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First detection of the invasive protozoan *Haplosporidium pinnae* in the critically endangered bivalve *Pinna nobilis* in the Southern Mediterranean Sea (Bizerte Lagoon, Tunis) and update of its current status

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Abstract

Pinna nobilis (Linnaeus, 1758) populations have been severely damaged in the last few decades, and since early autumn 2016, a mass mortality event (MME) has drastically impacted populations in the Mediterranean Sea. Accordingly, the aim of the present study was to improve the knowledge on the status of *P. nobilis* populations in the Bizerte Lagoon (Tunisia) between 2016 and 2022. Before the MME, *P. nobilis* was found in the lagoon at depths from 1.5 to 6 m, with a density ranging from 2 to 30 ind/100 m². After the MME, mortality reached 100% in the monitored area, except in the eastern part of the Bizerte Lagoon near the Menzel Jemil site, where some living specimens were detected. Moreover, in 2022, successful recruitment was observed in the lagoon. Additionally, our results revealed the presence of *Haplosporidium pinnae* and *Mycobacterium* sp. in some living specimens sampled in the Bizerte Lagoon after the MME. This finding constitutes the first detection of both pathogens in Tunisia and in the entire Southern Mediterranean Sea.

Keywords: Pinnidae; pen shell; mass mortality; *Haplosporidium pinnae*; Bizerte Lagoon; Tunisia.

Introduction

The pen shell or fan mussel *Pinna nobilis* (Linnaeus, 1758) is an emblematic marine bivalve endemic to the Mediterranean Sea. It is the largest bivalve in this area and one of the largest in the world, reaching up to 120 cm in length (Zavodnik et *al.*, 1991). It can live more than 50 years (Rouanet *et al.*, 2015). It occurs at depths of 0.5–60 m (Butler *et al.*, 1993) on soft or sandy sea bottoms covered with seagrass meadows of *Zostera*, *Cymodocea* and especially *Posidonia oceanica* (Vicente & Moreteau, 1991). *P. nobilis* populations have greatly declined in the past (Vicente & Moreteau, 1991) for many reasons: reckless fishing, incidental killing by trawling and anchoring, poaching, degradation of eggs, and regression of its common habitat (Katsanevakis & Thessalou-Legaki, 2009). It has become a rare species over the last few decades and is therefore subject to enhanced protection, and its preservation is urged mainly through Annex II of the Barcelona Convention (SPA/BD Protocol 1995) and Annex IV of the EU Habitats Directive (European Council Directive 92/43/EEC).

Since 2016, a mass mortality event (MME) caused by a parasite has led to a sharp decline in *P. nobilis* populations throughout the Mediterranean Sea (Vázquez-Luis *et al.*, 2017a; Katsanevakis *et al.*, 2019; Panarese *et al.*, 2019; Čižmek *et al.*, 2020; Zotou *et al.*, 2020; Katsanevakis *et al.*, 2021). This MME caused concern and a status change, and *P. nobilis* was included in the IUCN Red List as "Critically Endangered" (Kersting *et al.*, 2019). The term MME was first used for *P. nobilis* in 2017 in Spain (Darriba, 2017). Then, several studies were performed to track mass mortality in several countries (e.g., Spain, Italy, France, Greece, Portugal) (García-March *et al.*, 2020). The main causative agent of the mass mortality of *P. nobilis* is *Haplosporidium pinnae* (Grau *et al.*, 2022).

Coastal lagoons are shallow water bodies separated from the sea or the ocean by sedimentary barriers and connected through inlets (Kjerfve, 1994). These areas are some of the most used and valuable ecosystems on Earth, providing a wide range of ecosystem goods and services (Newton *et al*., 2018). In addition, coastal lagoons across the Mediterranean Sea are key conservation areas for *P. nobilis*; in the present context, some of them act as putative sanctuaries that are apparently "pathogen-free" areas (Nebot-Colomer *et al*., 2021; Donato *et al*., 2021; Prado *et al*., 2021; Moro-Martínez *et al*., 2023). Accordingly, the Bizerte Lagoon ecosystem has great importance related to its contribution to safeguarding the environment and biodiversity (Khammassi *et al*., 2019; Khammassi *et al*., 2021) and revenues from fishing and aquaculture (Béjaoui, 2009).

In Tunisia, the distribution and density of *P. nobilis* populations have been estimated in the Kerkennah Islands (Tlig Zouari, 1993), Ghar El Melh Lagoon (Zakhama-Sraieb *et al*., 2011), and Northeastern Tunisian coasts (Rabaoui *et al*., 2007; Rabaoui *et al*., 2010). However, there is no updated study of the status and eco-biology of *P. nobilis* in the Bizerte Lagoon (Northern Tunisia). Moreover, histological and molecular analyses of sampled fan mussels, to confirm the presence or absence of the protozoan *H. pinnae* have not yet been performed.

To fill this gap, the present study aims to: 1) determine the *P. nobilis* population status in the Bizerte Lagoon based on surveys conducted before and after the MME to provide reliable knowledge on the trends, actual situation and current conservation status of the species; and 2) monitor the presence of *H. pinnae* and *Mycobacterium* sp. pathogens in *P. nobilis* populations from the Bizerte Lagoon.

Materials and Methods

Study area

The Bizerte Lagoon (37.14 372°N, 9.78 994°E) is an area of considerable biogeographical interest due to its location between the Mediterranean Sea and Ichkeul Lake. It is situated in Northern Tunisia and covers an area of 150 km^2 with an average depth of 11 m (Fig. 1). The lagoon receives salt water from the Mediterranean Sea through a transition channel and fresh water from Ichkeul Lake and 18 other sources, with the principal tributaries being the Tinja, Garek, Mrezig, Gueniche and Ben Hassine Rivers (Triki *et al.,* 2014).

