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Status, distribution, and threats of the last surviving fan mussel populations in Greece

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Abstract

Since the first confirmed records of mass mortality events (MME) in the Aegean Sea in 2018, *Pinna nobilis* populations in Greek seas have been decimated. To bolster recovery efforts, this study aimed to assess the status of fan mussel populations in the Aegean and Ionian seas and investigate potential recolonization through natural recruitment. From May 2022 to May 2023, 163 independent underwater visual surveys were conducted across various locations and depths along the Greek coastline. A total of 4348 *P. nobilis* individuals was recorded, of which 87.3% were found dead and 12.7% were alive. The sole surviving fan mussel populations were located in the semi-enclosed gulfs of Amvrakikos in the Ionian Sea and of Kalloni in the Aegean Sea, with estimated recent mortality rates (excluding potential poaching) of 7.7% and 6.3%, respectively. To track potential new recruitment, a network of larvae collectors was deployed in multiple locations. Additionally, an ocean circulation model (OCM) was developed to predict the export and fate of larvae from the surviving populations in the Marmara Sea towards the Aegean Sea. Beyond the MME, this study identified several other threats, which significantly endanger fan mussel survival. The findings of this study underscore the urgent need to implement protection measures and restoration actions to enhance the chances of *P. nobilis* survival and recovery in the Greek seas.

Keywords: *Pinna nobilis*; mass mortality; conservation; Aegean; Ionian; Mediterranean.

Introduction

The fan mussel, *Pinna nobilis* (Linnaeus, 1758), is a large bivalve species endemic to the Mediterranean Sea and one of the world's largest bivalves. It can grow up to 120 cm in length (Zavodnik *et al.,* 1991) and has a maximum reported lifespan of 45 years (Rouanet *et al*., 2015). *Pinna nobilis* is a successive hermaphrodite with asynchronous gamete maturation (De Gaulejac, 1995; De Gaulejac *et al*., 1995; Deudero *et al*., 2017), attaining sexual maturity by two years of age (Richardson *et al*., 1999; Richardson *et al*., 2004). Despite its ecological significance, *P. nobilis* larval phase and dispersal potential remain poorly understood (Kersting & Garcia-March, 2017; Trigos *et al.,* 2018; Kersting *et al.,* 2020). The species primarily inhabits soft-bottom areas within seagrass meadows (Prado *et al.,* 2014; Kersting & García-March, 2017), but it can also be found in other substrates, such as unvegetated sandy areas, boulders, detritus beds, and marl beds (Katsanevakis, 2006; Basso *et al*., 2015; Kersting & Garcia-March, 2017; Tsatiris *et al*., 2018). *Pinna nobilis* plays a pivotal ecological role, actively filtering large amounts of detritus from the water and contributing to water clarity (Trigos *et al.,* 2014). Moreover, it provides a hard surface for other benthic species to colonize, thereby enhancing habitat diversity (Giacobbe, 2002; Rabaoui *et al.,* 2009).

Pinna nobilis populations have declined significantly due to various anthropogenic and environmental threats. Habitat degradation, illegal trawling, coastal construction, boat anchoring, illegal extraction, and pollution have all contributed to the decline of the species (Katsanevakis, 2007; Hendriks *et al.,* 2013; Sureda *et al*., 2013; Basso *et al.,* 2015; Alomar *et al.,* 2015). Consequently, *P. nobilis* has been listed as an endangered and protected species under various legislative instruments since the 1990s, such as the EU Habitats Directive (92/43/EEC, Annex IV), the Protocol for Specially Protected Areas and Biological Diversity in the Mediterranean of the Barcelona Convention (Annex II), and national legislation in most Mediterranean countries.

A severe blow to *P. nobilis* populations occurred with the emergence of the likely non-indigenous parasite *Haplosporidium pinnae* in autumn 2016 (Catanese *et al.,* 2018). This previously unknown pathogen triggered mass mortality events (MMEs) in the western Mediterranean, resulting in the loss of approximately 90% of Spanish *P. nobilis* populations by June 2017 (Vázquez-Luis *et al*., 2017). The mortality rates soon reached 100% in the south and central Mediterranean coasts of the Iberian Peninsula and the Balearic Islands (Vázquez-Luis *et al*., 2017; Prado *et al.,* 2021; Garcia-March, 2020). The parasite subsequently spread through ocean currents, affecting the bivalves on Italian and French coasts (Catanese *et al.,* 2018; Cabanellas-Reboredo *et al.,* 2019; Carella *et al.,* 2019; Panarese *et al.,* 2019) and eventually reaching all Mediterranean regions (Kersting *et al.,* 2019; Katsanevakis *et al.,* 2022). The situation was further complicated by the presence of other pathogens in some individuals, such as *Mycobacterium* sp. (Box *et al*., 2020; Carella *et al.,* 2020; Lattos *et al*., 2020; Saric *et al.,* 2020) and *Vibrio mediterranei* (Prado *et al.,* 2020; Scarpa *et al.,* 2020; Carella *et al.,* 2023), which intensified pathogenicity and mortality rates of *P. nobilis*. Although not fully understood, the disease has been associated with 100% mortality rates in most Mediterranean populations (Katsanevakis *et al.,* 2022). While the involvement of *H. pinnae* as the primary pathological agent with a preeminent role in the onset of the MMEs is widely recognized (Grau *et al*., 2022), further investigations suggested that concurrent polymicrobial infections could have been pivotal in some MMEs, even in the absence of *H. pinnae* (Carella *et al.,* 2023). Furthermore, *H. pinnae* is highly species specific, affecting only *P. nobilis* and not its congeneric species *Pinna rudis* (Linnaeus, 1758) (Vázquez-Luis *et al*., 2017; Catanese *et al*., 2018).

The severity of the MMEs and the lack of substantial recruitment (Kersting *et al.,* 2020) have resulted in *P. nobilis* being classified as Critically Endangered by the International Union for the Conservation of Nature (IUCN) in 2019 (Kersting *et al.,* 2019). The ongoing MME caused by *H. pinnae* threatens *P. nobilis* with total extinction, as its natural recolonization is hampered by low population numbers and the isolation of surviving individuals (Kersting *et al*., 2020; Katsanevakis *et al*., 2022). Urgent conservation actions are required to prevent the species' extinction, with a focus on the few remaining unaffected populations acting as potential reservoirs for conservation efforts.

Since the first confirmed records of mass mortality events (MME) of *P. nobilis* populations in the Aegean Sea in 2018 (Katsanevakis *et al.,* 2019), several studies have been conducted which showed the MME escalation in the Greek seas (Zotou *et al*., 2020; Zotou *et al*., 2021; Katsanevakis *et al*., 2022). By the end of 2021, fan mussel populations had collapsed in most of Greek waters, with only two known surviving populations located in Kalloni Gulf (Aegean Sea) and Amvrakikos Gulf (Ionian Sea) (Zotou *et al.,* 2021).

Pinna nobilis is absent from the Black Sea, with the Istanbul Strait considered its northern distribution limit. However, the population status of this species in the Sea of Marmara (connecting the Aegean Sea and the Black Sea) had not been sufficiently studied before the MME. The Marmara Sea has a relatively lower temperature and salinity in comparison to the Mediterranean Sea (Chiggiato *et al.,* 2012), conditions that can either control or delay the possible expansion of the pathogen, resulting in a natural refugium (Cabanellas-Reboredo *et al*., 2019; Prado *et al.,* 2021). High mortality rates were observed in the Çanakkale Strait (Dardanelles Strait), connecting the Aegean and the Sea of Marmara (Özalp & Kersting, 2020; Acarli *et al*., 2021), where *H. pinnae* and multiple *Vibrio* species were detected (Künili *et al*. 2021). However, *H. pinnae* was never detected in the Marmara Sea, where dense populations of *P. nobilis* were recently reported in several locations (Öndes *et al*., 2020; Cinar *et al*., 2021a; Karadurmuş *et al.,* 2022). An MME in the Sea of Marmara in the spring of 2021 was not attributed to pathogen infection but rather to a massive mucilage outbreak (Cinar *et al*., 2021b; Acarli *et al*., 2022). The remaining healthy *P. nobilis* populations of the Sea of Marmara can play a very crucial role in the recolonization of the species in the Aegean Sea by potential larvae dispersal (Kersting *et al*., 2020).

Unaffected *P. nobilis* populations serve as natural refuges due to either particular local abiotic conditions that inhibit the spread of the pathogen (Cabanellas-Reboredo *et al*., 2019; Prado *et al.,* 2021) or enhanced host resistance to infection (Katsanevakis *et al.,* 2019). These populations can function as sources for natural (larval dispersal through ocean currents) or assisted (through translocations) recolonization in areas where the species has become locally extinct or pathogen presence is low (Katsanevakis, 2016; Kersting & Garcia-March, 2017; Kersting *et al*., 2020). *Pinna nobilis* larvae, with larval stages potentially exceeding one month (Deudero *et al*., 2017; Kersting & García-March, 2017; Trigos *et al*., 2018), can be transported by ocean currents over vast distances, even hundreds of kilometers away (Kersting *et al*., 2020).

Larval collectors are valuable tools for assessing *P. nobilis* recruitment (Cabanellas-Reboredo *et al.,* 2009; Kersting & Garcia-March, 2017; Kersting *et al*., 2020). Collected juveniles can be reared *ex situ* or transplanted to pathogen(s)-free locations for restocking or restoration actions in impacted populations (Katsanevakis, 2016; Kersting & Garcia-March, 2017; Kersting *et al*., 2020). These juveniles can also be utilized to assess resistance or repopulate habitats with high survival potential, such as lagoons (Katsanevakis *et al.,* 2022). Larval collectors have also been used to study genetic connectivity, revealing source and sink populations (Wesselmann *et al.,* 2018).

In light of the grave situation faced by *P. nobilis* populations, this study aims to provide a comprehensive report on the extent of the MME in the Greek Seas. The investigation involves exhaustive censuses in the remaining areas with surviving populations, mapping their distribution, and conducting molecular analyses to identify the presence of pathogenic parasites. Additionally, the study focuses on assessing *P. nobilis* recruitment through larval collectors and predicting larval dispersal using ocean circulation models. These findings are vital for understanding the current status of *P. nobilis* populations in Greece and informing urgent conservation actions to ensure the survival and recovery of this critically endangered species.

Methods

Study area

Between May 2022 and May 2023, we conducted 163 independent underwater visual surveys in 13 marine areas along the Greek coastline to assess the status of *P. nobilis* populations (Fig. 1). The selection of study areas was based on three main criteria: (a) possible sanctuary areas (such as coastal lagoons, semi-enclosed gulfs, and river estuaries) with potential optimal abiotic conditions to limit the spread of *H. pinnae* (Cabanellas-Reboredo *et al*., 2019; Grau *et al*., 2022), (b) areas previously known for large *P. nobilis* populations that have not been surveyed since the initiation of MMEs in Greece, and (c) anecdotal information on the presence of living individuals. The surveys covered a wide geographical area, including a case study in Amvrakikos Gulf, a case study in Kalloni Gulf, and several other areas along the Greek coastline (Fig. 1). Additionally, we investigated the north and north-eastern Aegean Sea as potential receivers of *P. nobilis* larvae, originating from surviving populations in the Sea of Marmara and transported by ocean currents through the Dardanelles Strait.

Case Study 1: Amvrakikos Gulf

Amvrakikos Gulf, situated in north-western Greece, is a semi-closed sea covering approximately 400 km^2 (excluding marshes and lagoons). The gulf, with a maximum length of 35 km and a maximum width of 20 km, reaches a depth of 62 m. It has limited connection to the open Ionian Sea through the narrow Preveza Channel (370 m wide and 5 m deep at its narrowest point). Two rivers, Louros and Arachthos, flow into the gulf from the north, creating various habitats that sustain diverse aquatic ecosystems, including extensive shallow lagoons.

The gulf is one of the most important wetland systems in Greece and has been designated as a Ramsar site in 1975, a Special Protection Area (SPA) under the EC Directive 79/409 on the conservation of wild birds, and a Specially Protected Area (SPA) under Protocol 4 of the Barcelona Convention. The broader area of Amvrakikos

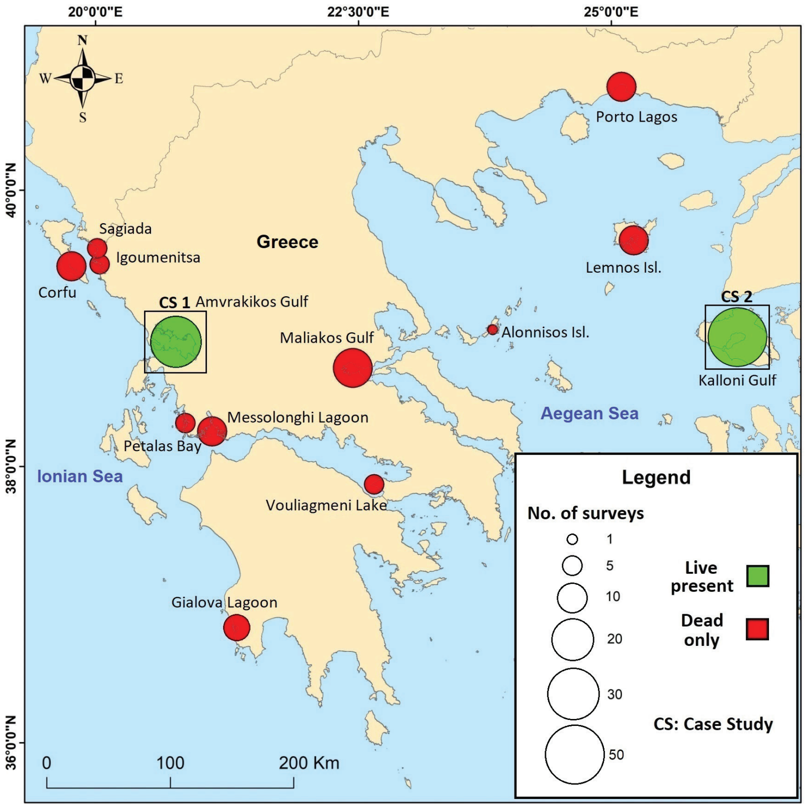

Fig. 1: Map of Greece depicting the distribution of the underwater visual surveys conducted to assess the status of *Pinna nobilis* populations between May 2022 and May 2023. The green boxed circles indicate the areas where live individuals were found. The sizes of the circles indicate the number of surveys conducted at each area.

