotechnology*. Springer 2020:7777–7785.

29. Coates CJ, Söderhäll K. The stress–immunity axis in shellfish. 2021; *JIP* **186**:107492.

30. Cunningham AA, Daszak P, Wood JLN. One health, emerging infectious diseases and wildlife: Two decades of progress? Vol. 372, *Philosophical Transactions of the Royal Society B: Biological Sciences*. Royal Society 2017:

31. Darriba S. First haplosporidan parasite reported infecting a member of the Superfamily Pinnoidea (*Pinna nobilis*) during a mortality event in Alicante (Spain, Western Mediterranean). 2017; *JIP* **148**:14–19.

32. Dennis M, Becker A, Freeman M. Pathology of multifocal purple spots, a nonspecific lesion morphology of Caribbean sea fans Gorgonia spp. 2020; *Disease Aquatic Organism*, **141**:79–89.

33. Dianzani MU, Dianzani I, Dianzani U. *Istituzioni di patologia generale*. UTET scienze mediche 2004.

34. Dimitriadis VK, Koukouzika N (2003) Effect of sampling procedures, transportation stress and laboratory maintenance on the structure and function of the digestive gland epithelium of the mussel *Mytilus galloprovincialis*. Mar Biol 142:915–924

35. European Centre for Disease Prevention and Control (ECDC). Towards One Health preparedness. 2018:22.

36. Feidantsis K, Giantsis IA, Vratsistas A, et al. Correlation between intermediary metabolism, Hsp gene expression, and oxidative stress-related proteins in long-term thermal-stressed *Mytilus galloprovincialis*. 2020; *Translational Physiology* **319**(3):R264–R281.

37. Feist SW, Thrush MA, Dunn P, Bateman K, Peeler EJ. The aquatic animal pandemic crisis. 2019; Rev Sci Tech **38**(2):437–457.

38. Fey, S. B. et al. 2015. Recent shifts in the occurrence, cause and magnitude of animal mass mortality events. – Proc. Natl Acad. Sci. USA 112: 1083–1088.

Fyhn, H. J. & Costlow, J. D. Anaerobic sampling of body-fluids in bivalve mollusks. Comp. Biochem. Phys. A 52, 265–268 (1975).

823 39. Ford SE, Haskin HH. History and epizootiology of *Haplosporidium nelsoni* (MSX), an oyster
824 pathogen in Delaware Bay, 1957-1980. 1982; JIP **40**(1):118–141.

825 40. Ford SE, Stokes NA, Alcox KA, et al. Investigating the Life Cycle of *Haplosporidium Nelsoni* (Msx):
826 A Review. 2018; *Journal of Shellfish Research* **37**(4):679–693.

827 40. Foulquie M, Dupuy de la Grandrive R, Dalias N, Vicente N. Inventaire et état de santé des
828 populations de Pinna nobilis (L.1758) dans l'étang de Thau (Hérault, France), 2020. Inventory and
829 health status of the populations of *Pinna nobilis* (L.1758) in Thau lagoon (Herault, France), 2020.
830 *marinelife-revue.fr* 2020:1–25.

831 41. Fulda S, Gorman AM, Hori O, Samali A. Cellular stress responses: Cell survival and cell death.
832 *International Journal of cell Biology*. 2010.

833 Galvis F, Ageitos L, Rodríguez J, et al. *Vibrio neptunius* Produces Piscibactin and Amphibactin and Both
834 Siderophores Contribute Significantly to Virulence for Clams. 2021; *Front Cell Infect Microbiol*
835 **11**:1049.

836 42. García-March JR, Hernandis S, Vázquez-Luis M, et al. Age and growth of the endangered fan mussel
837 *Pinna nobilis* in the western Mediterranean Sea. 2020; *Marine Environmental Research*
838 **153**:104795.

839 43. Gardner IA, Whittington RJ, Caraguel CGB, et al. Recommended reporting standards for test
840 accuracy studies of infectious diseases of finfish, amphibians, molluscs and crustaceans: The
841 STRADAS-Aquatic checklist. 2016; *Disease Aquatic Organism* **118**(2):91–111.