The major cities bordering the lagoon are Menzel Bourguiba (which has a naval port and a metal factory), Menzel Abderrahman and Menzel Jemil (which dump urban and industrial residues into the lagoon). All of these zones discharge various types of pollutants in significant quantities (Yoshida *et al*., 2002; Ben Salem *et al*., 2017; Alves Martins *et al*., 2016; Wakkaf *et al*., 2020; El Zrelli *et al.,* 2021). The localities around the Bizerte Lagoon described in this work were Canal (CA), Echaara (Ech), Menzel Abdrahmen (MA), Menzel Jemil (MJ), Jwawda (JW), Menzel Bourghuiba (MB) and Faroua (Fr) (Fig. 1).

Sampling

A field survey was conducted in the Bizerte Lagoon in early spring 2016 when the density and growth of vegetation in the lagoon was minimal, allowing the detection

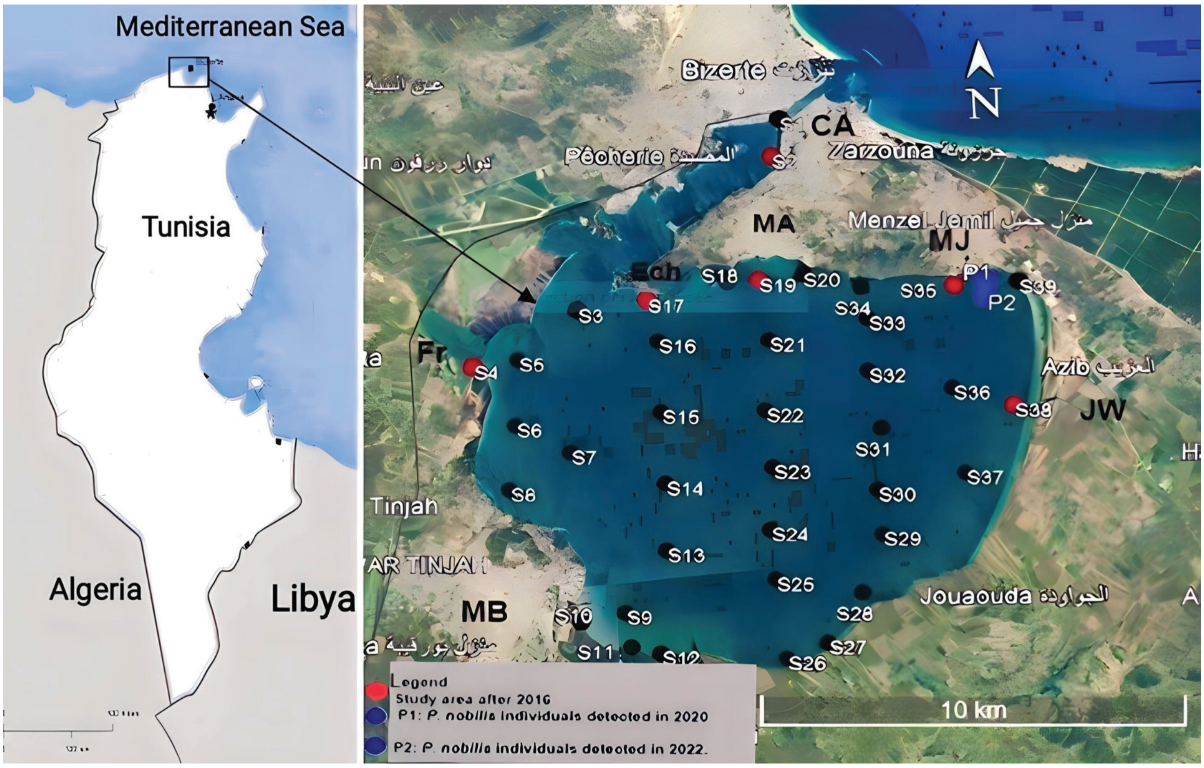

Fig. 1: Geographic situation of Bizerte Lagoon with sampling sites in 2016 and 2022. Black dots: sampling sites in 2016; red dots: monitoring sites from 2016 repeated in 2022. (MA: Menzel Abdrahmen; MJ: Menzel Jemil; JW: Jwawda; MB: Menzel Bourghuiba; Fr: Faroua); blue dots: sites that we found *P. nobilis* in 2020 and 2022.

of even small individuals of *P. nobilis*. A total of 39 sites were studied (Fig. 1), which were selected according to their bathymetry and proximity to anthropized areas. The method adopted to evaluate the density of individuals consisted of counting the number of living *P. nobilis* in 100-m^{2}quadrant surfaces subdivided into four equal sectors.

To monitor the status of *P. nobilis* in the Bizerte Lagoon, after 2016, a total of six sites were investigated through underwater visual surveys in 2018, 2020, and 2022 (S2, S4, S17, S19, S35 and S38; Fig. 1). The sites were chosen according to existing information on the presence of *P. nobilis* populations. Moreover, local ecological knowledge (LEK) was also recorded through interviews with fishers and scuba divers to collect as much information as possible on the status of pen shells in the lagoon and associated changes between 2018 and 2022. Based on these interviews, two additional sites were investigated: P1 and P2 (Fig. 1). At each site, a randomly placed quadrant with 100 m^{2}of surface area was established, and the number of live *P. nobilis* was counted.

Data collection and statistical analysis

At each sampling station, two samples of surface sediment (to 5 cm depth) were collected using a 0.1-m² Van Veen grab sampler. The sediment samples were conserved for grain size analysis. Simultaneously, the water depth was recorded with sonar,and the position of each station was recorded with a GPS device.

The granulometric analyses consisted of placing 100 g of sediment samples in a glass container and drying them in an oven at 70 °C for 48 hours. Grain size was determined by dry-screening of the coarse fraction for 20 minutes in AFNOR sieves with meshes varying from 4000 µm to 45 µm in diameter (Buchana, 1984). Additionally, water temperature and salinity were measured using a thermometer (WTW LF 196, Suntex, Weilheim, Germany) and a salinometer (WTW Cond 315i), respectively.