Gulf was declared a National Park in 2008, with its marine area falling within two Natura 2000 sites (GR2110001 & GR2110004).

The gulf acts as a typical dilution basin from winter to early summer. In the summer and early autumn, evaporation surpasses freshwater input from rivers, yet remains insufficient to raise salinity above that of the open sea (Friligos *et al*., 1997). The annual salinity levels in the top water layer (< 12 m) fluctuate between 11 psu in winter and 33 psu in summer, with corresponding temperature levels from 11 to 29°C (Kountoura & Zacharias, 2013, 2014; Georgiou *et al*., 2021). Human interventions, including a harbor at Preveza Strait and an underwater tunnel between Aktio and Preveza, restrict water exchange leading to water quality degradation, including hypoxic and anoxic conditions in the bottom layer (Ferentinos *et al*., 2010; Kountoura & Zacharias, 2014; Georgiou *et al*., 2021).

Case study 2: Kalloni Gulf

Kalloni Gulf, situated in the southern-central part of Lesvos Island in the north Aegean Sea, is an elongated, shallow, and semi-enclosed bay. The gulf covers an area of 110 km^2, with a maximum length of 22 km and a width of 10 km. It has an average depth of 10 m, reaching a maximum depth of 25 m, and is connected to the Aegean Sea through a 4-km-long channel. Kalloni Gulf is one of the most productive coastal regions in the eastern Mediterranean, with rich biodiversity (Panayotidis *et al*., 1999; Debenay *et al*., 2005), and is included in the Natura 2000 European network of protected areas (GR4110004 & GR4110007). Salinity levels in the Gulf vary seasonally between 37 psu in the winter to >40 psu in the summer (Petalas *et al.,* 2020), with some localized extreme low (25 psu) and high (45 psu) values observed near the river estuaries and the salt pans of the Gulf respectively (Evagelopoulos & Koutsoubas, 2008; Kefalas *et al.*, 2016). Temperature in the Gulf also exhibits significant variation, ranging from 6.5 ^{o}C in January to 30.5 ^{o}C in August (2020–2023, unpublished data). Before the MME, Kalloni Gulf supported a large healthy fan mussel population, estimated to exceed 1 million individuals (unpublished data).

Other Greek sites

Several areas were surveyed along the Greek coastline to investigate the presence of live individuals (Fig. 1). In the Aegean Sea, the surveyed areas were Lemnos Island (with a focus on the Gulf of Moudros), Alonnisos Island, Porto Lagos, and Maliakos Gulf. In the Ionian Sea, the surveyed areas were Gialova Lagoon, Messolonghi Lagoon, Petalas Bay, Igoumenitsa, Sagiada, Vouliagmeni Lake, and Corfu Island.

Population assessment

Between May 2022 and May 2023, we conducted 156 independent underwater visual surveys, following a 45-min protocol, across 13 marine areas along the Greek coastline to assess the status of *P. nobilis* populations (Fig. 1). Additionally, we performed seven fixed-area visual underwater surveys in Amvrakikos Gulf to estimate population density (individuals / 100 m^2). Experienced divers with knowledge of *P. nobilis* morphology and habitat preferences conducted the surveys, using scuba diving or snorkeling depending on depth and visibility. The surveyed sites were accessed from shore or by boat.

During each 45-min protocol survey, the divers systematically scanned the area parallel to the coast for *P. nobilis* individuals, using a stopwatch to track time. When a *P. nobilis* individual requiring measurement was detected, the stopwatch was paused, so that the overall search time remained unaffected. For the "fixed-area" surveys, a pair of divers swam side by side parallel to the coast, scanning a pre-defined shallow area (1 – 4 m depth). In both protocols, dead, freshly dead (assessed by the level of fouling in the inner shell), and living individuals were counted (Katsanevakis *et al*., 2019), while shell fragments and uprooted dead individuals on the seabed were not counted. We measured each live individual's shell width using a caliper and recorded the depth and habitat type where it was found.

In Amvrakikos Gulf, we conducted 36 45-min protocol surveys and seven fixed-area surveys at 21 different sites (Fig. 2, Table S1) between June and August 2022. The survey depths ranged from 0.5 to 28 meters. In Kalloni Gulf, we conducted 51 45-min protocol surveys (Fig. 3, Table S2) between October 2022 and May 2023, covering depths from 0.4 to 8 meters. For the remaining 11 marine areas, a total of 69 45-min protocol surveys were conducted at 36 sites (Table S3).

Recruitment

Ocean circulation model

Employing an Ocean Circulation Model (OCM), we predicted the potential export and fate of *P. nobilis* larvae, originating from the healthy population of the Sea of Marmara, to investigate how/if it contributes to northern Aegean repopulation via the Dardanelles Strait (Fig 4). For simulations, we selected the Regional Ocean Modeling System (ROMS: Shchepetkin & McWilliams, 2003; Shchepetkin & McWilliams, 2005). ROMS is a hydrostatic (Boussinesq assumption) ocean model, which solves explicitly the RANS (Reynolds Average Navier Stokes) equations. It has been applied in various coastal and basin-scale applications in the Mediterranean Sea (Dutour-Sikirić *et al*., 2012; Dutour-Sikirić *et al.,* 2013; Janeković *et al.,* 2014; Vilibić *et al*., 2016; Juza *et al*., 2016; Mamoutos *et al*., 2017; Mourre *et al*., 2018; Mamoutos *et al*., 2021; Petalas *et al*., 2022; Solodoch *et al*., 2023). An orthogonal curvilinear grid covering the

Fig. 2: Map of Amvrakikos Gulf, depicting 21 sites where the 36 45-min protocol surveys (black dots) and 7 fixed-area surveys (blue circles) were conducted between June and August 2022.

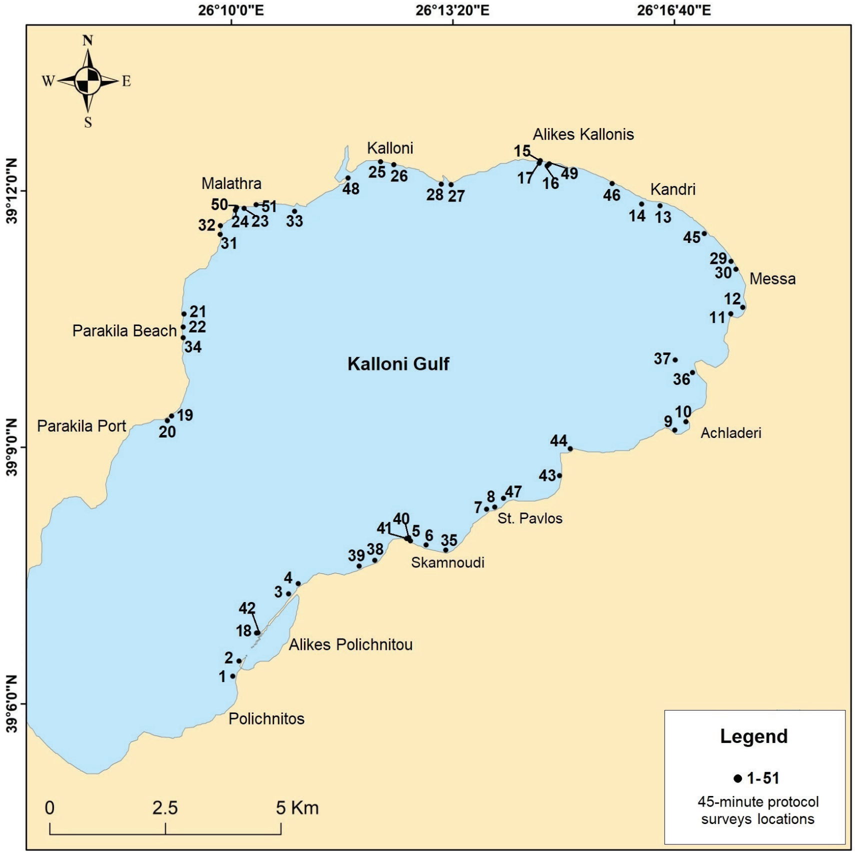

Fig. 3: Map of Kalloni Gulf, depicting the sites where the 51 underwater visual surveys were conducted between October 2022 and May 2023.

Fig. 4: *Pinna nobilis* larval collector network in North Aegean Sea, and Amvrakikos Gulf, Greece, 2022. Highlighted coastline represents the areas where the 42 larvae collectors (LC) were deployed, followed by the code and number of each site collectors. Ocean circulation model (OCM) optimal sites are depicted with blue color and the remainder with purple color.

Central and Northern Aegean (Fig. S1) was used, with a 2.5-km horizontal resolution and 20 vertical terrain following – also known as sigma (σ) coordinates levels with uneven spacing (dz). We derived bathymetry from the GEBCO (General Bathymetric Chart of the Oceans, Weatherall *et al*., 2015) dataset and interpolated linearly on the grid. Minimal smoothing was applied on the interpolated bathymetric field for hydrostatic consistency reasons. Boundary conditions along the southern limit of the grid were retrieved from the Mediterranean basin scale MED-MFC model (Escudier *et al*., 2020) freely distributed by CMEMS (Copernicus Marine Service). For the eastern open boundary, the Turkish Straits System (TSS), data were retrieved from Maderich (2015).

ECMWF ERA5 data (Hersbach *et al*., 2020) informed atmospheric forcing for reanalysis runs, while SKIRON data (Kallos *et al*., 1999) were used for forecast runs in 2022. Major river outflows came from the SHMI E-HYPE hydrological model results (Lindstrom *et al*., 2010). A four-year re-analysis from 1 January 2018 to 31 December 2021 employed the model's 4DVAR data assimilation capabilities (Moore *et al*., 2011a, b, c), specifically the incremental, strong constrain dual formulation of 4DVAR using the Restricted B-preconditioned Lanczos (RBL4DVAR, formerly known as PSAS) formulation of the conjugate gradient method in which the cost function is minimized directly in the observational space (Gürol *et al*., 2014). A detailed description of the Central North Aegean (CNAG-ROMS) system in terms of capabilities and the validity of the reanalysis results is given in Mamoutos *et al*. (2023).

To obtain a first estimate for the trajectory of *P. nobilis* larvae, a daily 3D velocity climatology for a standard/typical year (365 days) was created, from the aforementioned reanalysis dataset and used for an offline Lagrangian simulation (Kersting *et al.,* 2020). The Lagrangian tool of our choice was ROMS Path (Hunter *et al*., 2022) and the run started on 1 May and ended on 1 November of the climatological year covering 185 days. The simulation was conducted using 5000 particles, released on 1 May of the climatological year, as a cluster into the Turkish Straits System (TSS) to flow as neutral/passive particles in the north and central part of the Aegean Sea to investigate the possible trajectories of the larvae and propose sites for the deployment of the larval collectors. The next step was to incorporate the release of the same number of particles (5000) in each 5-day real-time forecast cycle of the CNAG ROMS operational model, covering the period from 1 May 2022 until 1 November 2022. This choice provided the ability to assess, through an indirect method using ROMSPath tool, the capability of the model to predict, to some extent, the larvae pathways in real-time conditions during the deployment of the collectors.

Larval collectors

To explore the potential of surviving populations as sources for recolonization, we employed a network of 26 larvae collectors, strategically placed in the North Aegean Sea, based on the results of the ocean circulation model, between May and June 2022. During the same period, 14 additional larval collectors were deployed at various locations along the North Aegean coastline, at sites not selected based on the model's predictions. Furthermore, two larval collectors were deployed in Amvrakikos Gulf, Ionian Sea, in late July 2022 (Fig. 4). All larval collectors were installed either by scuba diving or boat deployment, and the settled communities were retrieved and sorted by the end of December 2022.

The deployment of the North Aegean Sea included ten units in the eastern coastline of Lemnos Island (an OCM-identified near-optimal location), ten along the southern coasts of Samothrace Island (identified by OCM as optimal), six along the southern Coastline of Thasos Island (OCM near-optimal location), and nine in Kalloni Gulf (Lesvos Island), home to a significant remaining fan mussel population. Additionally, one larval collector was placed in the east coastline of Lesvos and four along the coast of Alexandroupoli (Fig. 4; Table S4).

Each larval collector of the North Aegean Sea network comprised a cement weight anchoring it to the seafloor, a primary rope supporting 2–4 settlement bags, and a buoy maintaining the structure's upright positioning within the water column (Fig 5b, c). The collector's bags consisted of a polyethylene onion mesh bag (dimensions of 40 × 60 cm), which were filled with 4–6 identical entangled bags, serving as substrate for *P. nobilis* larvae settlement (Buttler, 1987; Cabanellas-Roboredo *et al.,* 2009; Kersting *et al*., 2020) (Fig 5a). Bag placement maintained a minimum vertical separation of 1 m, with the deepest bags situated at least 1.5 m above the seafloor. Ranging from 4–10 m in height, the larval collectors were deployed at depths between 5 and 12 m, ensuring complete submersion (> 1 m below the surface) to obviate surface visibility and potential entanglement with boat propellers (Fig 5c). The larval collectors in Amvrakikos Gulf comprised of three settlement bags each, which consisted of an onion mesh bag, filled in with entangled nylon mussel-packaging nets (Kersting & Garcia-March, 2017; Kersting *et al*., 2020) and were installed on fish farming facility buoys, at depths of 6 m, 8 m, and 10 m (Table S4).

Molecular analysis

Confirming the identity of Pinna nobilis in Amvrakikos Gulf

Sampling took place in the subtidal zone of Amvrakikos Gulf (western Greece) during summer 2022 by SCUBA diving. In order to minimize disturbance and damage to the live specimens, a small section (3–5 mm) of the outer upper part of the shell was cut with scissors and placed in a plastic vial with sea water. After the end of the dive, a total of 13 samples was transferred immediately in Eppendorf tubes with pure ethanol. Additionally, three freshly dead juvenile specimens were collected from larval collectors in Amvrakikos Gulf and stored in pure ethanol.

