UN environment programme

Mediterranean Action Plan
Barcelona Convention

SPA/RAC

Draft RESTORATION PROGRAMME for *Pinna nobilis*

Tunis, Tunisia June 2022

Legal Notice

The designations employed and the presentation of the material in this document do not imply the expression of any opinion whatsoever on the part of Specially Protected Areas Regional Activity Centre (SPA/RAC) and United Nations Environment Programme concerning the legal status of any State, Territory, city or area, or of its authorities, or concerning the delimitation of their frontiers or boundaries.

United Nations Environment Programme / Mediterranean Action Plan (UNEP/MAP)
Specially Protected Areas Regional Activity Centre (SPA/RAC)
Boulevard du Leader Yasser Arafat B.P. 337 - 1080 Tunis Cedex - Tunisia
E-mail: car-asp@spa-rac.org

For bibliographic purposes this volume may be cited as:

SPA/RAC-UNEP/MAP, 2022. Draft restoration programme of Pinna nobilis. By Saul CIRACO, carlo FRANSOSINI, Atef OUERGHI, Ed. SPA/RAC, Tunis: Ed. SPA/RAC, Tunis: pages 15 pp+ Annexes.

Table of contents

FOREWORD

1. Elaborating and implementing action plans to conserve one species or group of species and or restoration programme is an effective way of guiding, coordinating and strengthening the efforts that the Mediterranean countries are making to safeguard the natural heritage of the region and fulfil their obligation under the new 1995 Barcelona Convention Protocol Concerning Specially Protected Areas and Biological Diversity in the Mediterranean (SPA/BD Protocol).

2. Pinna nobilis is a long-lived Mediterranean endemic species, considered one of the biggest bivalve molluscs in the Mediterranean Sea. It has a wide distribution across coastal areas, occurring mainly in seagrass meadows, but also present in other habitats such as rocky bottoms or rhodoliths beds.

3. A mass mortality event affecting Pinna nobilis populations was first detected in 2016 along the Spanish coast. The still ongoing mortality outbreak has been found to be caused by a pathogen, which rapidly spread throughout the Mediterranean Sea causing mortality rates of 80-100% across many regions.

4. A First online meeting of Mediterranean partners to coordinate a response to Pinna nobilis crisis, facilitated by IUCN-Med, to present the latest mortality data and progress to recover the Critically Endangered (CR) populations of Pinna nobilis, now included on the IUCN Red List of Threatened Species. The role of unaffected populations for a potential recovery, established with a network of larval collector stations to enhance larval dispersal from unaffected sites and potential recolonization through recruitment of resistant juveniles was also discussed

5. In this context, the Specially Protected Areas Regional Activity Centre (SPA/RAC) of the United Nations Environment Programme / Mediterranean Action (UNEP/MAP) Barcelona Convention, is implementing a project funded by the UNEP Regional Seas Programme - 2021 Swedish International Development Cooperation Agency (SIDA) allocation in the Mediterranean sub-basin, to contribute to the restoration of Pinna nobilis a species of the Annex II “List of endangered or threatened species” of the Protocol concerning Specially Protected Areas and Biological Diversity in the Mediterranean of the Barcelona Convention.

6. This project has two major actions. The first is related to the elaboration of draft restoration programme for Pinna nobilis and its discussion and validation during a two-day regional workshop (Tunisia, 20-21 June 2022). The second action is related to the organisation of a regional hands-on training on juveniles’ collection from identified sites and their transportation in rearing sites (Kerkennah Islands, Tunisia, 28-30 June 2022).

7. In the implementation of its project, SPA/RAC is partnership with the Life Pinna Project consortium “Conservation and re-stocking of the Pinna nobilis in the western Mediterranean and Adriatic Sea” coordinated by the regional agency for the protection of the Ligurian environment (Italy) and supported by the European Union (EU) Life Programme, drafted the present proposal for a restoration programme for Pinna nobilis, which should be discussed and validated during a regional workshop, before being speared and disseminated to the Mediterranean region, through the adequate fora of the Barcelona Convention, for endorsement and implementation.