Principal component analysis (PCA) was performed using XLSTAT version 1.6 to evaluate the influence of different parameters (depth and granulometry) on *P. nobilis* density in 2016.

Cartography

GIS mapping was applied as a basic analysis tool for the assessment of the spatial distribution of *P. nobilis* densities in the Bizerte Lagoon. Maps were prepared using inverse distance weighted (IDW) interpolation using ArcGIS software (version 10.4.1), which is a local deterministic interpolation technique that calculates the value as a weighted average based on the distance of the sampled points in a defined neighbourhood (Burrough, 1986). The database structure of the GIS system included the geographic coordinates of each location as well as the *P. nobilis* density at each station in spring 2016.

Collection of biological materials

To explore the presence of pathogens in the lagoon, and because *P. nobilis* is a strictly protected species (Annex II of Barcelona Convention and Annex IV of the European Council Directive 92/43/EEC), sample collection was carried out with the permission of regional authorities (APAL). The goal was to detect the aetiological agent of the MME both by histological and molecular analyses. For this reason, only 4 individuals were sacrificed and collected from the Menzel Jemil area (P1 and P2; Fig. 1) by scuba diving at depths between 1.5 and 2.5 m. Asymptomatic specimens were processed directly after sampling. One individual was collected in January 2021 and 3 were collected in May 2022. Tissue samples of two *P. nobilis* individuals (one sampled in January 2021 and one sampled in May 2022) were collected from the digestive gland, mantle, gills and muscle and preserved in Bouin's fixative for histological analysis, while mantle samples from the 3 specimens sampled in May 2022 were preserved in absolute ethanol for molecular analysis.

Histological analysis

After fixation, the samples collected from the two individuals for histological analysis were dehydrated in an increasing ethanol gradient, cleared with X-free ethanol (Bio-Optica), embedded in paraffin (Paraplast Plus, Kendall), sectioned at 3-4 µm, and stained with Mayer's haematoxylin and eosin (MHE) for routine light microscopy examination and with Mayer's haematoxylin-VOF (Gutiérrez, 1967) to detect sporulation stages of *H. pinnae* (Catanese *et al.*, 2018). Some additional sections were stained with the Brown and Brenn gram and Ziehl-Neelsen (ZN) staining procedures to facilitate the detection of bacteria and acid-fast bacteria (Luna, 1968), respectively.

Molecular analysis

The samples of mantle tissue dissected from the 3 pen shells collected in 2022 and stored in absolute ethanol were used for DNA extraction. Total genomic DNA was purified using the DNA NucleoSpin® Tissue extraction kit (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions. The quality and concentration of the DNA were measured using a Nanodrop ND1000 (Thermo Scientific, Waltham, MA, USA). To detect the presence of *H. pinnae*, fragments of the small subunit ribosomal DNA (SSUrDNA) gene were amplified using the primer pairs HPNF3/HPNR3 (Catanese *et al.*, 2018) and HpF3/HpR3 (López-Sanmartín *et al.*, 2019). For *Mycobacterium* spp., we used the specific primers mycgen-f/mycgen-r described by Böddinghaus *et al.* (1990) and the PCR conditions indicated by Carella *et al.* (2019) to detect its presence in *P. nobilis*. All PCRs were performed in a total volume of 20 µL containing 10 µL of Kapa Taq Ready mix (Sigma–Aldrich, Burlington, MA, USA), 8.2 µL of sterile water, 0.4 µL of each primer (stock 20 Mmol) and 1 µL of DNA at 150 ng/µL. The PCR products were separated on 1.5% agarose in TAE 1× buffer gels (w/v), stained with Gel Red® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) including a Low Ranger 100-bp DNA ladder size standard (Norgen, Thorold, Canada) and visualized on a UV transilluminator. The amplicon bands were cut from the agarose gel, purified using a mi-Gel Extraction Kit (Metabion International, Planegg, Germany) and bidirectionally sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, United States). The obtained sequences were analysed with Peak Scanner™ software v1.0 (Life Technologies), edited using BioEdit software and compared for similarity with GenBank sequences using the BLAST algorithm at the NCBI server for species identification.

Results

P. nobilis *distribution in the Bizerte Lagoon in different years*

In 2016, *P. nobilis* was rare and only detected in 17 of the 39 sites between 1.5 and 6.1 m depth. It was present in all littoral areas of the Bizerte Lagoon, except for the sites close to Menzel Bourguiba in the southwest, and it was most widespread in shallow water in the northeast and northwest of the lagoon (Fig. 2) on coarse sand, fine sand, or muddy sand. The mean density of living individuals for all 39 sites was 4.6±7.32 ind/100 m². The density values varied between 2 ind/100 m² (S20) and 30 ind/100 m² (S4). According to the Kruskal–Wallis test, significant differences in population density were observed among subareas (N=39, p<0.0001): the highest densities were recorded in northwest and east areas characterized by sandy and muddy sands, shallow depths, and low hydrodynamic stress.