Prior to extraction, all shells were thoroughly cleaned from debris and foreign material. Furthermore, shells were initially treated with PBS to hydrate. Total DNA was isolated with the salt-extraction procedure of Aljanabi & Martinez (1997) with modifications regarding the concentration of Proteinase K (final concentration 1mg/mL) and incubation time (samples left at 56°C overnight). After isolation, 1 μl DNA was used as a template in PCR with different sets of primers (Folmer *et al*., 1994; Katsares *et al*., 2008; Sanna *et al*., 2013 and primers designed for this study: PnCOI_F_MK22 and PnCOI_R_MK22) aiming to the amplification of Cytochrome Oxidase subunit I (COI) fragment. A second semi-nested PCR followed, using as template 1 μl of the PCR product from the initial PCR, with the forward newly designed primer. For the juvenile samples, a single PCR with the new primers was needed. The sequences of different primers used for PCR amplifi-

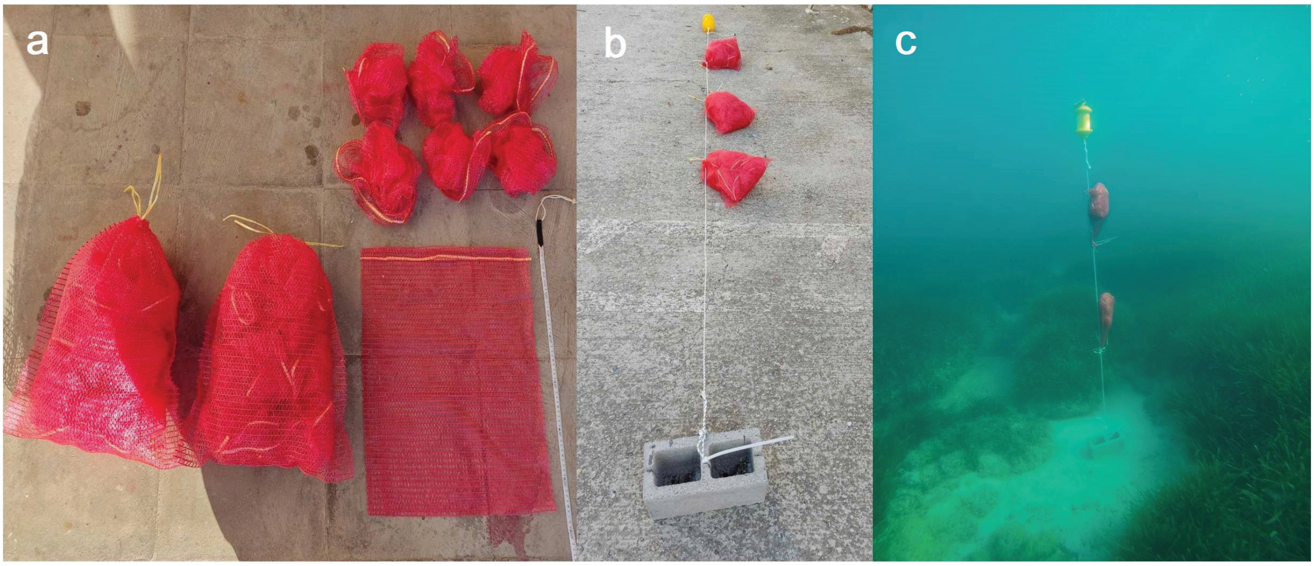

Fig. 5: The larval collector structures that were used in Northern Aegean Sea network in 2022. Larvae settlement bags made by nylon filament onion bags (a), a constructed larvae collector consisted of a weight, the main rope, the settlement bags, and a buoy (b) and a larvae collector deployed at 6m depth in Lemnos Island (c). (Photo credits: O. Papadakis).

cation are listed in Table S5. PCRs were conducted in 25 μL volumes with 1x KAPA2G Fast Multiplex PCR Mix (Kapa Biosystems), 10μM of each primer and approximately 10ng of genomic DNA (or PCR product for the semi-nested PCR), filled to 25 μL with double-distilled (dd H2O) water]. Cycling conditions comprised of an initial step of 95°C for 2 min, and 37 cycles comprising of 94°C for 30 sec, 48°C for 30 sec, 72°C for 1 min with a final extension step of 72°C for 10 min. PCR products were examined on a 1% agarose-TAE gel stained with GelRed® Nucleic Acid Gel Stain (Biotium, San Francisco, SF, USA). PCR products were purified using commercially available spin columns (Macherey-Nagel). The PCRs yielded a final product of approximately 400 bps. Sequencing was conducted on an ABI 3700 Genetic Analyzer (Applied Biosystems™, Waltham, MA, USA) using PnCOI_F_MK22 primer. The resulted chromatograms were manually inspected and the corrected sequences were subjected to multiple alignment with CLUSTAL-W v1.4 (Thompson *et al.*, 1994). For each specimen, the consensus sequence was extracted after alignments and superimpositions of sequencing results from various PCR amplifications, considering each base quality peak.

Investigating pathogens in Kalloni Gulf

We collected six mantle biopsies from freshly dead juvenile *P. nobilis* individuals in November 2023 at Alykes Polichnitou, Kalloni Gulf, and preserved them in absolute ethanol for subsequent molecular analysis. Total genomic DNA was extracted using the DNA NucleoSpin® Tissue extraction kit (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions. Different fragments of the ribosomal unit were amplified by PCR for *H. pinnae* detection. The primers HPNITSF and 1500R (Moro-Martínez *et al.*, 2023) were used to amplify a partial rDNA region (ITS2), while the primers HapF1/HapR2 (Renault *et al.*, 2000) and HpF3/HpR3 (López-Sanmartín *et al.*, 2019) were used to amplify partial regions of the 18S rDNA gene. Moreover, the primers mycgen-f/mycgen-r, described by Böddinghaus *et al.* (1990) with PCR conditions indicated by Carella *et al.*, (2019), were utilized to detect the presence of *Mycobacterium* sp. in the same samples.

PCR reactions were conducted in a total volume of 20 μL. The reaction mixture consisted of 10 μL of KAPA Taq Ready Mix PCR kit (Sigma-Aldrich, Burlington, MA, USA), 0.4 μL of each primer (stock 20 μM), 1 μL of DNA (approx. 200 ng/μL), and the remaining volume was made up with water. The temperature profile for the PCR reaction included an initial denaturation step at 94 °C for 2 minutes, followed by 40 cycles of denaturation at 94 °C for 30 seconds, annealing at 49-60 °C for 20 seconds, and extension at 72 °C for 30–60 seconds.

The PCR products were separated on 1.5% agarose gels in TAE 1x buffer (w/v). The gels were stained with GelRed (Biotium, Fremont, CA, USA), and a HighRanger 1kb DNA ladder size standard (Norgen, Thorold, Canada) was included. The amplified DNA fragments were visualized using a UV transilluminator. To ensure the specificity of the amplification, the PCR products were excised from the agarose gel and purified using the Metabion International mi-Gel Extraction Kit (Metabion International, Planegg, Germany), following the manufacturer's instructions. All the obtained PCR fragments were subjected to bi-directional sequencing using the 3130xl DNA automated sequencer (Applied Biosystems, Carlsbad, CA, USA) at the Secugen S.L. service in Madrid, Spain. The sequences were edited and aligned using the BioEdit v. 7.2.5 software (Hall, 1999) and MEGA X (Kumar *et al.*, 2018).

Results

Population assessment

Overall, across the 156 45-min protocol surveys and the seven fixed-protocol surveys, 4348 *P. nobilis* individuals were recorded, of which 87.3% were dead and 12.7% alive (Fig. 6). The only surviving fan mussels were found in the semi-enclosed Gulfs of Amvrakikos in the Ionian Sea and Kalloni in the Aegean Sea. Of the dead individuals, 1.0% were determined to have recently died, all of which in Amvrakikos and Kalloni Gulfs.

Case Study 1: Amvrakikos Gulf

In Amvrakikos Gulf, 241 live *P. nobilis* individuals were recorded through the 36 45-min protocol surveys, spanning depths from 0.6 to 9.7 m. By excluding potential poaching (which could not be estimated by this dataset) and by excluding dead-old individuals, the recent mortality rate was approximated at 7.7%, based on 20 recently dead individuals. Most live specimens were concentrated in a single location (Manna Gea), wherein 175 of the 241 live individuals were recorded. The mean shell width of the living individuals was 9 cm, with a median depth of 1.2 m (Fig. 7-Top; Table S1).

Over the seven fixed-area surveys conducted in Amvrakikos Gulf, encompassing a total surveyed area of 4.61 ha, 102 *P. nobilis* individuals were recorded, of which 41 were dead and 61 alive. Across all surveyed regions, the seabed was characterized by unvegetated substrates, covered by sand or gravel and in some cases rocks. By excluding dead-old individuals the mortality rate was estimated at 0.0%, with no freshly dead or alive juvenile individuals observed. The living individuals identified were all deemed young adults, with shell widths ranging between 8.4 and 13.2 cm, and a mean shell width of 10.1 cm. The highest density of live individuals was encountered at the Akrotiri site, estimated at 0.31 individuals per 100 m^2, followed by Menidi with an estimated density of 0.22 individuals per 100 m^2 (Fig. 7-Bottom; Table S1).

By merging the results of the two sampling methods employed in Amvrakikos Gulf, a total of 602 *P. nobilis* were recorded, among which 302 were alive, 280 were

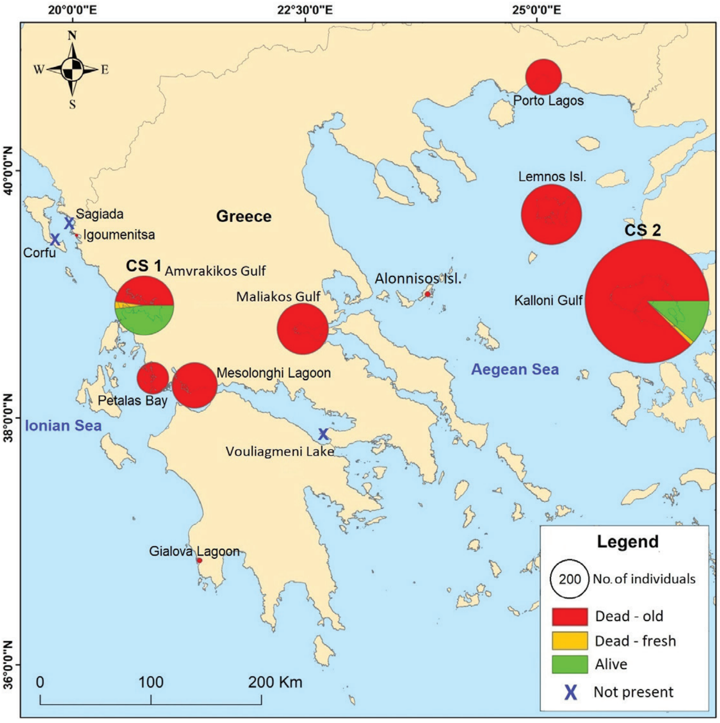

Fig. 6: Map of Greece depicting the assessment of *Pinna nobilis* populations at 13 marine areas, for 156 45-minute protocol surveys, conducted between May 2022 and May 2023. The sizes of the pie charts represent the number of recorded individuals. The red, yellow, and green colors represent the proportion of old-dead, freshly dead, and living individuals, respectively. "X" represents the areas where no individuals were recorded.

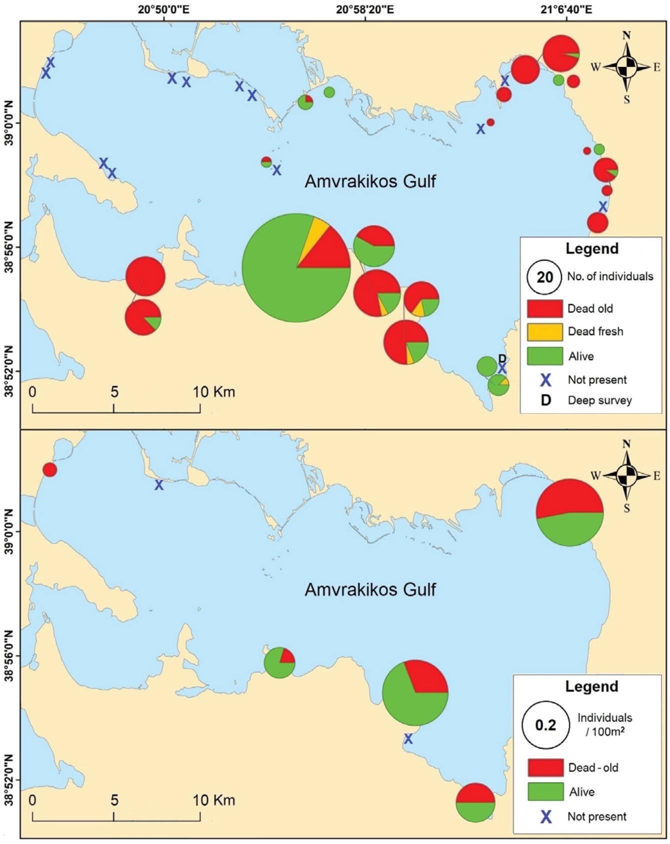

Fig. 7: Maps of Amvrakikos Gulf depicting the assessment of *Pinna nobilis* populations, during the 36 45-minute protocol surveys conducted in July-August 2022 (Top), and the assessment of *Pinna nobilis* populations, during the 7 Fixed-area surveys, conducted between June and August 2022 (Bottom). The sizes of the pie charts represent the number of recorded individuals (top), and the number of recorded individuals per 100m^2 (bottom). The red, yellow, and green colors represent the proportion of old-dead, freshly dead, and living individuals, respectively. "X" represents the sites where no individuals were recorded, and "D" the deep surveys (> 20 m).

old-dead and 20 were freshly dead, with an overall recent mortality rate estimated at 6.2% (by excluding old-dead individuals)

Case Study 2: Kalloni Gulf

In Kalloni Gulf, 2211 *P. nobilis* individuals were recorded, encompassing 252 live individuals (Fig. 8; Table S2). These live individuals included 70 adults (8.0 to 24.2 cm shell width) and 182 juveniles (2.2–8.0 cm shell width; the 8-cm threshold was based on Katsanevakis 2006: fig. 4), found at depths ranging from 1.4 to 5.6 m. The juveniles (Fig. 9a), accounting for 72.2% of the total living population, were inferred to have been recruited in 2022 (Richardson *et al*., 1999; Katsanevakis, 2016). Of the dead individuals, 17 (0.87% of the dead population) were estimated to have died recently, which leads to an estimate of a 6.3% recent mortality rate.