842 44. Giménez-Casalduero F, Gomariz-Castillo F, Alonso-Sarría F, Cortés E, Izquierdo-Muñoz A, Ramos-
843 Esplá AA. *Pinna nobilis* in the Mar Menor coastal lagoon: A story of colonization and uncertainty.
844 2020; *MEPS* **652**:77–94.

845 45. Giménez-Romero À, Grau A, Hendriks IE, Matias MA. Modelling parasite-produced marine
846 diseases: The case of the mass mortality event of *Pinna nobilis*. 2021; *Ecological Modelling*
847 **459**:109705.

848 46. Gorgoglione B, Bailey C, Fast MD, et al. Co-infections and multiple stressors in fish. In: *Bulletin of*
849 *the European Association of Fish Pathologists*. 2020:4–19.

850 47. Grau A, Villalba A, Navas JI, Hansjosten B, Valencia JM, García-March JR, Prado P, Follana-Berná G,
851 Morage T, Vázquez-Luis M, Álvarez E, Katharios P, Pavloudi C, Nebot-Colomer E, Tena-Medialdea
852 J, Lopez-Sanmartín M, Peyran C, Cižmek H, Sarafidou G, Issaris Y, Tüney-Kizilkaya I, Deudero S,
853 Planes S and Catanese G ˇ(2022) Wide-Geographic and Long-Term Analysis of the Role of
854 Pathogens in the Decline of *Pinna nobilis* to Critically Endangered Species. Front. Mar. Sci.
855 9:666640. doi: 10.3389/fmars.2022.666640

856 48. Gutierrez Y. *Diagnostic Pathology of Parasitic Infections with Clinical Correlations*. Oxford University
857 Press 2000.

858 49. Hamieh A, Tayyar R, Tabaja H, et al. Emergence of *Mycobacterium simiae*: A retrospective study
859 from a tertiary care center in Lebanon. 2018; *PLOS ONE* **13**(4).

860 50. Hashemi-Shahraki A, Darban-Sarokhalil D, Heidarieh P, et al. *Mycobacterium simiae*: a possible
861 emerging pathogen in Iran. 2013; *J Infect Dis*. **66**(6):475–479.

862 50. Haskins H, Andrews J. Uncertainties and speculations about the life cycle of the eastern oyster
863 pathogen *Haplosporidium nelsoni* (MSX). In the book: Disease Processes in Marine Bivalve
864 Molluscs 1988.

865 51. Hasson K.W. E.M. Wyld, Y. Fan, S.W. Lingsweiller, S.J. Weaver, J. Cheng, P.W. Varner
866 Streptoccosis in farmed *Litopenaeus vannamei*: a new emerging bacterial disease of penaeid
867 shrimp Dis. Aquat. Organ., 86 (2009), pp. 93-106

868 Holm A, Hill R, Farsang A, Jungbäck C. Diagnostics in the veterinary field: The role in
869 healthsurveillance and disease identification. In: *Biologicals*. Academic Press 2019:80–84.

870 Hooper, C Slocombe R, R Day and S Crawford Leucopenia associated with abalone viral
871 ganglioneuritis. Aust Vet J (2012);90:24–28 doi: 10.1111/j.1751-0813.2011.00877.x

872

873 52. van Ingen J, Boeree MJ, Dekhuijzen PNR, van Soolingen D. Clinical relevance of *Mycobacterium*
874 *simiae* in pulmonary samples. 2008; *Eur Respir J* **31**(1):106–109.

875 53. van Ingen J, Tortoli E, Selvarangan R, et al. *Mycobacterium sherrisii* sp. nov., a slow-growing non-
876 chromogenic species. 2011; *Int J Syst Evol Microbiol* **61**(Pt 6):1293.

877 54. Karassova V, Weissfeiler J, Krasznay E. Occurrence of atypical mycobacteria in *Macacus rhesus*.
878 1965; *Acta Microbiol Acad Sci Hung* **12**(3):275–282.

879 55. Katsanevakis S. The cryptogenic parasite *Haplosporidium pinnae* invades the Aegean Sea and
880 causes the collapse of Pinna nobilis populations. 2019; *Aquatic Invasions* **14**(2):150–164.