INTRODUCTION

1. The fan mussel *Pinna nobilis* (Linnaeus, 1758) is the largest endemic bivalve of the Mediterranean Sea. *P. nobilis* occurs in soft-bottom habitats of transitional water ecosystems and in marine coastal zones at depths between 0.5 and 60 m, mostly in seagrass meadows of *Posidonia oceanica* or *Cymodocea nodosa* (Zavodnik et al. 1991, Richardson et al. 1999, García March et al. 2007, Orfanidis et al. 2007, Coppa et al. 2010; 2013, Prado et al. 2014), but also in bare sandy bottoms (Katsanevakis 2005). This species is an important benthic filter feeder contributing to water clarity, and a "conservation species", playing the roles of flagship, key and umbrella species.

2. Due to its ecological relevance, P. nobilis has recently been suggested as being a reliable bioindicator for benthic coastal ecosystems according to the Descriptor 1 "Biological diversity" and 4 "Status of the single structural components of ecosystems" of the EU Marine Strategy Framework Directive (MSFD 2008/56/EC). It could be also used in the implementation of the Integrated monitoring and assessment programme of the Mediterranean Sea and coast and related assessment criteria (IMAP)

3. The Pinna nobilis facies that could characterize the infralittoral sands or muddy sands is part of the reference list of species and habitats to be monitored in the framework of the Barcelona Convention's Integrated Monitoring and Assessment Programme of the Mediterranean Sea and Coast and Related Assessment Criteria (Decision IG.22/7).

4. In addition, the fan mussel represents the host for two crustacean symbionts (i.e., Pontonia pinnophylax and Nepinnotheres pinnotheres) (Rabaoui et al. 2008) and it is also predated by other species, such as for instance Octopus vulgaris, playing a key role in the trophic web.

5. During the 80s, populations of *P. nobilis* greatly declined due to several human activities (i.e., fishing, ornamental harvesting, anchoring, and trawl nets). As a consequence, *P. nobilis* is nowadays a protected species under Annex II "List of endangered or threatened species" to the Protocol concerning Specially Protected Areas and Biological Diversity in the Mediterranean of the Barcelona Convention and. the Annex IV of the EU Habitats Directive 92/43/EEC (EEC 1992) and

6. In a few decades, this full regime protection led to a complete recovery of the species in the whole Mediterranean, as it was also evidenced by molecular analyses (Sanna et al. 2013; 2014). Unfortunately, in early autumn 2016 a mass mortality event (MME) impacted P. nobilis populations in the south-western Mediterranean Sea (Vázquez-Luis et al. 2017). Since then, the situation has worsened, gradually affecting the Spanish, French, Italian, Tunisian, and perhaps Turkish coasts. In Italy for example, from Sardinia to Sicily, from Apulia to Tuscany, fan mussels are dying. The protozoan Haplosporidium pinnae, a pathogenic micro-organism that affects the digestive system of the mollusk progressively reducing the feeding of the animal and causing its death, was initially imputed as the main cause of this mass mortality (Catanese et al. 2018, Panarese et al. 2019). However, recently several bacteria species have been also invoked as pathogens involved in the mass mortality of this species (Carella et al. 2019, Prado et al. 2020, Scarpa & Sanna et al. submitted) suggesting that the real causes of the mortality are not completely understood and that a multifactorial disease may be the most probable responsible factor.

CASE STUDIES

MERCES project – Croatia, Italy, Turkey

7. MERCES project "Marine Ecosystem Restoration in Changing European Seas", coordinated by the Università Politecnica delle Marche (Italy), has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No-689518. The project was focused on the restoration of different degraded marine habitats, with the aim of: 1) assessing the potential of different technologies and approaches; 2) quantifying the returns in terms of ecosystems services and their socio-economic impacts; 3) defining the legal-policy and governance frameworks needed to optimize the effectiveness of the different restoration approaches. Specific aims include a) improving existing, and developing new, restoration actions of degraded marine habitats; b) increasing the adaptation of EU degraded marine habitats to global change; c) enhancing marine ecosystem resilience and services; d) conducting cost-benefit analyses for marine restoration measures; e) creating new industrial targets and opportunities. To achieve these objectives, MERCES created a multi-disciplinary consortium with skills in marine ecology, restoration, law, policy and governance, socioeconomics, knowledge transfer, dissemination and communication. MERCES started from the inventory of EU degraded marine habitats (WP1), conducted pilot restoration experiments (WP2, WP3, WP4), and assessed the effects of restoration on ecosystem services (WP5).