Eleven of the adults and 40 of the juveniles were tagged to monitor their survival and growth rate (Fig. 9b). Notably, a juvenile *P. nobilis* (2.9 cm shell width) growing on an experimental artificial surface (settlement plate) was carefully removed in order not to damage its byssus (Fig. 9c) (Katsanevakis, 2016; Acarli, 2021) and relocated to a protected cage (Kersting & Garcia-March, 2017) in a sanctuary area near the salt pans of Kalloni Gulf, where other healthy *P. nobilis* individuals were observed (Fig. 9d).

Other Greek sites

Throughout the surveys conducted at 11 marine areas, 1523 *P. nobilis* individuals were recorded, all of which were dead, with none determined to have died recently (Fig. 6, Table S3). The highest densities of dead individuals were observed in Lemnos Island (52 individuals per survey) (Fig. S2), followed by Petalas Bay (36 individuals per survey) (Fig. S5), Maliakos Gulf (34 individuals per survey) (Fig. S8), Messolonghi Lagoon (30 individuals per survey) (Fig. S5), and Porto Lagos (27 individuals per survey) (Fig S3). Conversely, neither dead not living fan mussels were recorded at Corfu Island, Sagiada, and Lake Vouliagmeni in the Ionian Sea (Fig. S6, S7).

Recruitment

Ocean circulation model

Notably, the highest estimated probability density of recruitment is located in the periphery of Samothrace Island Fig 10a). The maximum value of the probability density is recorded at the southern part of the island. Lower values are located north of Samothrace's coasts. Moderate values are also located between Thasos Island and north-west of Samothrace. For longitudes west of 24.75° E (WGS84 decimal degrees) the probability is even lower and as shown from the figure the larvae are

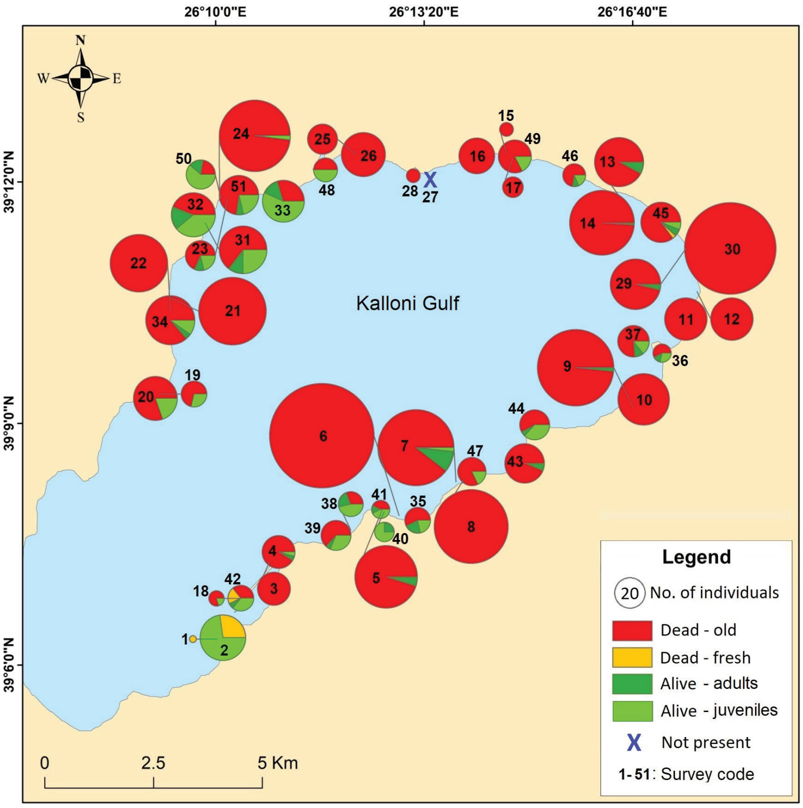

Fig. 8: Map of Kalloni Gulf depicting the assessment of *Pinna nobilis* populations, during the 51 45-minute protocol surveys, conducted between October 2022 and May 2023. The sizes of the pie charts represent the number of recorded individuals. The red, yellow, dark green, and pale green colors represent the proportion of old-dead, freshly dead, alive-adult, and alive-juvenile individuals respectively. "X" represents the sites where no individuals were recorded.

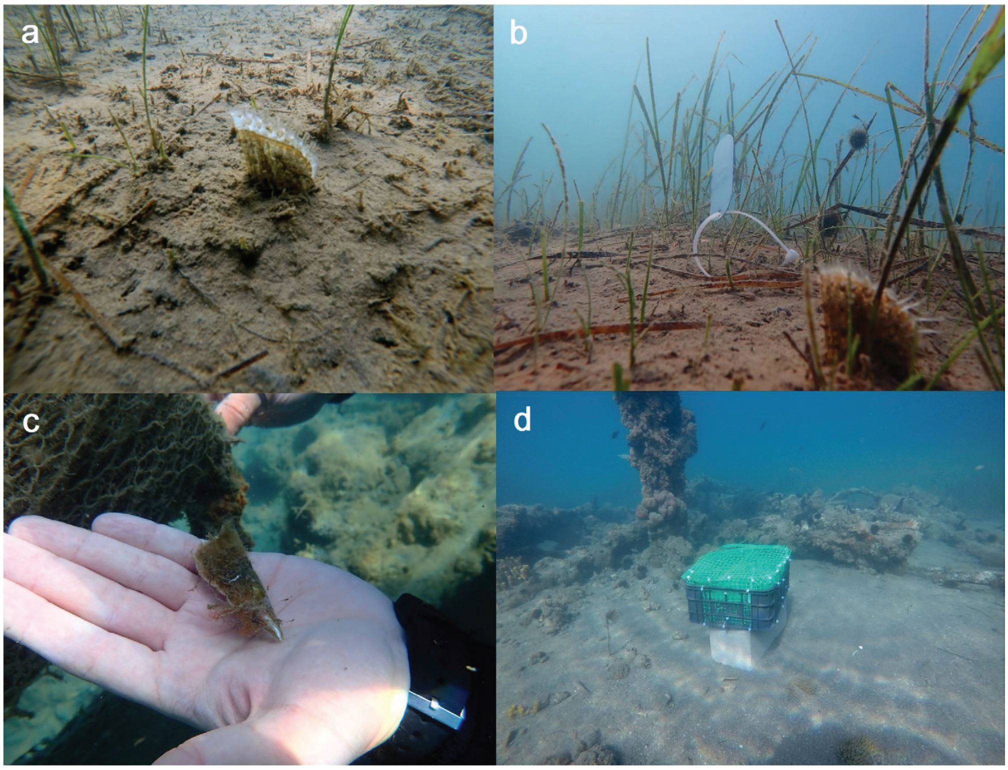

Fig. 9: *Pinna nobilis* juvenile (3.5 cm wide) (a) and a tagged juvenile (4 cm wide) (b), which were recruited in Kalloni Gulf, Lesvos Island in 2022. *Pinna nobilis juvenile* (2.9 cm wide) recruited on an artificial experimental surface in Kalloni Gulf, Lesvos Island in 2022 (c), placed in a weighted protected cage and translocated in a sanctuary area of the gulf (d). (Photo credits: O. Papadakis O.: a, d; E. Papadimitriou: b, c).

mainly located at the western part of the Central/North Aegean Sea. Figure 10b shows the accumulated probability density (logarithmic) of the simulating the *P. nobilis* larvae, using as input in ROMS Path, the results/output of each forecast cycle for the period from 1 May 2022 until 1 November 2022. As seen also for this case, the general pattern of the probability density distribution is similar with the climatological one. The most distinct differences have to do with almost the same number of particles, and eventually probability density, east and west of Thasos and higher, compared with the climatological case, concentration of larvae in the Singitic and Toronaic gulfs at Chalkidiki peninsula. A more thorough picture for the larval spatial coverage evolution, for 5000 particles seeded in the Dardanelles in May 2022, during the months from May–November 2022 (both included), is given in the map provided in Figure 11.

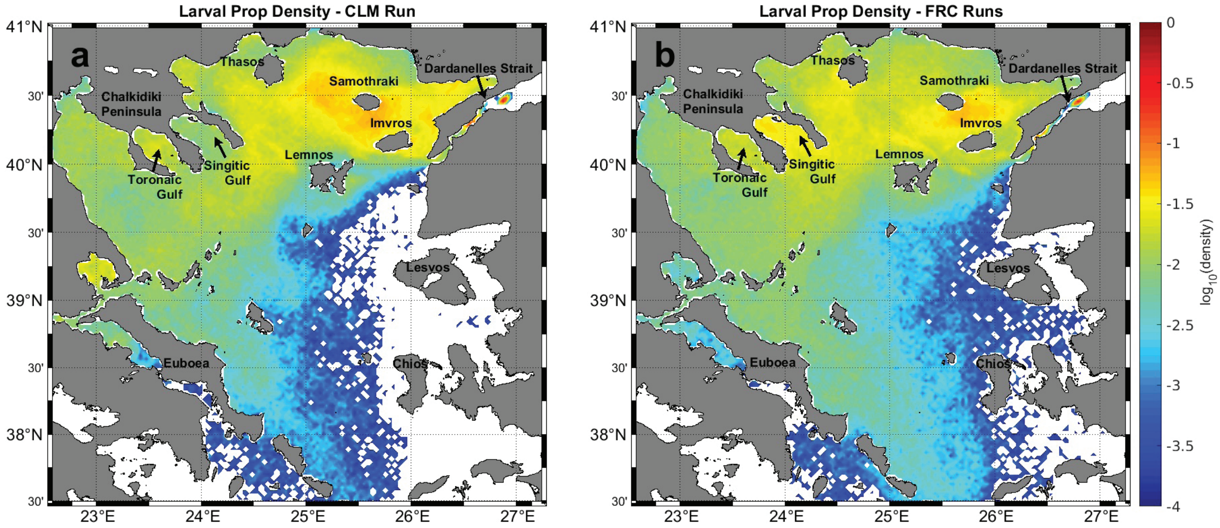

Fig. 10: Larval probability density maps. At panel (a) CLM refers to the climatological experiment that was conducted using the reanalysis data as input. It covers the period from 1 May to 1 November of the climatological year. Panel (b) refers to CNAG-ROMS verified forecasts (FRC) for the period from 1 May 2022 until 1 November 2022. For both panels, the color bar denotes the normalized probability density for the particles at each grid point of the model.

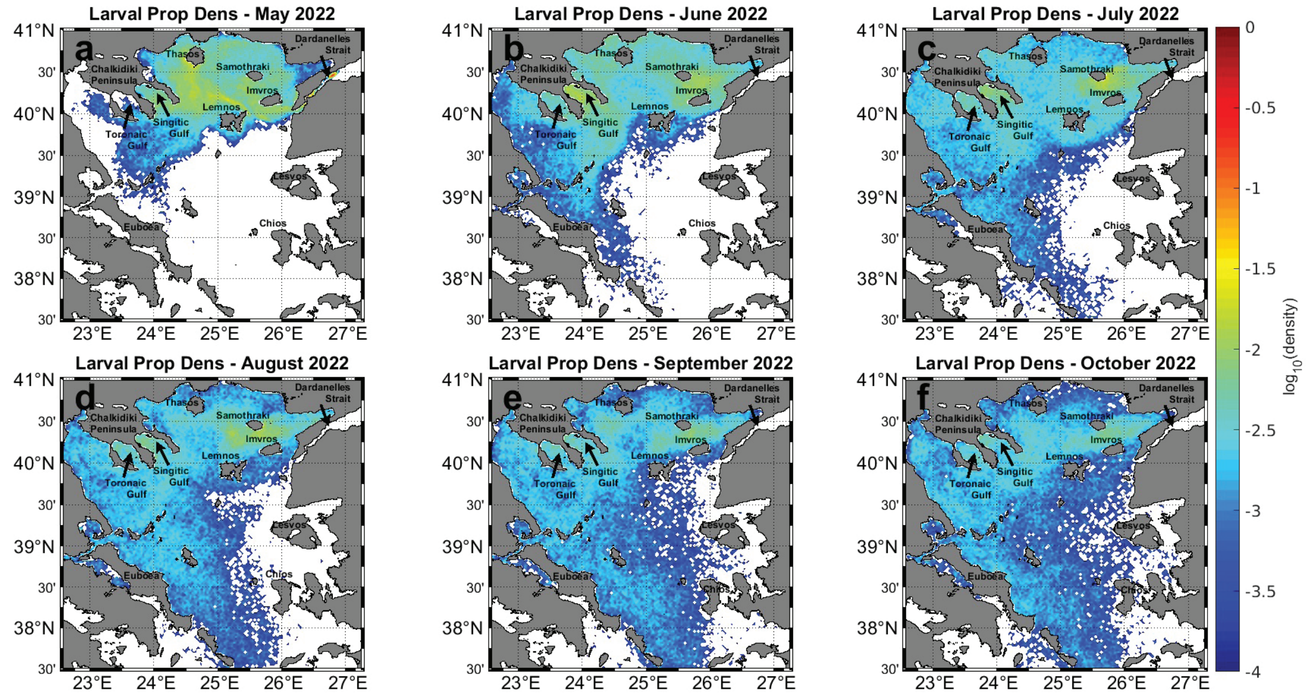

Fig. 11: Monthly larval probability density maps covering the months from May to November 2022. For all panels, the color bar denotes the normalized probability density for the particles at each grid point of the model.