881 56. Keragala BSDP, Gunasekera CN, Yesudian PD, et al. Disseminated *Mycobacterium simiae* infection
882 in a patient with adult-onset immunodeficiency due to anti-interferon-gamma antibodies - A case
883 report. 2020; BMC Infectious Diseases **20**(1):1–7.

884 57. Keller, and C.S. Smith. 2004. Histological techniques for marine bivalve mollusks and
885 crustaceans. NOAA Technical Memorandum NOS NCCOS 5. Oxford, MD. 218 pp.

886 57. Kim H, Kim SH, Shim TS, et al. Differentiation of *Myocobacterium* species by analysis of the heat-
887 shock protein 65 gene (hsp65). 2005; Int J Syst Evol Microbiol **55**(4):1649–1656.

888 58. Künili İE, Ertürk Gürkan S, Aksu A, et al. Mass mortality in endangered fan mussels *Pinna nobilis*
889 (Linnaeus 1758) caused by co-infection of *Haplosporidium pinnae* and multiple *Vibrio* infection in
890 Çanakkale Strait, Turkey. Biomarkers 2021:1–12.

59. Kushmaro A, Banin E, Loya Y, Stackebrandt E, Rosenberg E. *Vibrio shiloi* sp. nov., the causative agent of bleaching of the coral *Oculina patagonica*. 2001*; Int J Syst Evol Microbiol* **51**(4):1383–1388.

60. Kushmaro A, Loya Y, Fine M, Rosenberg E. Bacterial infection and coral bleaching [6]. Vol. 380, *Nature*. Nature Publishing Group 1996:396.

61. Kushmaro A, Rosenberg E, Fine M, Loya Y. Bleaching of the coral *Oculina patagonica* by *Vibrio* AK-1. 1997; *Marine Ecology Progress Series* **147**(1–3):159–165.

62. Lattos A, Bitchava K, Giantsis IA, Theodorou JA, Batargias C, Michaelidis B. The implication of vibrio bacteria in the winter mortalities of the critically endangered *Pinna nobilis*. 2021; *Microrganism* **9**(5):922.

63. Lattos A, Feidantsis K, Georgoulis I, et al. Pathophysiological Responses of *Pinna nobilis* Individuals Enlightens the Etiology of Mass Mortality Situation in the Mediterranean Populations. 2021;*Cells* **10**(11).

64. Lattos A, Giantsis IA, Karagiannis D, Michaelidis B. First detection of the invasive Haplosporidian and Mycobacteria parasites hosting the endangered bivalve *Pinna nobilis* in Thermaikos Gulf, North Greece. 2020; *Marine Environmental Research* **155**.

65. Lattos A, Giantsis IA, Karagiannis D, Theodorou JA, Michaelidis B. Gut symbiotic microbial communities in the iucn critically endangered *Pinna nobilis* suffering from mass mortalities, revealed by 16s rRNA amplicon ngs. 2020; *Pathogens* **9**(12):1–21.

66. López-Sanmartín M, Catanese G, Grau A, Valencia JM, García-March JR, Navas JI. Real-Time PCR based test for the early diagnosis of *Haplosporidium pinnae* affecting fan mussel *Pinna nobilis*. 2019; *Plos One* **14**(2):e0212028.

67. Magouras I, Brookes VJ, Jori F, Martin A, Pfeiffer DU, Dürr S. Emerging Zoonotic Diseases: Should We Rethink the Animal–Human Interface? 2020; *Front. Vet. Sci.***7**:582743.

68. Marcogliese DJ. The Distribution and Abundance of Parasites in Aquatic Ecosystems in a Changing Climate: More than Just Temperature. In: *Integrative and Comparative Biology*. Oxford University Press 2016:611–619.

69. Matozzo V, Pagano M, Spinelli A, Caicci F, Faggio C. *Pinna nobilis*: A big bivalve with big haemocytes? 2016; *Fish Shellfish Immunol* **55**:529–534.