8. MERCES Work Package 2 (WP2) focuses on shallow soft-bottom habitats, especially seagrass meadows and bivalve reefs. Using a combination of field surveys, aquarium and field experiments, and case studies, WP2 aimed to:
(a) determine the factors affecting seagrass restoration success,
(b) test whether integrating feedbacks and interactions in restoration increases success rates, and
(c) provide recommendations for managers and policymakers.

9. MERCES WP2 included 9 research groups in 7 countries (Croatia, Estonia, Finland, Italy, Netherlands, Norway, Turkey). In Northern European seas (Baltic Sea, North Sea, Wadden Sea), test species include eelgrass (Zostera marina), dwarf eelgrass (Z. noltii), blue mussels (Mytilus edulis) and Baltic clams (Macoma balthica). In Southern Europe (Adriatic Sea, Eastern Mediterranean), researchers are restoring the seagrasses Cymodocea nodosa and Posidonia oceanica and the endangered noble pen shell Pinna nobilis.

10. Considering the Southern Europe pilot actions several activities have been conducted. Among them very interesting was the Seagrass-bivalve co-restoration using *Pinna nobilis*, Cymodocea and Zostera. The main question was if planting seagrass and *P. nobilis* together could increase the survival and growth of either or both species? Can transplantation of *P. nobilis* in existing meadows increase the growth/survival of the seagrasses? The experiments were conducted in two different sites (Italy and Croatia).

11. In Italy, *P. nobilis* transplanting was performed using U-shaped stainless-steel rods. First of all, a housing for the transplanting bivalve was prepared in the seabed using a corer. After that, the hole was partially filled with pebbles and the bivalve was anchored with the steel rod. Nine *P. nobilis* specimens have been transplanted in three experimental plots (1x1m): three specimens in bare sediments, three specimens in natural seagrass meadows and three specimens in transplanted seagrasses. *P. nobilis* abundance: 1 ind./m2 per each experimental plot. Seagrass transplantation using biodegradable bags. The experimental treatments included transplanting seagrass, transplanting seagrass and *P. nobilis* and existing seagrass as a control. Each experimental plot (1x1 m, n=3). The presence of seagrass favoured the survival of *P. nobilis* specimens while the severe hydrodynamic conditions occurred immediately after the beginning of the experiment have limited the success of the seagrass transplanting. The proposed method of anchorage for *P. nobilis* specimens resulted to be efficient. Plots with *P. nobilis* into existing seagrass meadows showed higher organic matter concentrations immediately after the translocation of bivalves. No differences among experimental plots in terms of meiofaunal abundance and diversity were observed immediately after the beginning of the experiment. Environmental conditions immediately after translocation play a key role in the survival of *P. nobilis* and transplanted seagrasses. The presence of natural seagrass acts as a barrier for *P. nobilis* reducing the severe hydrodynamic conditions and avoiding possible burial effects. The presence of *P. nobilis* may increase the availability of food for benthic fauna associated

with seagrasses meadows. Considering the results of Croatian site transplanting *P. nobilis* within seagrass meadow enhances its survival in exposed areas, given that transplantation is (ideally) carried out during early summer, thus providing enough time for pen shells to regenerate byssus and anchor well, prior to winter storms. Furthermore, transplanting pen shells in high density (e.g. 5 ind./m2) may enhance C. nodosa growth through a putative fertilization effect.