Larval collectors

Of the 40 larval collectors that were deployed in the North Aegean, 30 were successfully retrieved and sorted between October and December 2022 and the remainder were either lost or severally damaged. No *P. nobilis* recruitment was recorded in any of the sorted larval collectors. In Amvrakikos Gulf, only one collector was retrieved and the other was lost. A total of 12 *P. nobilis* juveniles were found in the collector bags, with an average shell length of 3.8 cm (2.2–5.1 cm). Nine of the collected juveniles were placed in a protected cage (Kersting & Garcia-March, 2017) and kept in their natural environment. The remaining three juveniles died soon after they were collected and were stored in pure ethanol for molecular analysis (Table S4).

Molecular analysis

Pinna nobilis identification in Amvrakikos Gulf

All specimens were successfully identified as *Pinna nobilis*. The identification procedure was based on the sequencing of PCR-amplified ca. 400 bps COI region. This particular region is widely used to distinguish between the generality of *Pinna* species known and thus, allows for the classification and phylogenetic identification of unknown and/or juvenile specimens. The genetic divergence of the COI mtDNA marker has been proposed as a species delimitation threshold within the DNA-barcoding framework (Hebert *et al.,* 2003). After inspection of the chromatograms, the corrected sequences were compared by BLAST search (National Center for Biotechnology Information Basic Local Alignment Search Tool NCBI BLAST; http://www.ncbi.nlm.nih.gov/) of the COI region of all submitted *Pinna* sp. in GenBank database, aiming for accurate and rapid identification. Analyzed sequences showed a similarity of more than 98% to KY321794.1 or KY321775.1 samples in the GenBank database. Furthermore, sequences were translated to amino acids using Table S5 (Invertebrate Mitochondrial Code), starting at the 3rd codon position on the reverse strand, with no stop codons, and utilizing Sequence Manipulation Suite (Stothard, 2000). All sequences determined for this study have been deposited in GenBank with Accession Numbers OR479010-OR479025.

Pathogens present in Kalloni Gulf

By utilizing various PCR tests with different amplification lengths (approximately 900 bp and 350 bp), we aimed to analyze the samples for the presence of specific pathogens. Interestingly, one of the samples exhibited a remarkably strong positive detection of *H. pinnae*, indicating a significant presence of this particular organism. However, for four other samples, the PCR results were potentially positive but weak, suggesting the possibility of degraded DNA or a lower parasitic load in those specimens. However, the resulting sequences for both fragments showed 100% identity with the consensus sequence obtained from *H. pinnae* isolated in *P. nobilis* individuals (accession number LC637522).

Conversely, our attempts to amplify *Mycobacterium* sp. DNA using PCR were unsuccessful, indicating the absence or possibly an extremely low concentration of this specific pathogen in the analyzed samples. On a similar note, one sample did not yield any type of PCR amplification, likely due to the lower quality of the extracted

DNA compared to the other samples. It is possible that the DNA in this particular sample was compromised during the extraction process, leading to the lack of amplification.

Discussion

Current status of **Pinna nobilis** *populations in Greece*

The present study confirmed the complete collapse of *P. nobilis* population in most of the areas investigated across the Greek coastline and, in agreement with the recent study of Zotou *et al*. (2021), the only remaining live fan mussels were found in Amvrakikos and Kalloni gulfs, while 100% mortality was recorded in all the other areas.

The efforts for detecting live fan mussels in previously uninvestigated areas with relatively high chances of holding surviving populations (such as coastal lagoons, semi-enclosed gulfs, and river estuaries) were unsuccessful, highlighting the severe impact of the pathogen(s) on the species in the Greek Seas. Characteristic examples of such areas included Moudros Gulf (Lemnos Isl.), Petalas Bay, Maliakos Gulf, and Messolonghi Lagoon (Table S3) which, prior to the MME, held large fan mussel populations (unpublished data). Another notable example was Vouliagmeni Lake, where *P. nobilis* populations had been well studied in the past (Katsanevakis, 2006, 2007, 2009), but during the present study, a large percentage of the Lake's seabed was entirely covered by shell fragments, and although no upright dead individuals were recorded during the deep surveys (Fig. S7, Table S3), a large number was present at depths < 10 m. The complete absence of fan mussels (dead, alive, or shell fragments) at the visited sites of Corfu Island and Sagiada indicates that either the biotic and abiotic conditions did not favor their prior colonization, or the hydrodynamic conditions and wave activity had swept away any dead individuals.

It appears that the MME, after decimating fan mussel populations in Greece, has now reached a state of stability, as the living populations of Amvrakikos and Kalloni Gulfs that were documented in 2021 by Zotou *et al*. (2021) are still surviving. At the moment, these gulfs hold the last known surviving populations in the Ionian and the Aegean seas, and most probably the last in the Greek Seas. The presence of surviving populations in these two semi-enclosed gulfs indicates that specific environmental conditions, such as extreme temperature and salinity, could limit *P. nobilis* mortality by *H. pinnae.* Based on observations in other areas of the Mediterranean Sea, different authors have suggested specific tolerance of *H. pinnae* to temperature and salinity (temperatures above 13.5°C and a salinity range between 36.5–39.7 psu) (Cabanellas-Reboredo *et al*., 2019; García-March *et al*., 2020; Prado *et al*., 2021; Nebot-Colomer *et al*., 2021).

In Amvrakikos Gulf, *H. pinnae* has not been detected (Y. Issaris, pers. comm.), indicating that the fan mussels of Amvrakikos represent the only known population that remains unaffected by the pathogen in Greece. The absence of the parasite in Amvrakikos is probably a consequence of the low salinity levels, prevalent mostly in the top-water layer of the Gulf (Kountoura & Zacharias, 2014; Georgiou *et al.,* 2021). In contrast, the molecular identification of *H. pinnae* in specimens obtained from Kalloni confirms the sustained presence of this infectious parasite inside the Gulf, four years after its first detection in 2018 by Katsanevakis *et al*. (2019). However, the recent mortality rates both in Amvrakikos (6.2%) and Kalloni (6.3%) gulfs may be regarded as comparable to mortality rates observed in healthy populations similar to the annual mortality rates documented prior to the MME events, e.g., in Vouliagmeni Lake, Greece (5.3%, Katsanevakis, 2007) and in Port-Cros, France (mostly between 5-15% for adult individuals < 20 years old, Rouanet *et al.,* 2015). The findings of the molecular analysis performed on the samples from Kalloni Gulf emphasize the need for further investigation and assessment to obtain a comprehensive understanding of the pathogen dynamics in the area but also the importance of the variations in PCR amplification outcomes, highlighting the potential differences in pathogen abundance and DNA quality among the tested samples.

Natural refugia like Amvrakikos and Kalloni gulfs are crucially vital to the species' survival, as they represent pockets of parasite-resistant or unaffected populations. Such areas can have a substantial impact on the restoration of fan mussel populations, as they can serve either as source of recruits to other areas or as a repository of healthy adult individuals, used for management actions, such as transplantations into other suitable areas (Katsanevakis, 2016; Kersting *et al.,* 2020). The successful recruitment of 2022, represented by the 12 juveniles found on one larval collector in Amvrakikos Gulf, and especially the substantial number of juveniles recorded in the field in Kalloni Gulf (72.2% of the living population), provide encouraging prospects for the future survival of the species in these regions. Furthermore, the influx of offspring in these gulfs may indicate a future potential for recolonization in nearby areas, further enhancing the overall natural recovery prospects of the species (Kersting *et al.,* 2020) in the broader Ionian and Aegean Sea regions.

Threats and protection measures

In Amvrakikos Gulf, several environmental stressors have taken a toll on water quality during the recent decades. These stressors include pollution from agricultural chemicals and fertilizers, the accumulation of solid wastes, sewage contamination, the management of freshwater flows for irrigation, and organic wastes from numerous large-scale aquaculture facilities (Ferentinos *et al*., 2010; Kapiris & Conides, 2009; Andrea *et al*., 2020). Given that the two rivers from the north serve as the primary conduits for pollutants, the near-absence of fan mussels (dead or alive) in the north-western region and their sparse presence in the other northern areas of the Gulf might be attributed to these pressures, similar to what has been documented in the Mar Menor refugia in

Spain (Giménez-Casalduero *et al.,* 2020).

During the underwater surveys in Amvrakikos, numerous intense anthropogenic pressures impacting fan mussels and the wider marine ecosystem of the Gulf were documented. Of utmost concern are the compelling indications of widespread fan mussel poaching by scuba divers, coupled with other illegal fishing methods, such as improvised shallow-water dredges and the deployment of heavy fishing gear on shallow seabeds, such as fish-pot clusters. Crushed fan mussels (some of which were still alive) were also recorded in the shallow waters (< 1 m depth) of public beaches, seemingly caused by swimmers.

Kalloni Gulf attracts various coastal activities leading to significant anthropogenic impacts on the marine ecosystem. These impacts encompass pollution and eutrophication, overfishing, and habitat degradation caused by legal and illegal fishing activities, as well as habitat degradation due to coastal development. (Diapoulis *et al.,* 1998; Kefalas *et al.,* 2003; Spatharis *et al.,* 2007). The highest nutrient and pollution levels primarily accumulate in the inner parts of the Gulf, where limited water circulation allows for the accumulation of agricultural runoffs from nearby river estuaries, as well as domestic sewage from the town of Kalloni (Panayotidis & Klaudatos, 1997; Zanou & Kopke, 2001; Evangelopoulos & Kotsoubas, 2008). During the underwater surveys undertaken in Kalloni Gulf, several activities posing major threats to the surviving *P. nobilis* populations were documented, some of which were extensive fishing with bottom-nets in very shallow waters (< 4 m depth) across the entire coastline of the Gulf, fish-pot clusters deployed in shallow waters (< 10 m depth), boat anchoring, and possible fan mussel poaching.

Incorrect enforcement of the current legislation, inadequate surveillance, and insufficient enforcement of penalties appear to contribute to widespread incidents of illegal commercial and recreational fishing, resulting in significant impacts on both fan mussel populations and other marine life. As Amvrakikos and Kalloni Gulfs are marine areas of unique importance for biodiversity and also represent the last hope for the conservation of *P. nobilis* in Greece, urgent action is required to implement necessary protection and conservation measures. In order to protect these last surviving populations, it is essential to establish new zones of strict protection in both Amvrakikos and Kalloni gulfs. Within these zones, all fishing activities should be strictly prohibited, while boat anchoring and recreational activities should be regulated, so that the surviving fan mussel populations are adequately protected. Finally, it is crucial to intensify and reinforce the control of illegal exploitation and marketing of fan mussels, especially in the case of Amvrakikos Gulf.

Recruitment and larval collectors

In Kalloni Gulf, a significant successful recruitment occurred in 2022, with extensive dispersal across the entire coastal area of the Gulf. Surprisingly, no larval settlement was recorded on any of the larval collectors in the Gulf, despite that recruitment in the natural substrate was observed just a few meters away from some larval collectors. In contrast, the successful recruitment on the different types of larval collector bags, used in Amvrakikos Gulf consisting of entangled nylon nets (Kersting & Garcia-March, 2017; Kersting *et al*., 2020), could indicate that the type of bags utilized in the North Aegean larval collector network (entangled onion bags), despite being successfully tested in the past by Buttler (1987) and Kersting *et al*. (2020), might have structural weaknesses that hindered larval settlement. However, similar larval collector bags to the ones used in Amvrakikos were also used in Kalloni prior to the present study (2021); nevertheless, no successful recruitment was recorded. Similarly unsuccessful settlement on larval collectors had also been documented by Kersting *et al*. (2020), even though they were installed close to a healthy adult population. Although the North Aegean larval collector network was installed prior to the larval collectors of Amvrakikos Gulf (anticipating potential early-season recruitment), possible abiotic conditions such as the water temperature, could vary locally, shifting *P. nobilis* spawning and settlement periods (Richardson *et al*., 2004; Kersting & Garcia-March, 2017; Trigos *et al.,* 2018) prior to the larval collector deployment. The successful recruitment of fan mussel larvae on collectors probably depends on a multitude of factors, such as the availability of larvae, the structural elements of the collector, environmental conditions, and the behavior of larvae.

In the North Aegean, the estimated distribution of the particles/larvae for this time of year, i.e., May to October, is consistent with the mean circulation pattern of the area from mid-spring to mid-autumn according to the available literature (Nittis *et al*., 2002; Olson *et al*., 2007; Tzali *et al*., 2010; Androulidakis *et al*., 2011). The fact that the higher probable density for larvae is located at the periphery of Samothrace's coast agrees with the intensification of Samothrace gyre that time of year due to the higher outflow of Black Sea waters from the Dardanelles straits (Kanarska *et al*., 2008; Maderich *et al*., 2015).

The maps presented in Figures 10 & 11 offer compelling evidence supporting the strategic placement of larvae collectors in our study. As depicted in Figure 11b-f, the probability density is high along the periphery of Samothrace Island. Moreover, high probability densities are observed mainly in the south-eastern and, to a lesser extent, western marine regions of Thasos during June, August, September, and October 2022. In contrast, the northern part of Lemnos Island exhibits moderate probability density values, primarily located offshore. An interesting finding concerning the probability density during these months in 2022 is the elevated values observed in both the Singitic and Toronaic gulfs of the Chalkidiki peninsula, combined with low values in the eastern part of Lemnos. One plausible explanation may be linked to modifications in the southern branch of the Dardanelles flow into the North Aegean Sea (Androulidakis *et al*., 2011). These modifications could be attributed to a lower than climatological average intensity of the Etesian winds. Furthermore, another potential reason for the lack of larvae/particles landing on the east coast of Lemnos could be timing: the larval population in our experiments left the Dardanelles in May, which is two months prior to the onset of the Etesian winds. These winds play an essential role in the bifurcation and dynamics of the southern branch of the Dardanelles current. Thus, by the time that the Etesian winds commenced, the particles had already traversed to the Sea of Thrace through the Strait between Lemnos and Imbros islands, where they were predominantly captured by the Samothrace anticyclone. Furthermore, an additional factor potentially influencing the relatively low probability density of particle occurrence along the coasts of Lemnos Island is the Coriolis force-induced northward displacement of the Dardanelles outflow current core. This displacement occurred along the southern coast of Imbros Island during westward flow in the absence of constant Etesian winds, as substantiated by observations from the DARDANOS Hf radar (Kokkini *et al*., 2014; Kokkini *et al*., 2017). These intricate dynamics highlight the multifaceted interplay of environmental factors in shaping larval distribution patterns within the North Aegean Sea.