McDowell WG and Sousa R (2019) Mass Mortality Events of Invasive Freshwater Bivalves: Current Understanding and Potential Directions for Future Research. Front. Ecol. Evol. 7:331. doi: 10.3389/fevo.2019.00331

924 70. Mihaljević Ž, Pavlinec Ž, Zupičić IG, et al. Noble Pen Shell (*Pinna nobilis*) Mortalities along the
925 Eastern Adriatic Coast with a Study of the Spreading Velocity. 2021; J. *Mar. Sci. Eng* **9**(7):764.

926 71. Mohamed AM, Kuyper DJ, Iwen PC, Ali HH, Bastola DR, Hinrichs SH. Computational approach
927 involving use of the internal transcribed spacer 1 region for identification of *Mycobacterium*
928 species. 2005; *J. Clinical Microbiol.* **43**(8):3811–3817.

929 72. Nunan LM, Lightner D v., Pantoja CR, Stokes NA, Reece KS. Characterization of a rediscovered
930 haplosporidian parasite from cultured *Penaeus vannamei*. 2007*; Dis. Aquat. Organ.* **74**(1):67–75.

931 73. Öndes F, Kaiser MJ, Güçlüsoy H. Human impacts on the endangered fan mussel, *Pinna nobilis*.
932 2020; *Aquatic Conservation: Mar. Freshwat. Ecosyst.* **30**(1):31–41.

933 74. Panarese R, Tedesco P, Chimienti G, et al. *Haplosporidium pinnae* associated with mass
934 mortality in endangered Pinna nobilis (Linnaeus 1758) fan mussels. 2019; *J. Invert. Path*. **164**:32–37.

935 Pensa, D., Fianchini, A., Grosso, L. *et al.* Population status, distribution and trophic implications
936 of *Pinna nobilis* along the South-eastern Italian coast. 2022*npj biodivers* **1**, 3
937 https://doi.org/10.1038/s44185-022-00002-2

938 75. Prado P, Carrasco N, Catanese G, et al. Presence of *Vibrio mediterranei* associated to major
939 mortality in stabled individuals of *Pinna nobilis* L. 2020; *Aquaculture* **519**.

940 76. Prado P, Grau A, Catanese G, et al. *Pinna nobilis* in suboptimal environments are more tolerant
941 to disease but more vulnerable to severe weather phenomena. 2021; *Mar. Environ. Res.* **163**.
942 :105220.

943 77. Pujalte MJ, Garay E. Proposal of *Vibrio mediterranei* sp. nov.: A new marine member of the genus
944 Vibrio. 1986; *Intern. J System. Bacteriol*. **36**(2):278–281.

945 78. Rodríguez S, Balboa S, Barja J. Search for an assay model for marine potential microbial
946 pathogens: the case of Pinna nobilis. In: *VI International Symposium on Marine Sciences (ISMS*
947 *2018)*. 2018:

948 79. Rodríguez S, Balboa S, Olveira G, et al. First report of mass mortalities in natural population of
949 *Pinna nobilis*. A microbial perspective. In: *7th Congress of European Microbiologists (FEMS 2017)*.
950 2017:FEMS7-3233.

951 80. Rosenberg E, Falkovitz L. The *Vibrio shiloi/Oculina patagonica* model system of coral bleaching.
952 Vol. 58, *Annual Review Microbiol.*2004:143–159.

953 81. Rubio-Portillo E, Santos F, Martínez-García M, et al. Structure and temporal dynamics of the
954 bacterial communities associated to microhabitats of the coral *Oculina patagonica*. 2016; *Environ.*
955 *Microbiol*. **18**(12):4564–4578.

82. Šarić T, Župan I, Aceto S, et al. Epidemiology of noble pen shell (*Pinna nobilis* l. 1758) mass mortality events in adriatic sea is characterised with rapid spreading and acute disease progression. 2020; *Pathogens* **9**(10):1–21.

83. Scarpa F, Sanna D, Azzena I, et al. Multiple non-species-specific pathogens possibly triggered the mass mortality in *Pinna nobilis*. 2020; *Life* **10**(10):1–18.

84. Selvarangan R, Wu WK, Nguyen TT, et al. Characterization of a Novel Group of Mycobacteria and Proposal of *Mycobacterium sherrisii* sp. nov. 2004; *J Clin Microbiol*.**42**(1):52.