12. A further question was addressed by the project: Can covering with cage help Pinna establish after translocation? For the experiment conducted in Turkey, *P. nobilis* translocation was done by collecting small individuals from the vicinity and digging out with 50 cm radius and 50-60 cm deep sediment to protect the byssus as much as possible. All individuals were then transferred by covering the attached sediment with a plastic bag and carried underwater. They were placed and covered with their original sediment, and no support was used. After 1x1x0.5 m cages were used to cover the individuals. Transplanted *P. nobilis* individuals were alive and healthy after the winter and spring periods. Some new individuals were observed in spring on both cage covered and uncovered plots and few on the frame of the cages. However, in July 2018, due to parasite infection all individuals were either looking unhealthy (slowly closing their shell) or even dead. It was observed that cages help pen shells to anchor after translocations and promote recruitment of new individuals, but a solid conclusion cannot be made due to disease outbreak that wiped out a large portion of the Mediterranean *P. nobilis* population.

13. Main conclusion for MERCES (Manual of restoration measures in soft bottoms based on surveys and experiments WP2 Deliverable 2.1) was that in southern European habitats (Mediterranean), mutual facilitation of *P. nobilis* and a seagrass was observed and transplanting *P. nobilis* within seagrass meadow enhances seagrass survival, especially in exposed areas. Furthermore, transplanting *P. nobilis* at a density of 5 ind./m2 may enhance C. nodosa growth through fertilization. The presence of natural seagrass acts as a barrier reducing the severe hydrodynamic stress for P. nobilis and avoiding possible burial effects. Conversely, the presence of *P. nobilis* may increase the availability of food for benthic fauna associated with seagrasses meadows. In other words, bivalve facilitation may not only enhance seagrass restoration, but the interactions between bivalves and seagrass proved positive for both species.

RESTORFAN project – Italy

14. Thanks to the MedPAN Small Projects financial contribution, in 2019 the RESTORFAN project was carried out within the Miramare Marine Protected Area (MPA), in Italy. All the specific objectives of the project were based on the currently available information and the experts knowledge gathered during several meetings; the proposal aimed to satisfy all the IUCN recommendations and results of the first meeting of Mediterranean partners to coordinate a response to Pinna nobilis crisis (online, Fabruary 2021), as the Northern Adriatic Sea and particularly the Gulf of Trieste (Italy) represent key areas for early action and rapid implementation of conservation measures.

15. The specific objectives were:

1. Increasing international scientific knowledge (by means of new research and papers) on the species.
2. Test of an experimental hatchery/culture, with specimens coming from mussel farms, finalized to the organization of a Rescue Programme as requested by IUCN Guidelines. Indeed, according to IUCN guidelines, the development of a rescue programme close to the affected areas is paramount and it should be developed as soon as possible in areas where there is an important density of Pinna nobilis and the parasite has confirmed not arrived.
3. According to the goal - “raise the issue at national level and advocate for the development of a rescue programme”, Miramare MPA was proponent of several meetings among all the local main actors, to promote the development of a rescue programme. Within this context RESTORFAN developed a protocol, in compliance with IUCN guidelines, for the local/basin rescue programme for Pinna nobilis.
4. “Collaborate in the identification of Pinna nobilis hotspots” in the entire region. A density map has been prepared to represent the most relevant hotspots at Friuli Venezia Giulia

scale to support the future evaluations. A proposal of a monitoring programme for these "hot sites" has been produced and delivered to regional authorities (Friuli Venezia Giulia, Italy).

16. Among the main results of the project is certainly the development of the protocol for the recovery and transplantation of the juvenile specimens collected in the mussel farmers' longlines. The arrival of mass mortality during the project strongly influenced the actions by pushing for a strong action of awareness raising and search for survivors. The data collected were used for the realization of thematic maps of the gulf of Trieste. A further result of the project was the network of relationships with researchers and MPAs that led to the preparation of the LIFE Pinna project, which was then financed by the LIFE programme.

LIFE PINNA project – Italy, Slovenia

17. Funded by the contribution of the LIFE programme, the European Union's financial instrument supporting environmental, nature conservation and climate action projects. The aim of the LIFE PINNA 1project is to repopulate the areas identified in the project with healthy individuals, survivors of the mass die-off that started in 2016. In particular, the areas involved are the Gulf of Trieste, as a donor site, the MPA of Bergeggi (Liguria, Italy) and the MPA of Asinara (Sardinia, Italy) as recipient sites. Survivors are likely to be characterized by natural resistance to the pathogens responsible for the disease outbreak. Some analysis of the level of pathogenic infection in the tissues of surviving or dying individuals will be conducted to identify microorganisms that are involved in the disease. In addition, considering that proper identification of the pathogens causing mass mortality is a crucial point in setting up adequate recovery plans for this species, it is also important to assess the level of contamination/infection occurring where the mussels died and where they survived. Repopulation actions will be carried out with transplantation of juvenile organisms, and in parallel protocols for captive breeding of adult organisms will be developed. The organisms derived from this artificial insemination will be used to repopulate the affected areas.