The hydrodynamic model CNAG ROMS and the Lagrangian tool (ROMSPath package) results, which attempted to simulate *P. nobilis* larvae dispersal, adequately reproduced the mean surface circulation of the Central/Northern Aegean Sea in both simulations setups, climatological and forecasting runs. The results of the offline Lagrangian experiments were positive proof of the validity of the simulated surface velocities fields. Potential discrepancies in the simulated particle positions, compared with the dispersal of *P. nobilis* larvae, can be attributed to our limited understanding of their behavior (Trigos *et al.,* 2018). This is especially pertinent considering that ROMS Path has the capacity to integrate such behavioral data in simulations, if available. Kersting et al. (2020) used an offline Lagrangian particle tool (backwards) to track possible larvae sources in the western Mediterranean. Although their results cannot be directly compared with ours (different areas, backward tracking instead of forward) the aforementioned study indicates/poses the potentially significant role of ocean circulation models and Lagrangian methods in order to investigate not only the fate of larvae but also, using the combined data, the sources, and possible unaffected populations.

Continuous monitoring and ongoing actions

Considering the thriving *P. nobilis* population in the Sea of Marmara and the remaining live population in Kalloni Gulf, 50 larval collectors were deployed in various locations in the North Aegean Sea in 2023, in an effort to track this year's recruitment. To increase the chances of successful settlement in 2023, two distinct types of settlement bags were employed on these larval collectors (entangled onion bags and entangled nylon fishing nets). The search for surviving *P. nobilis* populations is crucial to guide conservation measures and increase the species' chances of survival in Greek waters. To document the potential natural recovery of *P. nobilis* populations, continuous and systematic monitoring is essential, particularly focusing on the search for juveniles and recruits. Early detection of successful larval recruitment and juvenile survival in different areas is indispensable for assessing the species' population dynamics (Caley *et al.,* 1996; Kersting *et al.,* 2020).

In sites of Kalloni Gulf, where juvenile and adult *P. nobilis* have been observed growing under adverse conditions (such as depths shallower than 1 m with a high risk of stranding due to wave activity or in areas with intense boating or recreational activities), 28 vulnerable individuals were carefully transplanted deeper at the same sites or at sites characterized by more favorable environmental conditions, thus decreasing the risk of parasitic infection. The transplanted individuals, in addition to 40 juveniles and 11 adult *P. nobilis* individuals, were tagged to monitor their mortality and growth rates (Fig 9b). Additional vulnerable individuals will also be translocated to safer areas or introduced into potential new sanctuaries identified through ongoing monitoring efforts.

A series of temperature loggers has been deployed in Kalloni Gulf, aiming to gain a better understanding of the spatial variability of the temperature regime, the conditions that may trigger parasite spread and mortality, as well as identify conditions conducive to the survival of fan mussels. The University of the Aegean also maintains a permanent buoy in Kalloni Gulf, fully equipped with various sensors (such as CTD, pH, chlorophyll, nitrates, gamma-radiation), which will provide a time-series of the average conditions in the Gulf. In addition, tissue samples from surviving individuals, obtained through non-lethal sampling and from freshly dead individuals, will continue to be collected to monitor the presence of the haplosporidian parasite as well as other pathogens such as *Mycobacteria* sp. and *Vibrio mediterranei*.

Acknowledgements

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Appendix

Table S1. List of the 20 sites in Amvrakikos Gulf of Case Study 1, surveyed for mortality assessment of *P. nobilis* populations during the 36 45-minute protocol surveys (number of individuals) and the 7 Fixed-area surveys (individuals per 100m^2), conducted between June and August 2022. The date of the surveys and the data of dead-old, dead-fresh, and living individuals recorded at each site are presented. The red font of the data refers to the deep surveys (> 20 m). n/a: not applicable.

Site Coordinates (DD)		Site Name	Date of surveys (D/M/Y)	Dead - old	Dead -fresh	Alive	Mortality (%)
Latitude (N)	Longitude (E)						
39.032385	20.760184	St. Nikolaos	27/07/2022	0	0	0	n/a
39.030674	20.759546			0	0	0	n/a
39.039965	21.075786	Koperna		0	0	0	n/a
39.033606	21.077213			4	0	0	100.0
39.042325	21.115291	Menidi		3	0	0	100.0
39.042585	21.114267			0	0	2	0.0
38.921082	20.837293	Kamarina	28/07/2022	21	0	3	87.5
38.922493	20.838859			29	0	0	100.0
38.932596	20.943438	Manna Gea		31	12	175	19.7
39.006840	21.063220	Arachthos	30/07/2022	0	0	0	n/a
39.017577	21.069314			1	0	0	100.0
39.021795	20.940933	Fidokastro		1	0	3	25.0
39.028030	20.956855			0	0	2	0.00
38.976862	20.808422	St. Apostoloi		0	0	0	n/a
38.981549	20.804388			0	0	0	n/a
38.986069	20.919862	Vouvalos Isl		0	0	0	n/a
38.988337	20.916724			1	0	1	50.0
39.005056	21.144435	Katafourko (N)	31/07/2022	1	0	0	100.0
39.003547	21.145572			0	0	2	0.0
38.877940	21.090372	Vlycho		0	0	7	0.0
38.876754	21.086499			0	1	7	12.5
38.877838	**21.088430**		02/08/2022	**0**	**0**	**0**	**n/a**
38.921256	20.996054	Rouga		13	0	18	41.9
38.920534	20.998988			32	2	7	82.9
38.897610	21.019625	Drymos		15	3	5	78.3
38.896479	21.021028			28	2	7	81.1
39.032110	20.854226	Salaora	03/08/2022	0	0	0	n/a
39.031252	20.847574			0	0	0	n/a
39.019420	20.910166	Koronisia		0	0	0	n/a
39.022512	20.908428			0	0	0	n/a
39.047427	21.095180	Divaria		24	0	1	96.0
39.047077	21.090856			15	0	0	100.0
38.964779	21.145052	Arapis	04/08/2022	0	0	0	n/a
38.967390	21.147434			8	0	0	100.0
38.993566	21.151758	Katafourko (S)		2	0	0	100.0
38.995997	21.150690			10	0	1	90.9
38.903192	21.020127	Drymos	08/06/2022	0.0/100m^2	0/100m^2	0.0/100m^2	n/a
39.030034	21.122964	Menidi		0.25/100m^2	0/100m^2	0.22/100m^2	53.1
38.870929	21.071149	Loutraki		0.08 /100m^2	0/100m^2	0.08/100m^2	50.0
38.927807	21.024817	Akrotiri	28/07/2022	0.14/100m^2	0/100m^2	0.31/100m^2	30.8
38.939107	20.930089	Skorpios		0.02/100m^2	0/100m^2	0.08/100m^2	20.0
39.034321	20.762942	St. Nikolaos	11/09/2022	0.02/100m^2	0/100m^2	0.0/100m^2	100.0
39.031418	20.839816	Salaora		0.0/100m^2	0/100m^2	0.0/100m^2	n/a

Table S2. List of the 51 sites in Kalloni Gulf of Case Study 2, surveyed for mortality assessment of *P. nobilis* populations during the 45-minute protocol surveys conducted between October 2022 and May 2023. The coordinates, the codes, and the dates of the surveys as well as the data of dead-old, dead-fresh, living adults and living juvenile individuals, recorded at each site are presented. n/a: not applicable.

Site Coordinates (DD)		**Survey code**	**Date of surveys (D/M/Y)**	**Dead – old**	**Dead -fresh**	**Living adults**	**Living juveniles**	**Mortality (%)**
Latitude (N)	**Longitude (E)**							
39.105763	26.168593	1	24/10/2022	0	1	0	0	100.0
39.108712	26.170108	2		0	12	0	32	27.3
39.121897	26.182338	3		23	0	0	0	100.0
39.123895	26.184731	4		21	0	1	1	91.3
39.133074	26.212325	5		79	0	4	0	95.2
39.131676	26.216670	6		232	0	0	0	100.0
39.138745	26.231750	7	25/10/2022	111	0	11	1	90.2
39.139155	26.233788	8		116	0	0	0	100.0
39.154456	26.278736	9		123	0	1	0	99.2
39.156137	26.281504	10		56	0	0	0	100.0
39.177254	26.292497	11		38	0	0	0	100.0
39.178506	26.295480	12		38	0	0	0	100.0
39.198183	26.274548	13	26/10/2022	47	0	4	0	92.2
39.198482	26.269956	14		88	0	1	0	98.9
39.206805	26.244478	15		4	0	0	0	100.0
39.205812	26.246189	16		9	0	0	0	100.0
39.206300	26.244223	17		27	0	0	0	100.0
39.114226	26.174433	18	02/11/2022	4	0	0	1	80.0
39.156374	26.152673	19	03/11/2022	10	0	0	4	71.4
39.155459	26.151579	20		33	0	0	8	80.5
39.176271	26.155515	21		97	0	0	0	100.0
39.173738	26.155349	22		70	0	0	0	100.0
39.196984	26.170332	23		13	0	2	4	68.4
39.196614	26.168165	24		107	0	0	1	99.1
39.206339	26.204453	25	11/11/2022	19	0	0	0	100.0
39.206106	26.205987	26		41	0	0	0	100.0
39.201087	26.220559	27		0	0	0	0	n/a
39.202051	26.219701	28		4	0	0	0	100.0
39.187469	26.292486	29		52	0	2	0	96.3
39.186271	26.293237	30		178	0	0	0	100.0
39.186271	26.293237	31	06/03/2023	31	0	5	12	35.4
39.191881	26.164425	32		18	0	7	16	56.1
39.193564	26.16445	33		11	0	5	21	70.3
39.196506	26.182981	34		44	0	2	5	13.7
39.171667	26.155350	35	07/03/2023	8	0	3	3	42.9
39.130706	26.221622	36		4	0	1	2	42.9
39.165708	26.283067	37		16	0	2	3	23.8
39.168156	26.278661	38	12/04/2023	4	0	3	6	69.2
39.128591	26.203871	39		12	0	1	6	36.8
39.127449	26.199980	40		0	0	2	6	100.0
39.132428	26.212778	41	13/04/2023	3	0	1	3	57.1
39.132915	26.211800	42		5	3	1	5	42.9
39.114259	26.174799	43	14/04/2023	31	0	2	0	6.1
39.145406	26.249928	44	20/04/2023	11	0	1	7	42.1
39.150680	26.252554	45		29	1	2	2	11.8
39.192823	26.285703	46		8	0	1	2	27.3
39.202434	26.262532	47	21/04/2023	14	0	0	3	17.7
39.140901	26.235967	48		6	0	0	6	50.0
39.203103	26.196341	49	09/05/2023	19	0	0	4	17.4
39.206202	26.246702	50		4	0	3	11	77.8
39.197150	26.168471	51		24	0	2	7	27.3

Table S3. List of 11 areas of Case Study 3, surveyed for mortality assessment of *P. nobilis* populations for 69 45-minute protocol surveys conducted between May and September 2022. The dates, the numbers of surveys per site, and the data of dead-old, dead-fresh, and living individuals recorded at each site are presented. The red font color of the data refers to the deep surveys (>20 m). n/a: not applicable.

Sites Coordinates (DD)		**Site Name**	**Area**	**Date of surveys (D/M/Y)**	**No. of surveys**	**Dead - old**	**Dead -fresh**	**Alive**	**Mortality (%)**
Latitude (N)	**Longitude (E)**								
39.959799	25.326274	Ancient Ifestia	Lemnos Isl.	30/05/2022	2	1	0	0	100
39.891338	25.203753	Nea Koutali		31/05/2022	6	207	0	0	100
39.908217	25.264788	Lychnos			1	315	0	0	100
39.883929	25.269603	Koukonisi			1	0	0	0	100
40.974262	25.043244	Mandra	Porto Lagos	04/06/2022	3	12	0	0	100
40.999466	25.118174	Porto Lagos			1	0	0	0	n/a
40.976028	25.129375	Fanari		05/06/2022	3	175	0	0	100
36.949646	21.700298	Gialova	Gialova Lagoon	21/07/2022	2	0	0	0	n/a
36.954476	21.670194	Divari			2	0	0	0	n/a
36.914136	21.691759	Pylos			2	0	0	0	n/a
38.348057	21.314232	West Lagoon	Messolonghi Lagoon	23/07/2022	2	247	0	0	100
38.332060	21.383787	East Lagoon			2	11	0	0	100
38.326530	21.407948	Port			2	28	0	0	100
38.323916	21.427361	Salt Pans			2	0	0	0	n/a
38.303175	21.255962	Louros		25/07/2022	2	0	0	0	n/a
38.393757	21.116542	East Bay	Petalas Bay	24/07/2022	1	80	0	0	100
38.399508	21.108731	West Bay			1	54	0	0	100
38.409319	21.105862	North Bay			2	10	0	0	100
39.528910	20.140329	Kalamas Delta	Igoumenitsa	06/08/2022	2	0	0	0	n/a
39.517390	20.169061	Erimitis			2	1	0	0	100
39.627253	20.177627	Sagiada	Sagiada	07/08/2022	2	0	0	0	n/a
39.465298	**19.979853**	Kalami	Corfu Isl.	08/08/2022	**1**	**0**	**0**	**0**	**n/a**
39.472754	19.940243	Alykes		09/08/2022	2	0	0	0	n/a
39.444251	20.083783	Psaras			2	0	0	0	n/a
39.422130	19.954865	St. George		10/08/2022	2	0	0	0	n/a
38.031450	**22.887953**	NE Lake	Vouliagmeni Lake	07/09/2022	**1**	**0**	**0**	**0**	**n/a**
38.030580	**22.878984**	NW Lake			**1**	**0**	**0**	**0**	**n/a**
38.026820	**22.891945**	SE Lake		08/09/2022	**1**	**0**	**0**	**0**	**n/a**
38.025392	**22.884242**	SW Lake			**1**	**0**	**0**	**0**	**n/a**
38.829772	22.615802	Zoodohou Pigis	Maliakos Gulf	10/09/2022	2	17	0	0	100
38.833976	22.643096	Molos			2	36	0	0	100
38.886871	22.569223	W. St. Marina		11/09/2022	2	30	0	0	100
38.889919	22.579801	E. St. Marina			2	109	0	0	100
38.904976	22.626339	Stylida			2	186	0	0	100
38.781890	**22.809323**	Kamena Vourla		12/09/2022	**1**	**0**	**0**	**0**	**n/a**
39.188855	23.926754	St. Petros	Alonnisos	19/09/2022	1	4	0	0	100

Table S4. *Pinna nobilis* larvae collector network and the recruitment data in Northern Aegean Sea, Greece and Amvrakikos Gulf in 2022.