85. Serrano W, Tarazona UI, Olaechea RM, Friedrich MW. Draft genome sequence of a new Vibrio strain with the potential to produce bacteriocin-like inhibitory substances, isolated from the gut microflora of scallop (*Argopecten purpuratus*). 2018; *Genome Announc*.**6**(20).

Shield J.D. Collection techniques for the analyses of pathogens in crustaceans Journal of Crustacean Biology 2017, 37(6), 753–763,. doi:10.1093/jcbiol/rux077 2017

86. Simide R, Couvray S, Vicente N. Présence de *Pinna nobilis* (L.1758) dans l'étang littoral de Diana (Corse). 2019: *marinelife-revue.fr* –1–4.

87. Simmons SO, Fan CY, Ramabhadran R. Cellular stress response pathway system as a sentinel ensemble in toxicological screening. 2009; *Toxicol. Scien*. **111**(2):202–225.

88. Song L, Wang L, Qiu L, Zhang H. Invertebrate immunity. In: *Invertebrate Immunity*. Austin: Springer Science+Business Media 2010:44–65.

89. Sureda A, Natalotto A, Álvarez E, Deudero S. Increased antioxidant response and capability to produce ROS in hemocytes of *Pinna nobilis* L. exposed to anthropogenic activity. 2013; *Environ. Pollut.* **181**:321–324.

90. Tarazona E, Lucena T, Arahal DR, Macián MC, Ruvira MA, Pujalte MJ. Multilocus sequence analysis of putative *Vibrio mediterranei* strains and description of *Vibrio thalassae* sp. nov. 2014; *Syst. Appl. Microbiol.* **37**(5):320–328.

91. Torres M, Reina JC, Fuentes-Monteverde JC, et al. AHL-lactonase expression in three marine emerging pathogenic Vibrio spp. reduces virulence and mortality in brine shrimp (*Artemia salina*) and Manila clam (*Venerupis philippinarum*). 2018; *Plos One* **13**(4):e0195176.

92. Utari HB, Senapin S, Jaengsanong C, Flegel TW, Kruatrachue M. A haplosporidian parasite associated with high mortality and slow growth in *Penaeus (Litopenaeus) vannamei* cultured in Indonesia. 2012; *Aquaculture* **366–367**:85–89.

93. Vaumourin E, Vourc'h G, Gasqui P, Vayssier-Taussat M. The importance of multiparasitism: examining the consequences of co-infections for human and animal health. 2015; *Parasit Vectors* **8**(1):1–13.

94. Villalba A., Mourella S.G., Lopez M.C., Carballal M.J. & Azevedo C., Marteiliasis affecting cultured mussels *Mytilus galloprovincialis* of Galicia (NW. Spain). I. Etiology, phases of the infection, and temporal and spatial variability in prevalence. 1993; Dis. Aquat. Org., 16, 61–7295. Vittecoq M., Thomas F., Jourdain E., Moutou F , Renaud F. and Gauthier- M. Clerc Risks of Emerging Infectious Diseases: Evolving Threats in a Changing Area, the Mediterranean Basin 2014 Transbound Emerg Dis, Feb;61(1):17-27. doi: 10.1111/tbed.12012.

96. de Vico Gionata, Carella Francesca. *Elementi di Patologia Comparata dei Molluschi*. Paolo Loffredo 2016.

97. Waller DL, Cope WG. The Status of Mussel Health Assessment and a Path Forward2019; *BioONE* **22**(2):26.

98. Zaroogian G, Yevich P. Cytology and biochemistry of brown cells in *Crassostrea virginica* collected at clean and contaminated stations. 1993; *Environ.l Pollution*. **79**(2):191–197.

99. Zotou M, Gkrantounis P, Karadimou E, et al. *Pinna nobilis* in the Greek seas (NE Mediterranean): On the brink of extinction? 2020; *Medit. Marine Sci.* **21**(3):558–574.

98. Aquatic Code Online Access - OIE - World Organisation for Animal Health.

99. Role of Veterinary Services - OIE - World Organisation for Animal Health.