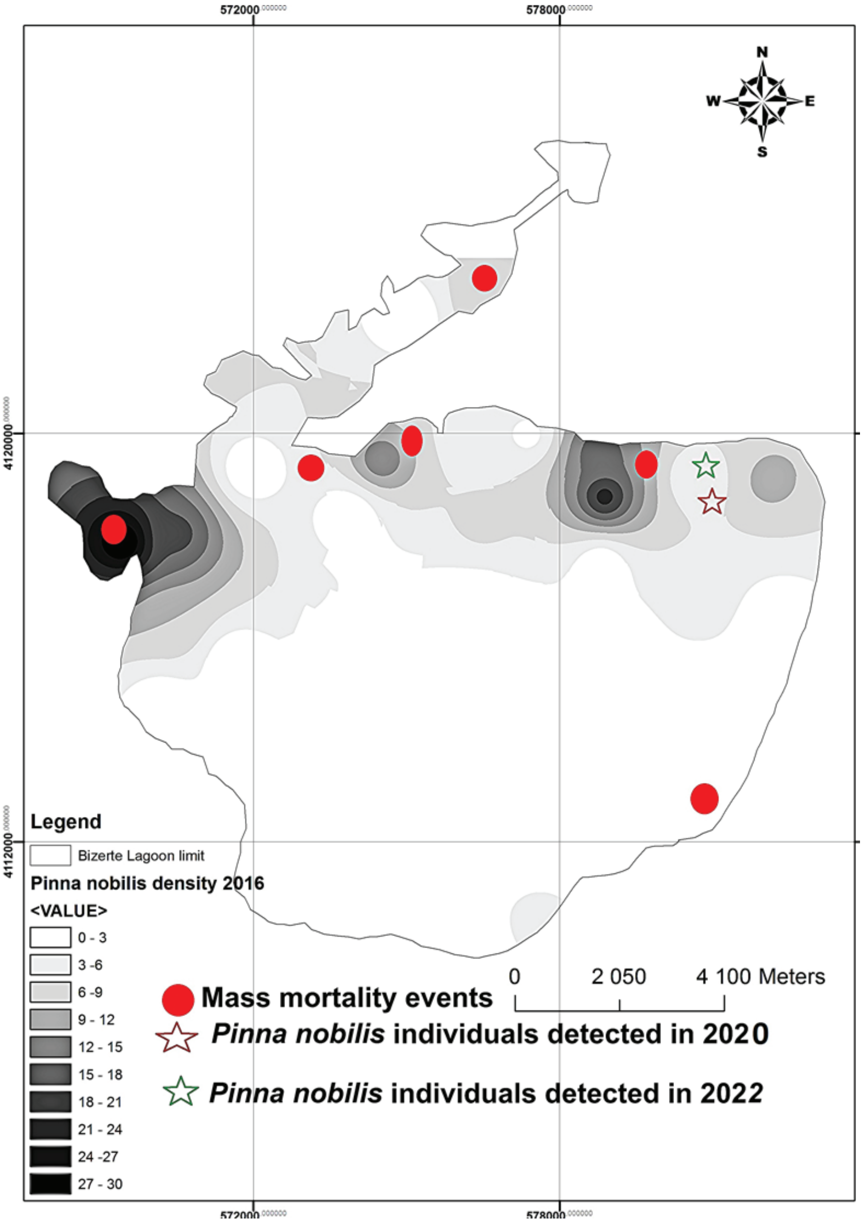

Fig. 2: Distribution of *Pinna nobilis* density in Bizerte Lagoon in 2016 and sites with mass mortality events (MME) recorded in 2018.

By the beginning of 2018, a great mortality of individuals of *P. nobilis* was detected across several locations along the Bizerte Lagoon (Fig. 2). The MME of the pen shell was first reported in the northern and northwestern parts of the lagoon in March 2018. A few months later (June 2018), the MME was detected in the west and southwest of the lagoon. Affected specimens were size/age-independent (juveniles and adults) and found across all environmental conditions (depth, granulometry, habitat type). Moreover, sick pen shells were observed and presented gross signs of gaping, slow closing and loss of valve force and mantle recession. In 2019, mortality affected almost all the populations, since 100% of dead individuals were detected in monitoring sites.

In 2020, two years after the MME observed in the lagoon, the *P. nobilis* status in the Bizerte Lagoon was studied again. No recruitment was observed after the MME in S2, S4, S17, S19, S35 and S38. However, during the survey, some live individuals were found in the eastern part of the lagoon (P1) in a new ecosystem incorporating sebkha and sand islets, with density values of 2 ind/100 m² (Fig. 2).

In 2022, four years after the MME, *P. nobilis* was detected near 1 of the 6 controlled sites (S35). It was found in the eastern part of the lagoon (P2) close to the Menzel Jemil area at 2.5 m depth, on the bottom covered with seagrass meadows of *Zostera noltii* and *Cymodocea nodosa*. The density of living individuals was 22 ind/100 m². The monitoring of the recruitment potential of *P. nobilis* by in situ visual surveys in S2, S4, S17, S19, S35 and S38 after the mortality outbreak revealed that local recruitment stopped.

Grain size distribution

Grain size analysis revealed that 38.46% of the sites were characterized by sandy sediments, 30.76% were muddy, 23.07% were muddy sand and 7.69% were sandy silt. The composition of the Bizerte Lagoon sediment can be determined according to the relative percentages of the coarse (>63 µm) and fine (<63 µm) fractions. The mean content of the fine fraction (FF) was 58.51±28.37%. Sandy facies (20% <FF <40%) characterized the north and south of the lagoon (Menzel Abderrahman, Menzel Jemil and Menzel Bourguiba). In the central region, there was a sandy band rich in fine fraction (40% <FF <50%). On the eastern side of the lagoon, near the Tinja Channel, sediments were also fine (50% <FF <60%). However, the central sector was very muddy, with the fine fraction exceeding 90%.

Principal component analysis (PCA)

The PCA performed with depth, granulometry and *P. nobilis* density (Fig. 3) revealed that the first two axes (F1 = 72.38%, F2 = 20.27%) accounted for 92.65% of the total percentage of cumulative variance expressed. The first axis (F1) was positively loaded by depth and granulometry and negatively loaded by *P. nobilis* density. Negative associations were observed between *P. nobilis* density and depth and granulometry.