18. The specific objectives include:

- Analysis and selection of marine or transitional areas appropriate for restocking;
- Molecular characterisation of surviving specimens and selection of the best candidates to be reproduced;
- Development and implementation of the most suitable repopulation techniques, through translocation of self-recruited juveniles and captive breeding of P. nobilis in order to release a large number of specimens into the wild in a few years;
- Maintenance of a good level of genetic variation among the individuals used for restocking in order to obtain offspring that will be the founders of new future populations with good fitness in the long term;
- Monitoring of donor sites to evaluate the status of P. nobilis (including citizen science actions);
- Monitoring of "sentinel" organisms for the infection level of pathogens responsible for mass mortality of P. nobilis, to quickly detect anomalous values that are potentially dangerous for the species' survival;
- Public engagement to increase awareness on P. nobilis and influence sea users' behavior; and
- Transfer and replication of skills and methodologies to areas where the fan mussel is decreasing.

[1] website: http://lifepinna.eu/

LIFE PINNARCA project – France, Greece, Italy, Spain

19. LIFE PINNARCA[2] is a European project devoted to the protection and restoration of the fan mussel Pinna nobilis populations in the Mediterranean Sea. It has been conducted with the contribution of the LIFE programme, the European Union's financial instrument supporting environmental, nature conservation and climate action projects.

20. To project team focus on three main objectives:

1) Increasing awareness on a global scale, to reduce the possibility of vandalism and illegal collection of the remaining fan mussels, but also to call for broad public collaboration. Actions will be oriented at schools and the general public, including the production of a video, international workshops and volunteering actions;
2) Gathering all existing information on the remaining populations and resistant individuals into a database integrated within the project's website, to provide information to other countries planning mitigation and recovery actions. This objective will be achieved by implementing a comprehensive census of areas where resistant individuals or unaffected populations are found, as well as installing larvae collectors to assist successful recruitment;
3) Developing active recovery actions, focused both on resistant individuals and the remaining non-resistant populations, to increase the probabilities of recovery of the species. This objective involves efforts to aggregate resistant individuals, translocate vulnerable individuals to safer areas, exchange genetic information among remaining populations, identify locations with optimal conditions to repopulate with healthy fan mussels, maintain individuals in indoor facilities, and develop active measures to improve the environments where healthy non-resistant individuals are still found.

21. All project selected areas host habitats appropriate for Pinna nobilis populations, including from healthy *Posidonia oceanica* meadows (in all of them except the Columbretes Islands, Spain) to enclosed bays with gentle hydrodynamic conditions or deeper maërl beds, with optimum substrate and conditions for maintaining fan mussels. These areas also hosted dense fan mussel populations before the mass mortality event (MME) and had some permanent monitoring stations that were periodically surveyed. Therefore, a priori information about the distribution of fan mussels is available and the probability of finding resistant fan mussels in these areas is higher than in other sites not considered Special Areas of Conservation (SAC).

The "Preservation of *P. nobilis* in the Adriatic Sea" – A Croatian national project

22. Nowadays, in the Mediterranean the most far-reaching national project is the one currently being carried out in Croatia: "Preservation of Pinna nobilis in the southern part of the Adriatic Sea". The project was launched in late 2020 harmonizing actions carried out by institutions involved in the protection of the mollusc along the Croatian Adriatic. The project is implemented within the framework of the national programme for the conservation of Pinna nobilis in the Adriatic Sea, coordinated by the Institute for Environmental and Nature Protection of the Ministry of Economy and Sustainable Development of the Republic of Croatia. The total value of the project is HRK 2,500,000.00 Croatian Kuna (ca. 312,500 €), of which the Fund for Environmental Protection and Energy Efficiency co-finances 80%, while 20% of funding is provided by project partners. The estimated duration of the project is until 2022.