Coordinates (DD)		Site	Code	Depth	No of bags	Installation Date	Recovery Date	Recruits
Latitude (N)	Longitude (E)							
25.358563	39.784671	Lemnos	LC L1	5m	2	29/05/2022	missing/damaged	×
25.352208	39.796144	Lemnos	LC L2	5m	2	29/05/2022	missing/damaged	×
25.356131	39.827646	Lemnos	LC L3	5m	2	29/05/2022	8/10/2022	0
25.351984	39.853477	Lemnos	LC L4	6m	2	29/05/2022	8/10/2022	0
25.342803	39.878091	Lemnos	LC L5	5m	2	29/05/2022	8/10/2022	0
25.367790	39.891839	Lemnos	LC L6	6m	2	29/05/2022	missing/damaged	×
25.412854	39.960946	Lemnos	LC L7	5m	2	30/05/2022	missing/damaged	×
25.415698	40.020542	Lemnos	LC L8	6.5m	2	30/05/2022	9/10/2022	0
25.414568	40.019911	Lemnos	LC L9	6.5m	2	30/05/2022	9/10/2022	0
25.368192	40.001668	Lemnos	LC L10	5m	2	30/05/2022	9/10/2022	0
25.452903	40.467558	Samothrace	LC S1	6m	2	02/06/2022	14/10/2022	0
25.472761	40.450500	Samothrace	LC S2	6m	2	02/06/2022	14/10/2022	0
25.505745	40.427089	Samothrace	LC S3	6m	2	02/06/2022	14/10/2022	0
25.524715	40.420736	Samothrace	LC S4	6m	2	02/06/2022	14/10/2022	0
25.540423	40.414532	Samothrace	LC S5	6m	2	02/06/2022	14/10/2022	0
25.556624	40.399242	Samothrace	LC S6	6m	2	02/06/2022	13/10/2022	0
25.609631	40.397975	Samothrace	LC S7	6m	2	02/06/2022	13/10/2022	0
25.641707	40.408223	Samothrace	LC S8	6m	2	02/06/2022	13/10/2022	0
25.682117	40.419300	Samothrace	LC S9	6m	2	02/06/2022	13/10/2022	0
25.697163	40.443277	Samothrace	LC S10	6m	2	02/06/2022	13/10/2022	0
24.512787	40.641622	Thasos	LC TH1	6m	2	07/6/2022	missing/damaged	×
24.579114	40.622663	Thasos	LC TH2	5m	2	07/06/2022	16/10/2022	0
24.633202	40.581620	Thasos	LC TH3	5m	2	07/06/2022	16/10/2022	0
24.639155	40.580814	Thasos	LC TH4	6m	2	08/06/2022	15/10/2022	0
24.723717	40.603160	Thasos	LC TH5	5m	2	08/06/2022	missing/damaged	×
24.766880	40.664903	Thasos	LC TH6	6m	2	08/06/2022	missing/damaged	×
25.712319	40.847499	Alex/poli	LC AP1	5m	2	02/06/2022	12/10/2022	0
25.730365	40.844447	Alex/poli	LC AP2	7m	2	02/06/2022	12/10/2022	0
25.744066	40.845981	Alex/poli	LC AP3	7m	2	02/06/2022	12/10/2022	0
25.761419	40.845722	Alex/poli	LC AP4	7m	2	02/6/2022	12/10/2022	0
26.174532	39.114191	Lesvos	LC K1	5m	2	22/05/2022	02/11/2022	0
26.244710	39.205210	Lesvos	LC K2	5m	2	01/05/2022	30/11/2022	0
26.222540	39.200970	Lesvos	LC K3	5m	2	01/05/2022	30/11/2022	0
26.190330	39.198740	Lesvos	LC K4	5m	2	01/05/2022	30/11/2022	0
26.269900	39.197180	Lesvos	LC K5	5m	2	01/05/2022	30/11/2022	0
26.290230	39.417114	Lesvos	LC K6	8m	3	01/05/2022	30/11/2022	0
26.208375	39.167743	Lesvos	LC K7	12m	4	16/06/2022	missing/damaged	×
26.114306	39.103820	Lesvos	LC K8	6.5m	2	16/06/2022	missing/damaged	×
26.115704	39.098885	Lesvos	LC K9	6.5m	2	16/06/2022	missing/damaged	×
26.572734	39.086194	Lesvos	LC U	5m	2	20/06/2022	23/12/2022	0
38.926346	21.026885	Amvrakikos	LC AV1	20m	3	28/7/2022	31/10/2022	12
38.941639	20.928798	Amvrakikos	LC AV2	22m	3	28/7/2022	missing/damaged	×

Table S5. Primers used for the amplification of COI gene fragment at *Pinna* sp. samples. Samples were collected at the site of Akrotiri (see Table S1).

Primer name	**Primer Sequence (5' – 3')**	**Reference**
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.*, 1994
HCO2198	TAAACTTXAGGGTGACCAAAAAATCA	Folmer *et al.*, 1994
PnCOI_F_KTSR08	CCCTGCCAAATTACACCAGT	Katsares *et al.*, 2008
PnCOI_R_KTSR08	TTTTGGCTTTTGCCTTCTTC	Katsares *et al.*, 2008
PnCOI_Fdg_KTSR08	CCCTAGCCAAAATTACACCAGT	Katsares *et al.*, 2008
PnCOI_Rdg_KTSR08	GAAGAAGGCAAWAGCCAAAA	Katsares *et al.*, 2008
PnCOI_F_MK22	CAACACAGGAAGAGAGACTACCA	this study
PnCOI_R_MK22	GGCAGGGTTTTTGGGGGA	this study
PnCOI_L_SAN13	GGTTGAACTATHTATCCNCC	Sanna *et al.*, 2013
PnCOI_H_SAN13	GAAATCATYCCAAAAGC	Sanna *et al.*, 2013
Pmur_COIF_KTSR08	GAAAGTGCCCGGTAACAAAA	Katsares *et al.*, 2008
Pmur_COIR_KTSR08	TGATAGGGGTTCCGGATATG	Katsares *et al.*, 2008
Pmur_COIFdg_KTSR08	GAAAGTGCCCRGTWACAAART	Katsares *et al.*, 2008
Pmur_COIRdg_KTSR08	CATATCYGGMACCCCTATCA	Katsares *et al.*, 2008

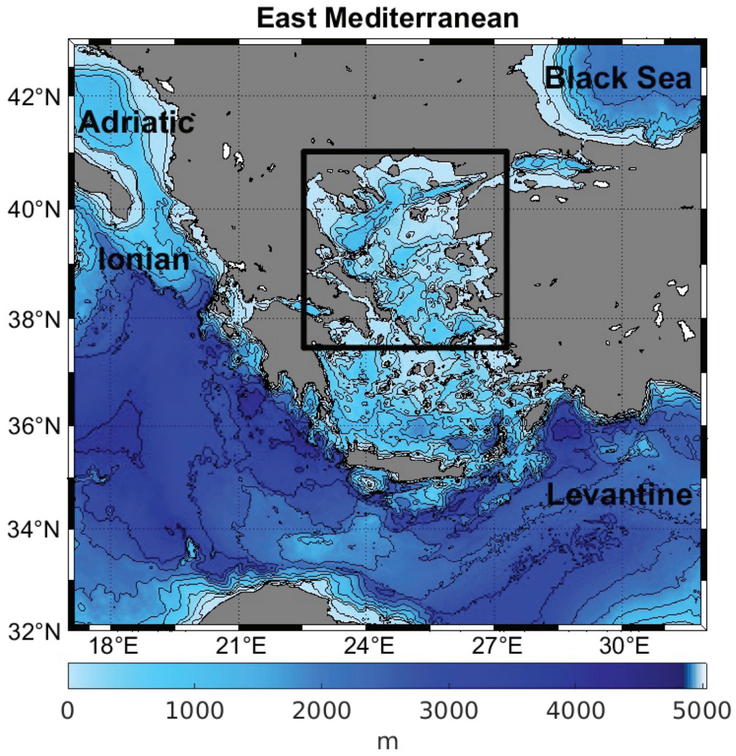

Fig. S1: Bathymetric map of the Eastern Mediterranean and its sub basins. The black rectangle denotes the model's geographical extent.

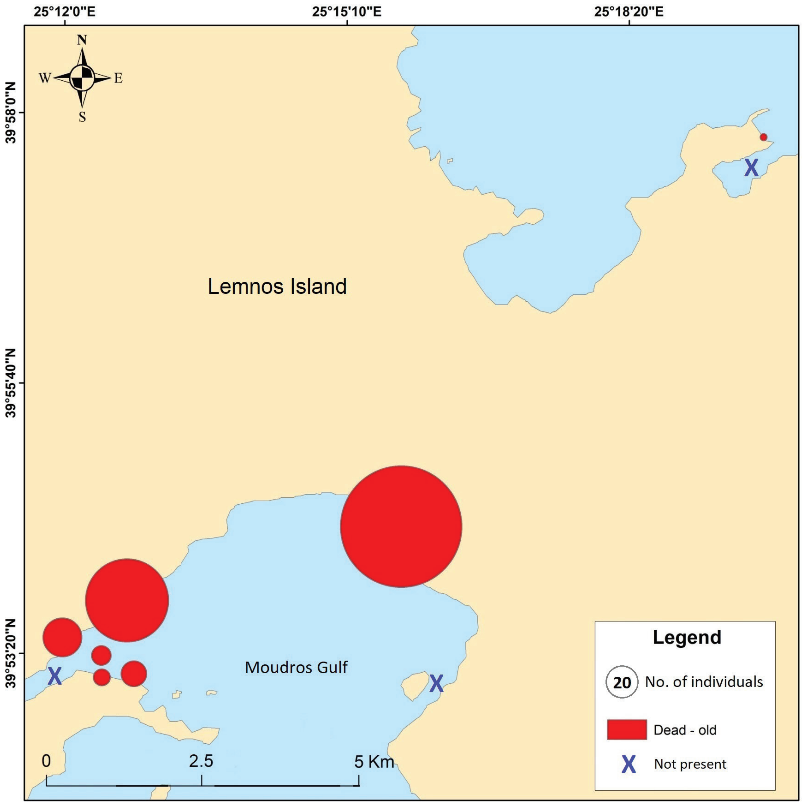

Fig. S2: Map of Lemnos Island depicting the assessment of *Pinna nobilis* populations, during 10 surveys conducted in May 2022. The sizes of the pie charts represent the number of recorded individuals. The red colour represents the old-dead individuals. "X" represents the sites where no individuals were recorded.

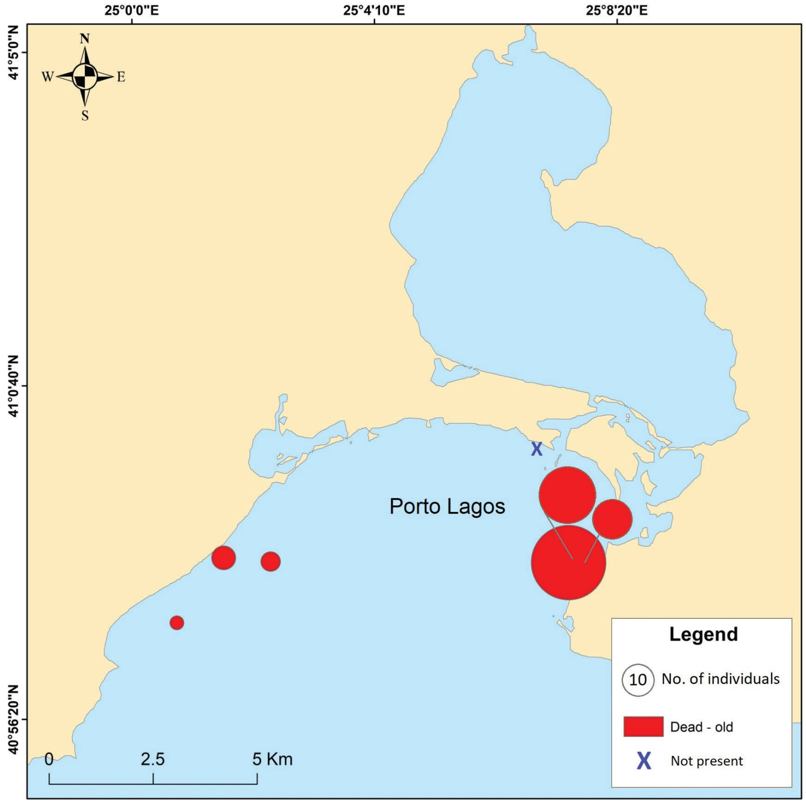

Fig. S3: Map of Porto Lagos depicting the assessment of *Pinna nobilis* populations, during 7 surveys conducted in June 2022. The sizes of the pie charts represent the number of recorded individuals. The red colour represents the old-dead individuals. "X" represents the sites where no individuals were recorded.