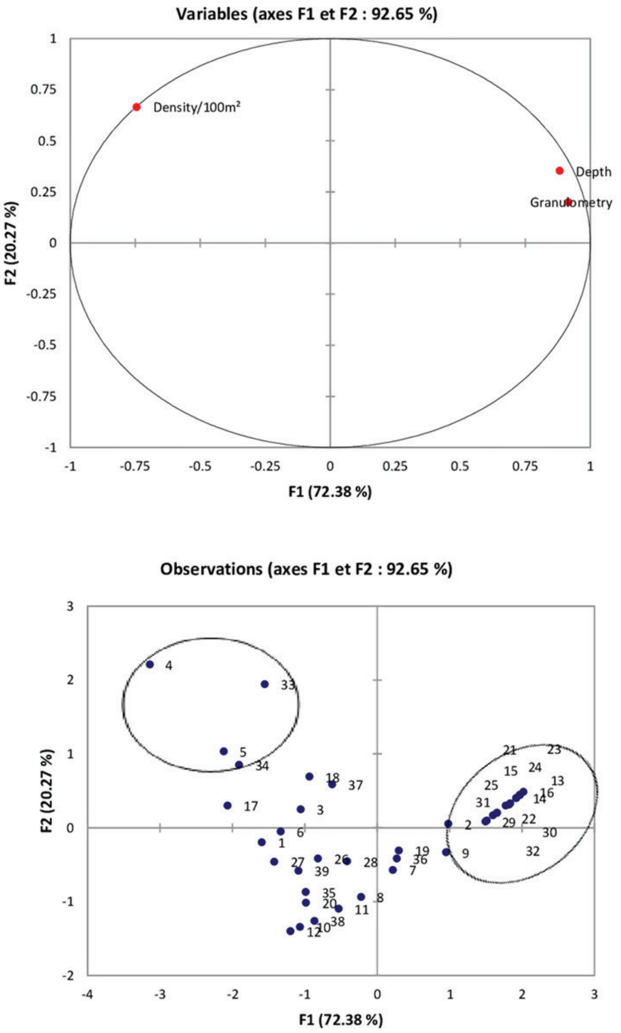

Fig. 3: Results of the Principal Component Analysis (PCA) conducted on data collected in Bizerte lagoon in spring 2016.

The samples from the different locations scored differently on the principal components (Fig. 3). According to the F1 axis, sampling sites S4, S5, S34 and S33 had high *P. nobilis* densities and were clearly separated from sites S13, S14, S15, S16, S21, S22, S23, S24, S25, S30, S31 and S32, which represented the deepest area with fine fractions.

Histological analysis

The two individuals collected in 2021 and 2022 and analysed showed a systemic infection of uninucleate cells resembling *H. pinnae* detected in the connective tissue throughout the visceral mass, gills and mantle as well as in the gut epithelium and haemolymph sinuses of both specimens (Fig. 4A,B). No sporulation stages were detected in the digestive gland epithelium. A heavy haemocytic inflammatory host response was associated with infection (Fig. 4A).

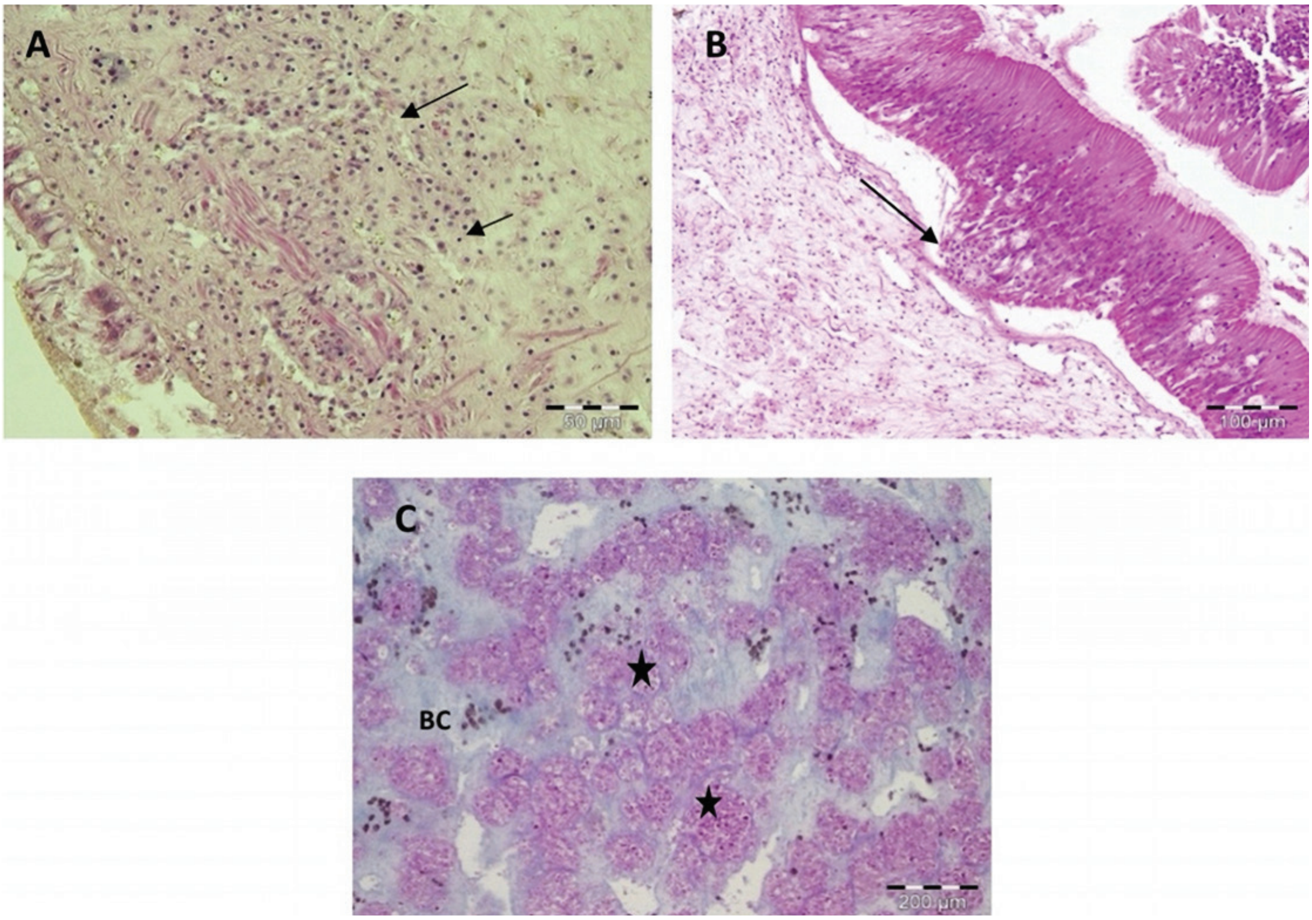

Fig. 4: Light micrographs of histological sections through the visceral mass and mantle of fan mussels *Pinna nobilis* from Bizerte Lagoon. A)Low magnification micrograph of the mantle showing numerous uninucleate cells of *H. pinnae* together with an haemocytic inflammatory infiltration host response (arrows, MHE); B) Section through the stomach showing numerous uninucleate cells located at the base of the host stomach epithelium (arrows, MHE), C) Section through the connective tissue surrounding stomach showing multiple nodular aggregations (stars) of haemocytes filled with mycobacteria (purple deposits) in aggregation with brown cells (ZN). BC, Brown cells.