23. The funds are intended for the implementation of in situ activities, such as setting up larvae collectors, protection of larvae and adult living individuals from predators and anthropogenic impact, marking sites for protection, monitoring of survivors' positions, maintenance of adult individuals and larvae in controlled conditions (ex-situ) and raising public awareness through various educational activities.

[2] website: https://www.lifepinnarca.com/

24.The coordinator is the Public institution for the management of protected parts of nature in the Split-Dalmatia County "Sea and Karst" and partners are Croatian Veterinary Institute, Institute of Oceanography and Fisheries, Public Institution for the Management of Protected Areas of Nature of the Dubrovnik-Neretva County, Public Institution "Lastovo Islands Nature Park", Public Institution "National Park Mljet", Public institution Lokrum Reserve, Natural History Museum and Zoo of the City of Split, University of Dubrovnik. By signing an agreement with the coordinators for the Northern and Central Adriatic (JU NP Brijuni and JU Telašćica), Aquarium of Pula officially became a partner of the project, as the main institution in Croatia in charge of maintaining juvenile and adult Pinna nobilis under controlled (ex-situ) conditions.

Other relevant or recent activities/studies – Malta, Spain, Turkey

25.The following other relevant or recent activities/studies are to be mentioned:

Country	Year	Activity / Title	Reference
Malta	2022	Ocean literacy and scientific data acquisition through citizen science campaigns: a mixed approach in the Maltese Islands to collect information on *Pinna nobilis* and *Pinna rudis*.	https://ejournals.epublishing.ekt.gr/index.php/hcmr-med-mar-sc/article/view/26623
Spain	2015	Embryological Development of *Pinna nobilis* in Controlled Conditions	https://link.springer.com/chapter/10.1007/978-3-319-13878-7_42
Spain	2021	Breeding, planktonic and settlement factors shape recruitment patterns of one of the last remaining major population of *Pinna nobilis* within Spanish waters	https://link.springer.com/article/10.1007/s10750-019-04137-5
Turkey	2011	Culture of fan mussel (*Pinna nobilis*, Linnaeus 1758) in relation to size on suspended culture system in Izmir Bay, Aegean Sea, Turkey	https://vetdergikafkas.org/uploads/pdf/pdf_KVFD_1032.pdf
Turkey	2021	Population, Aquaculture and Transplantation Applications of Critically Endangered Species *Pinna nobilis* (Linnaeus 1758) in the Mediterranean Sea	https://dergipark.org.tr/en/pub/masteb/issue/64818/627562

STATE OF THE ART

26.Considering the analysis of all the above projects and the preliminary results of some of the most recent research, it is not possible to indicate a single solution to facilitate the restoration of Pinna nobilis. Experiments conducted, but also the evolving knowledge on the causes of mass mortality, lead us to be cautious in proposing solutions that risk appearing to be decisive but are instead only illusory.

27.The table below shows the main actions undertaken in the different projects in order to better evaluate in a comparative way which strategy is the most shared and therefore what should be focused on in order to propose common actions not only on a national scale but also on a Mediterranean scale.

ACTION	MERCES	RESTORFAN	LIFE PINNA	PINNARCA LIFE	HR Project
Environmental status assessment of seagrass meadows and *Pinna nobilis* populations in donor and receiving areas	X	X	X	X	X
Molecular characterization of sentinel species in the putative pilot sites of restocking			X		
Molecular characterization of surviving individuals of *Pinna nobilis*		X	X	X	
Monitoring of pathogens in restocking sites by using sentinel species			X		
Monitoring of implanted juveniles	X	X	X		X
Monitoring of the project's impact on the *P. nobilis* status	X	X	X	X	X
Report with suggested correction measures that could be implemented	X	X	X	X	X
Location of optimum sites				X	
Collection and growth of *Pinna nobilis* self-recruited, collectors-recruited individuals		X	X	X	X
Adaptation, breeding and where possible reproduction for active restocking			X		X
Collection and transport (translocation) of specimens from self-capture to receptor sites	X	X	X	X	X
Installation of the specimens of *Pinna nobilis* at the pilot areas	X		X		X
Exhaustive shallow and deep census		X	X	X	X
Actions for environmental improvement in fan mussel sanctuary areas				X	
Treatment assays and analysis				X	