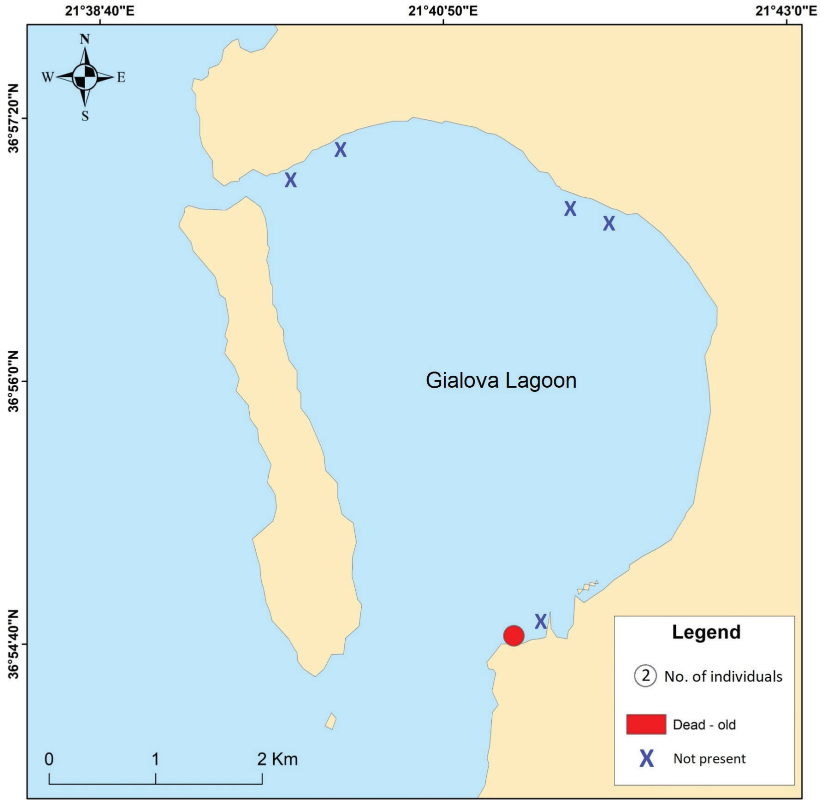

Fig. S4: Map of Gialova Lagoon depicting the assessment of *Pinna nobilis* populations, during 6 surveys conducted in July 2022. The sizes of the pie charts represent the number of recorded individuals. The red colour represents the old-dead individuals. "X" represents the sites where no individuals were recorded.

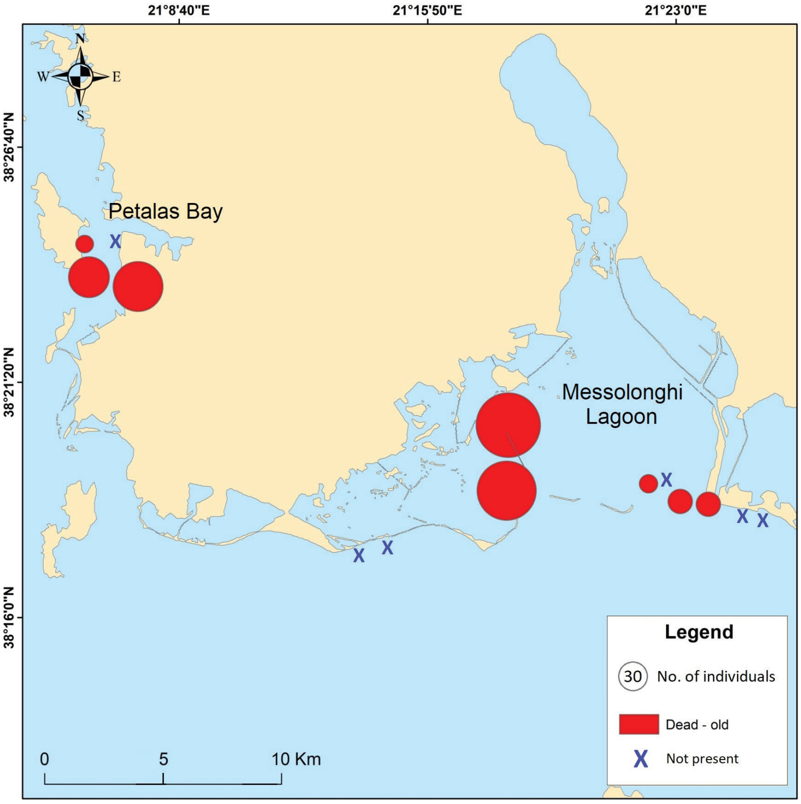

Fig. S5: Map of Messolonghi Lagoon and Petalas Bay, depicting the assessment of *Pinna nobilis* populations, during 10 & 4 surveys respectively, conducted in July 2022. The sizes of the pie charts represent the number of recorded individuals. The red, colour represents the old-dead individuals. "X" represents the sites where no individuals were recorded.

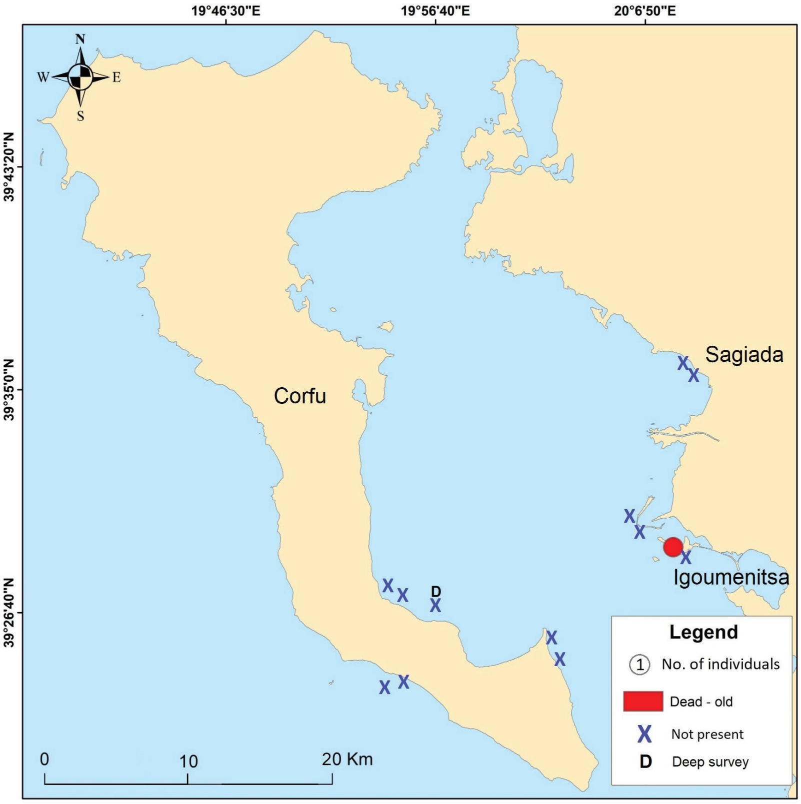

Fig. S6: Map of Igoumenitsa, Sagiada, and Corfu, depicting the assessment of *Pinna nobilis* populations, during 4, 2 & 7 surveys respectively, conducted in August 2022. The sizes of the pie charts represent the number of recorded individuals. The red colour represents the old-dead individuals. "X" represents the sites where no individuals were recorded, and "D" the deep surveys (> 20m).

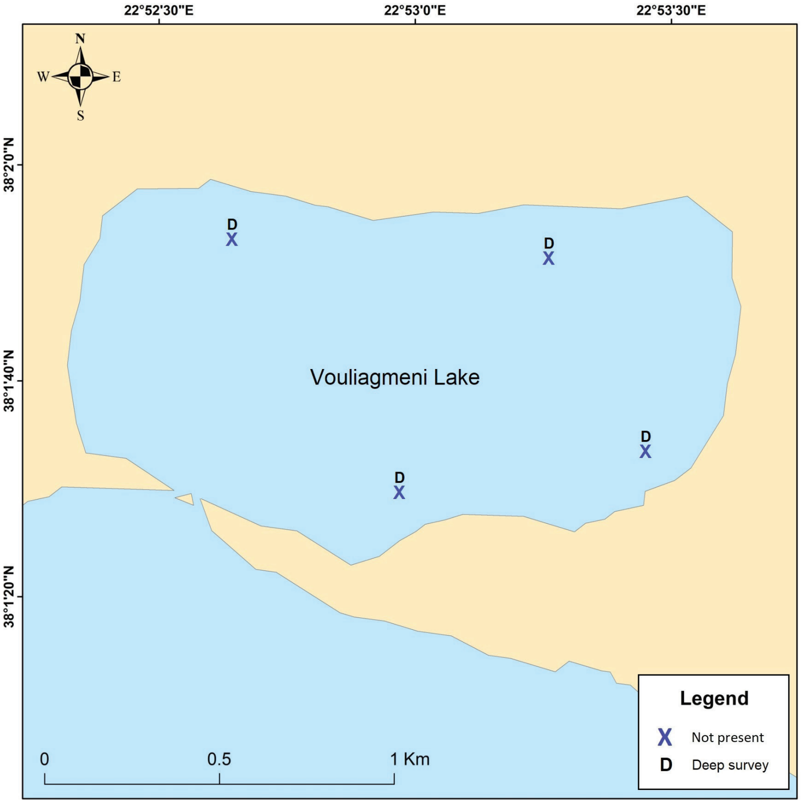

Fig. S7: Map of Vouliagmeni Lake, depicting the assessment of *Pinna nobilis* populations during 4 deep water surveys (> 20m), conducted in September 2022. "X" represents the sites where no individuals were recorded.

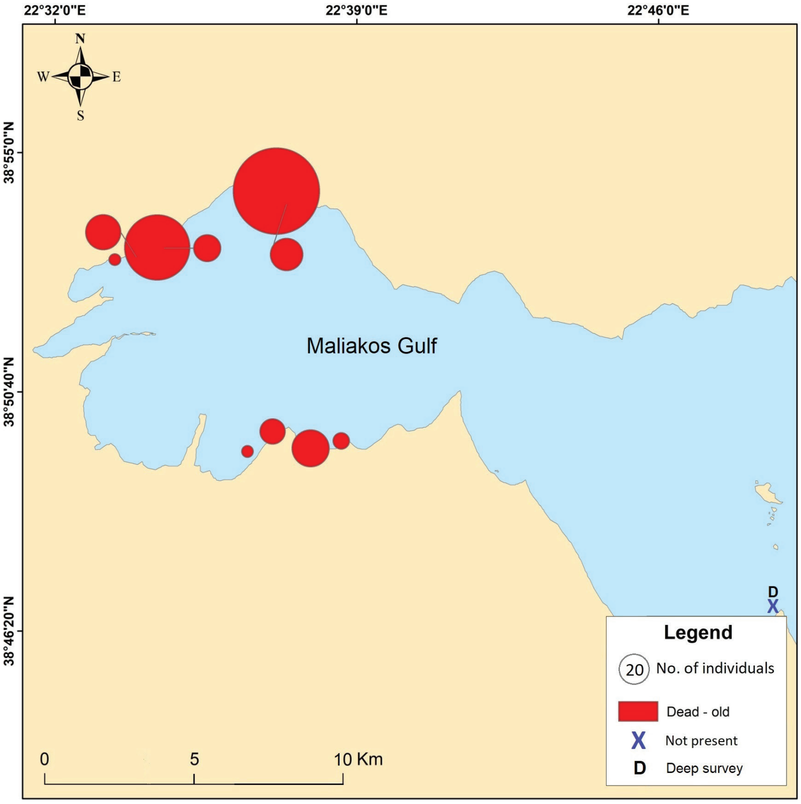

Fig. S8: Map of Maliakos Gulf, depicting the assessment of *Pinna nobilis* populations, during 11 surveys, conducted in September 2022. The sizes of the pie charts represent the number of recorded individuals. The red, colour represents the old-dead individuals. "X" represents the sites where no individuals were recorded, and "D" the deep surveys (>20m).

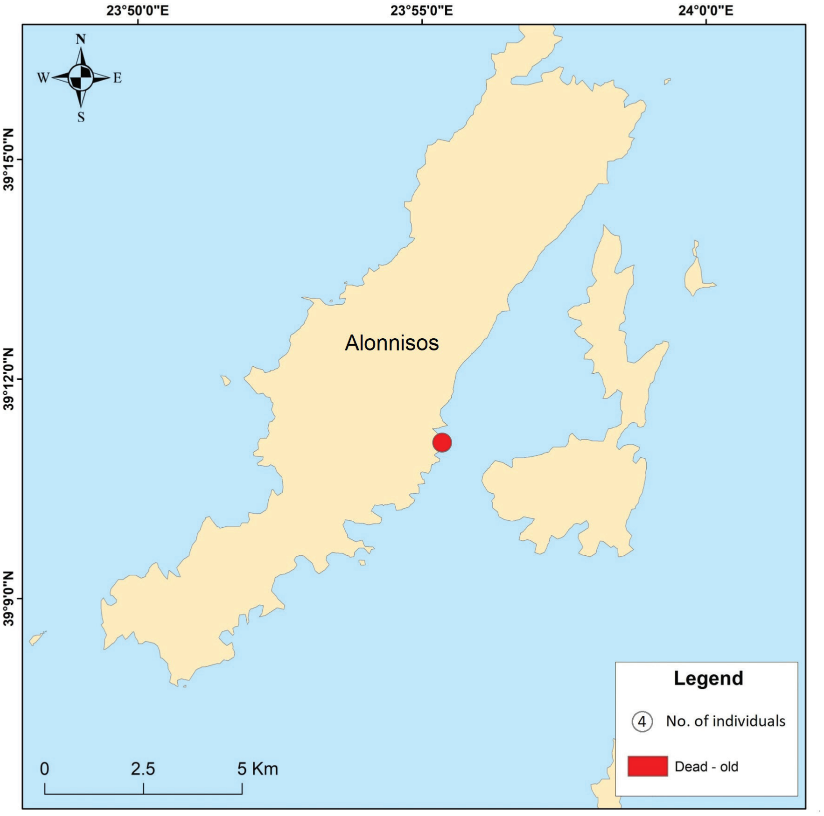

Fig. S9: Map of Alonnisos Island, depicting the assessment of *Pinna nobilis* populations during one survey, conducted in September 2022. The size of the pie chart represents the number of recorded individuals. The red colour represents the old-dead individuals.