In addition to the presence of *H. pinnae*, 1 of the 2 sampled individuals (the one from 2021) was positive for the presence of acid-fast bacteria (*Mycobacterium* sp.) by ZN staining (Fig. 4C). Mycobacteria-filled haemocytes located in nodular aggregates coupled with brown cells (Fig. 4C) were observed in the connective tissue surrounding the visceral mass, anterior stomach and gonadal follicles but also in the connective tissue of the mantle. No other bacteria were detected in the 2 studied samples. However, abundant ciliated opportunistic protozoa were observed attached to the gill in 1 individual collected in May 2022.

Molecular analysis

The results of the molecular analysis evidenced the presence of *H. pinnae* in 2 of the 3 individuals collected in 2022, while *Mycobacterium* sp. was detected in only 1 of the 3 sampled individuals. The third sample did not show any PCR amplification using the described primers. The PCR produced amplicons of the expected size for each of the two pathogens. All the sequences obtained from the PCR amplifications were subjected to BLAST analysis. The sequences of the positive individuals for *H. pinnae* reported 100% identity with the GenBank sequence with accession number LC338065. The sequence from the individual positive for *Mycobacterium* sp. was very closely related (100%) to that already described in *P. nobilis* (MH569645).

Discussion

The current study provides an updated report of the status of the *P. nobilis* population in the Bizerte Lagoon (2016-2022) (Fig. 5). We also describe the presence of a haplosporidan parasite in the Bizerte Lagoon, detected for the first time on Tunisian coasts and along the entire southern Mediterranean coast. Specifically, the presence of *H. pinnae* was confirmed by histopathological and molecular analyses.

The survey of the *P. nobilis* status in the Bizerte Lagoon before the MME in 2016 revealed a mean *P. nobilis* density for all 39 lagoon sites of 4.6±7.32 ind/100 m². The mean was calculated from all sites (including areas where *P. nobilis* was absent) since density could otherwise be overestimated (Coppa *et al.*, 2010; Galinou-Mit-