28.The actions implemented by the different projects have some shared points that deserve to be considered as priorities in the Pinna nobilis Restoration programme; in particular, these are actions concerning the setup of collectors for collecting larvae, environmental assessments of the health conditions of sites with live Pinna, monitoring of implanted juveniles (when replanting from the project is envisaged), continuous updating of all the methodologies used, growth of juveniles in aquaria and/or in facilities also at sea, transport of individuals to 'safe' sites and extensive monitoring actions also through Citizen Science. On some actions to be taken, on the other hand, there does not seem to be total agreement; however, these are choices determined by whether or not to have provided for transplanting individuals between different sites: in fact, where it has been decided to implement only collector collection practices, replanting has been favoured in places such as lagoons where individuals, not necessarily resistant, nevertheless seem to survive because of unfavourable conditions for pathogens. In these places, it would not make sense to implement monitoring techniques with environmental sentinels as envisaged when individuals are to be transferred between even very distant sites whose suitability must be evaluated in advance to avoid wasting valuable time and biological resources.

29.However, many things in common can be found in the harvesting, translocation and replanting protocols that are the result of the many completed or ongoing projects. Here are some of them that may be useful in the operational implementation phase of the Restoration Programme:

RESTORFAN protocol

30.A protocol for the handling, capture, and restoration of *Pinna nobilis* was developed during the project. The protocol is attached to this document (Annex 1). Specifically, the protocol is divided into 4 parts that deal respectively for uptake (1), for collection and extraction from sediment (2), for the housing and growth of organisms (3) and for the re-implantation of organisms (4). During the project larval collectors have been successfully realized and tested according to IUCN Protocol.

The IUCN protocol of larval collector (Kersting & Hendriks 2019)

31.Larval collectors consisted of a series of plastic mesh bags containing entangled nylon filament or onion bags (see De Gaulejac et al., 2003; Cabanellas-Reboredo et al., 2009; Kersting and García-March, 2017; Vicente, 2020, for more details). Thus covering the main reproduction and settlement period of the species (Cabanellas- Reboredo et al., 2009; Deudero et al., 2017; Kersting and García- March, 2017). Observation of P. nobilis recruits was undertaken with the naked eye, allowing the detection of recruits of sizes down to 0.3 cm antero-posterior length. Recruits extracted from the collectors were either installed in aquaria (García-March et al., 2020; Vicente, 2020) or in growth cages in the field following Kersting and García-March (2017). The complete protocol is attached to this document (Annex 2).

Paper on state of art in Greece, "Population, aquaculture and transplantation applications of critically endangered species P. nobilis (Linnaeus 1758) in the Mediterranean Sea"Acarli 2021

32.The population of fan mussel, Pinna nobilis across the Mediterranean Sea has been affected by factors such as overfishing, fisheries processes, environmental pollution, destruction of habitat, tourism, etc. Therefore, the species *P. nobilis* was taken under protection by the Decisions of the Council of Europe and the Barcelona Convention. However, its mortality rates of 100% have been reported to be due to *Haplosporidium pinnae*, a parasite in different Mediterranean regions. The status of *P. nobilis* has thus been revised to be reduced from "Vulnerable" to "Critically Endangered" and the importance of all the studies on the species further increased. The aim of the study is to present the current status of *P. nobilis*, the native to the Mediterranean, by combining the relevant studies on ecology, aquacultural process (larvae, spat settlement and rearing), culture methods and transplantation. The study has provided comprehensive knowledge on the current status of the *P. nobilis* population, aquaculture and transplantation activities. Except for studies to determine stocks, in particular, those on collecting young individuals from nature and planting and growing them in predetermined sites as well as their production through various cultures from their larval phase onwards are of great importance in terms of rehabilitation and sustenance of the damaged P. nobilis population. Therefore, alternative and potential habitats should be created thanks to transplantation and aquaculture. Marine protected areas should be determined to enable a healthy *P. nobilis* population to be sustained.