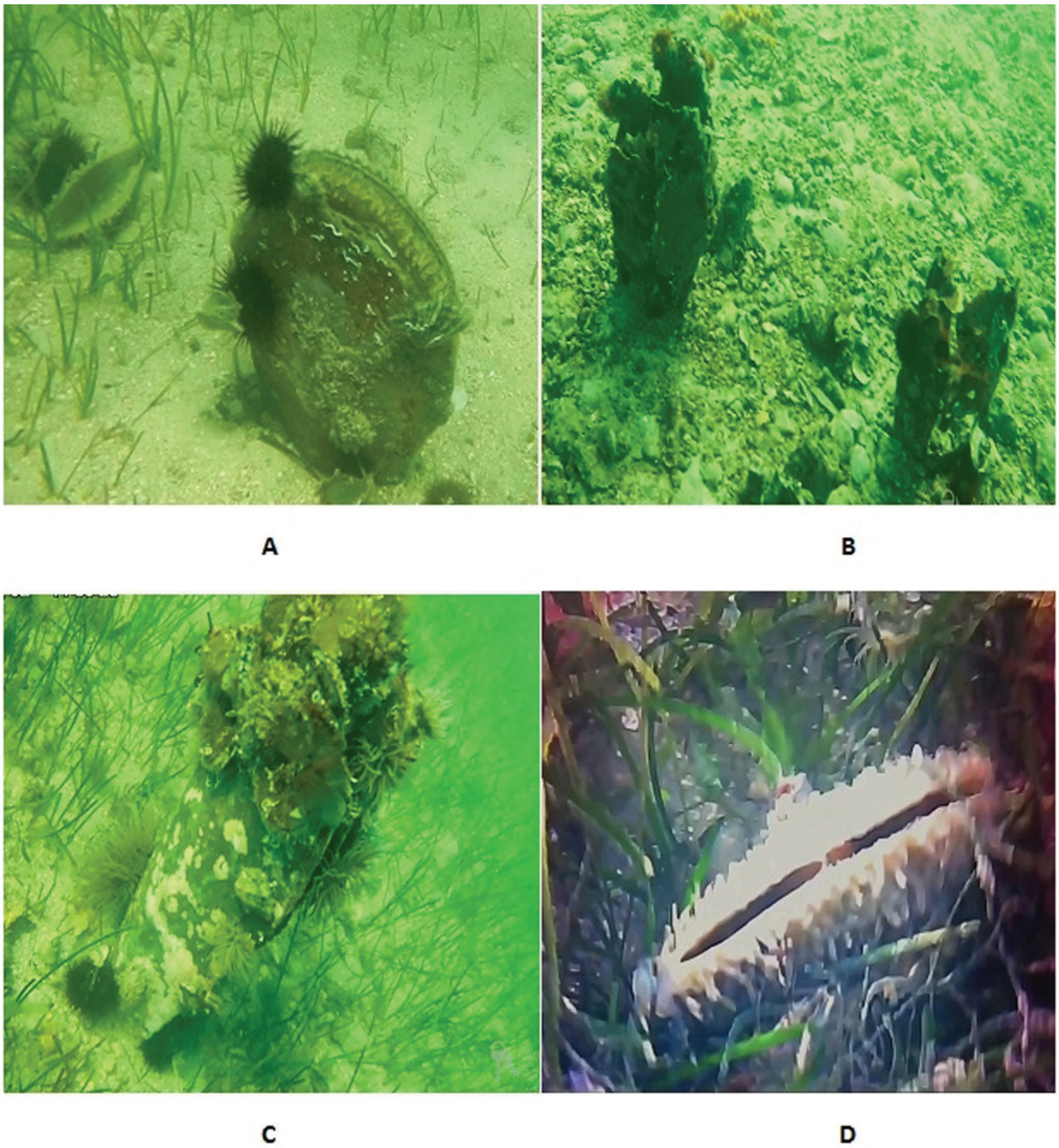

Fig. 5: Illustrations of the *P. nobilis*, observed during the 6 years of studies in Bizerte Lagoon (A: *P. nobilis* populations 2016; B: dead *P. nobilis* in 2018; C: alive *P. nobilis* in 2020, D: alive *P. nobilis* in 2022).

soudi *et al.*, 2006; the highest reported until now in the Mediterranean Sea. Mean length was 34.35 ± 11.78 cm and maximum length (L max Centoducati *et al.*, 2007). The mean density found was higher than that in most other studies conducted in other Mediterranean regions. In fact, the mean density of *P. nobilis* seems not to generally exceed 4 ind/100 m^2 in many areas, such as 0.45 ind/100 m^2 in Crete (Greece; Katsanevakis & Thessalou-Legaki, 2009), 0.97 ind/100 m^2 in the Gulf of Oristano (Italy; Coppa *et al.*, 2010), 2.5 ind/100 m^2 in the Northern and Eastern Tunisian Coasts (Ben Hassine & Rabaoui, 2008) and 3.79 ind/100 m^2 in the Western Mediterranean Sea (Vázquez-Luis *et al.*, 2017 b). However, the mean density of *P. nobilis* was reported as much higher in other studies, varying between 11.5 ind/100 m^2 in the Adriatic Sea (Siletic & Peharda, 2003), 70.83 ind/100 m^2 in the Northwestern Mediterranean Sea (France; Peyran, 2021a) and 104 ind/100 m^2 in the North Aegean Sea (Galinou-Mitsoudi *et al.*, 2006)the highest reported until now in the Mediterranean Sea. Mean length was 34.35 ± 11.78 cm and maximum length (L max . The variation in density among the Mediterranean ecosystems maybe attributed to both natural and human stressors. Among the natural factors, depth (Katsanevakis, 2004)Greece, hydrodynamics (Katsanevakis & Thessalou-Legaki, 2009), substratum, and trophic state (Vafidis *et al.*, 2014)it is under strict protection as a dramatic decline in its populations has been recognized, assigned to the combined effect of fisheries and habitat degradation. This study attempts to evaluate, by non-destructive sampling, the current status of P. nobilis populations in the south Aegean Sea (Dodecanese island complex have been found to influence *P. nobilis*, and anthropogenic factors, such as fishing pressure, anchoring (Vázquez-Luis *et al.*, 2015; Deudero *et al.*, 2015; Galinou-Mitsoudi *et al.*, 2006)the highest reported until now in the Mediterranean Sea. Mean length was 34.35 ± 11.78 cm and maximum length (L max and pollution (Centoducati *et al.*, 2007; Basso *et al.*, 2015) seem to be the most important. However, general comparisons of density must be done with caution, since different sampling strategies can produce different density results.

In the Bizerte Lagoon, the PCA results showed that bathymetry and granulometry were the factors that seem to influence the distribution of the protected species. A significant negative association was observed between *P. nobilis* density and bathymetry. Fan mussels were recorded from 1.5 to 6.1 m depths with a progressive decrease in density with increasing depth. Similar results were also obtained in Cabrera National Park, Balearic Islands (Spain) and the Western Mediterranean, where the density peaked at 9 m (population surveyed from 4.2 to 46 m depth (Vázquez-Luis *et al.*, 2014)). The decrease in density with increasing depth maybe correlated with the reduction in light, low temperatures in deep areas and reduction in food supply (García-March *et al.*, 2007). In the Maliakos Gulf, a small effect of depth was found, but it is important to consider that sampling was carried out only to a maximum depth of 2 m (Theodorou *et al.*, 2017).

Moreover, in Bizerte Lagoon, *P. nobilis* density and granulometry were also negatively associated, and *P. nobilis* was completely absent in muddy substrate. In fact, according to Katsanevakis (2004) Greece and García-March *et al.* (2008) nutrition, responses to environmental stimuli and follows rhythmic cycles in many species. Although the alternation of Sun and Moon is the major entraining agent for the daily living clock-driven rhythms, cyclic extrinsic environmental factors can also modulate gaping activity. Therefore, laboratory experimental conditions can alter the natural behaviour of bivalves, hindering the interpretation of observations. Many features of Pinna nobilis physiology are poorly known, gaping activity not being an exception. To reduce the knowledge gap on this regard, we performed a study on the species gaping activity. The research was designed to be carried out in situ, in order to avoid the interference of laboratory conditions in the observations. To this end, we designed and fabricated a new electronic system composed by a data logger and a sensor formed by multiple reed switches activated by a single magnet. The system is autonomous and can record gaping activity of subtidal bivalves in potentially any type of subtidal envi- ronment. Furthermore, it requires little framework for the installation. With this system, we monitored 10 individuals in periods ranging between 3 and 21 days, for up to a total of 98 days. The records have shown that far from being all day open, as previously suggested, *P. nobilis* follows marked circadian and circalunar rhythms of gaping Communicated by J.-M. Gili. J. R. García-March, the important percentage of fine fraction may damage the cilia of fan mussels, reducing their respiration and feeding activities.

The results of the present study after 2016 indicate that the MME affected the *P. nobilis* populations across a large area of the Bizerte Lagoon and removed 100% of the pen shell population in most cases. Mass mortalities of *P. nobilis* started to be detected along Mediterranean coasts in 2016 (Darriba, 2017; Vázquez-Luis *et al.*, 2017a). Thereafter, approximately 90% of the Spanish fan mussel populations were lost, and mortality rates reached 100% on the south and central Mediterranean coasts of the Iberian Peninsula and the Balearic Islands (Vázquez-Luis *et al.*, 2017b; García-March *et al.*, 2020). Later, important mortality of *P. nobilis* populations was also observed on the Italian and French coasts (Catanese *et al.*, 2018) and in the Çanakkale Strait, Turkey (Acarlı *et al.*, 2021). As result of this MME, *P. nobilis* has been classified as "critically endangered" (IUCN, 2019).

At the beginning of these mortalities, several studies indicated that they were caused by the parasite *H. pinnae* (Catanese *et al.*, 2018; Panarese *et al.*, 2019; Tiscar *et al.*, 2022). However, some studies emphasize that deaths in individuals occur due to co-infection of protozoa with bacteria such *Mycobacterium* sp. (Carella *et al.*, 2019; Carella *et al.*, 2020; Lattos *et al.*, 2020) or *V. mediterranei* (Prado *et al.*, 2020; Andree *et al.*, 2021). Our results confirmed the presence of *H. pinnae* and highlighted the presence of encapsulated mycobacteria in the connective tissue surrounding the gonads and intestine. In this regard, some studies have reported cases of co-infection of the two pathogens (Cizmek *et al.*, 2020; Box *et al.*, 2020), but a more recent study by Grau *et al.* (2022) highlights a preeminent role of *H. pinnae* relative to the other pathological agents, supporting that the onset of the MME was strongly associated with the detection of this pathogen.

In the Bizerte Lagoon, mortalities reached 100% in all the monitored areas, and *P. nobilis* populations disappeared in all the prospected sites (S2, S4, S17, S19, S35 and S38), except near the Menzel Jemil area (P1 and P2) in a neo-ecosystem (Shaiek *et al.*, 2018) that may constitute a natural refuge for the pen shell. In fact, in this area, infection was observed later, while recruitment was successfully achieved. It must be taken into account that the Menzel Jemil area is also characterized by low hydrodynamism (Béjaoui *et al.*, 2005), which could be the reason for the low spread of the disease, since currents have been identified as the main pathway for parasite dispersal (Cabanellas-Reboredo *et al.*, 2019; García-March *et al.*, 2020).

The spatial distribution of the disease, mortality percentage, and pathogenic effect are controlled by several factors, such as environmental stress, including variations in temperature and salinity (Arzul & Carnegie. 2015; Cabanellas-Reboredo *et al*., 2019). A study by Cabanellas-Reboredo *et al.* (2019) strongly suggests that the parasite has probably dispersed regionally by surface currents and that the disease expression seems to be closely related to temperatures above 13.5 °C and to a salinity range between 36.5–39.7 psu. High temperatures during the summer may have lowered the physiological capacity of *P. nobilis* to resist parasite infections due to a high respiratory demand at increased temperatures (Trigos *et al.*, 2015), which is also coincident with the reproductive and post-reproductive periods (Deudero *et al.*, 2017). In the Bizerte Lagoon, temperature and salinity fluctuated between seasons, as described by Afli *et al.* (2008). Salinity patterns along the Bizerte lagoon are influenced by two main influxes: marine water coming from the Mediterranean Sea and entering the lagoon through the Bizerte channel and freshwater coming from Ichkeul Lake, which enters the lagoon via the Tinja River. Therefore, favourable conditions for *H. pinnae* spread and infection can be found during some periods in the Bizerte Lagoon (Figure S1). Moreover, the presence of pollutants could have significant effects on the pathogens themselves, on the host and on the host–pathogen relationship. Hence, they could reduce the physiological capacity of *P. nobilis* to resist parasite infections.

In the context of the pandemic, only some lagoon populations remain unaffected and provide natural refuges compared to all open-water coastal areas in the Mediterranean Sea (Peyran *et al.*, 2021b). The dynamics of haplosporidan parasites are modulated by particular environmental conditions that make parasite proliferation difficult or impeded. Significant differences in the prevalence of *H. pinnae* between individuals living in the open sea, in harbours and in Mediterranean coastal lagoons have already been described (Grau *et al.*, 2022).

However, the conditions in Mediterranean coastal lagoons could become unfavourable, leading to the collapse of the last *P. nobilis* populations. For instance, in the largest coastal lagoon in Spain (Mar Menor), the cumulative impacts produced by intensive agricultural activity around the lagoon, together with a catastrophic meteorological phenomenon that occurred in 2019, have triggered a significant decline in fan mussel population densities and in population mean shell sizes over the years (Nebot-Colomer *et al.*, 2021). Additionally, fluctuations in salinity facilitated the infection of some fan mussels by *H. pinnae*. In Lake Faro (Sicily), the number of living *P. nobilis* specimens has decreased in the last 10 years in this biologically stressful environment, comprising now a low-density relict population lacking external larval supply (Donato *et al.*, 2021). Living individuals remain in the Bizerte Lagoon but some bear a high parasitic load of ciliated protozoa in the gill, as we observed in one analysed sample, hindering respiratory activity in these individuals. Accordingly, the current status of the population should be monitored since every *P. nobilis* aggregation is essential for the conservation of the species in Tunisia but also important for the conservation of the species in the wider Mediterranean Sea. Therefore, urgent management and conservation actions focused on improving the environmental quality and avoiding the extinction of this Mediterranean endemic bivalve are urgently needed.

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Supplementary Data

The following supplementary information is available online for the article:

Fig. S1: Seasonal variations of the main physico-chemical parameters in the Bizerte lagoon. Data collected in 2016/2017: (A) Temperature; (B) Salinity.

Appendix: Table S1. Salinity, temperature and *P. nobilis* density in Bizerte Lagoon in 2016.