RESTORATION PROGRAMME

33.The restoration programme aims to establish the main steps to be followed in order to start a recovery process for the penshell. The difficulties of operating with distances that are too great for actions such as transporting individuals make it necessary for the programme to have focal points that can carry out the main actions in each of the regions where it is intended to operate. The technical-scientific expertise also required for some of the proposed analyses makes it appropriate to identify one or more competent structures that can carry out this task for the benefit of the peripheral locations and lacking these skills. For all actions also, it will be necessary to initiate training, perhaps available online on a shared e-learning platform, to school the personnel who will be dedicated to

operations such as the setup of the collectors, their placement and the sorting of the collected material, as well as the collection and translocation of individuals in a practical and safe way.

34. The proposed programme is based on what was developed under the LIFE Pinna project and supplemented with the support of documentation collected from the other existing projects.

Objectives:

35. The main objective aimed at by the present Restoration programme are
    - Ensuring that *Pinna nobilis* recover according to their specifities and best available scinece
    - Avoiding loss and degradation of the seagrass meadows, and of other vegetal assemblages of importance for the marine environment, as marine habitats that are essential to the survival of many Mediterranean species and in particular *Pinna nobilis*, and keeping them in favourable conservation status;

Priorities and action required to attein the objective of the restoration programme :

36. At National Level :
    - Continuous mapping and monitoring of the situation throughout the Mediterranean coasts to determine the population's status and whether any recruitment is taking place even after mortality has occurred.
    - Precise mapping of resistant populations, implementation of systematic monitoring with sampling campaigns (by biopsies) for genetic studies, systematic marking campaigns for fan mussels in shallow areas and establishment of protective cages around the most exposed individuals
    - Identification and mitigation of anthropic pressures experienced by existing populations
    - Establish of marine protected areas or expansion of existing ones to aid in the preservation of new *Pinna nobi*lis individuals that appear to be resistant to the parasite's impact if certain protective measures are applied.
    - Establish ecological mooring systems in areas frequented by boaters to limit the impact of anchors on fan mussel populations and seagrass beds, where juveniles and sub-adults settle;
    - Elaboration and implementation of appropriate legislation
    - Develop public and professional awareness actions on the status of the species
    - Boost the captive breeding project

37. At Regional Level :

    - Establish a Pan-Mediterranean task force coordinated by SPA/RAC to implement the present restoration programme.
    - make sure updated information on the status of populations is well circulated and real-time
    - Strengthening cooperation and exchange of cooperation between Contracting Parties, concerned actors and project
    - Promote the installation of larval collectors in strategic locations
    - Organise regional training
    - Elaborate guidelines, recommendations and standardised Protocol to monitor, study populations, for translocation and/or rescue to ex-situ and captive breeding.

38. At population level :

The programme envisages development in phases and has two main targets for action: *Pinna nobilis* adult individuals and juveniles obtained by collectors or through search actions in places such as marinas or transition water zones where the chance of finding them seems greater. For each of the actions to be taken, it is considered appropriate to evaluate carefully and always whether it is preferable to leave the individuals in place or to relocate them based always on scientific analysis that justifies the move for safety reasons (the place for example might be subject to hazards such as mechanical threats due to anchoring) or for the improvement of the individual's health status: the

individual is in a place that still has a high presence of pathogens and therefore would benefit from being moved to a place that is pathogen-free.

Target ONE - juveniles

The main strategy and efforts of the restoration programme should be devoted to identifying locations free of the pathogens identified so far as causes of the mass mortality and collecting juvenile individuals and larvae also in order to increase the chances of restoration.

The actions to be taken, after checking that you are following the latest protocols, are as listed below:

1. **Search for juveniles**
    - Extensive action to search for juveniles; this involves initiating, also with the help of citizen science, an effective and extensive search for juvenile individuals that might be found in estuarine areas but also in places such as marinas and harbors where calm sea conditions seem to be favorable for settlement. A further excellent collection site has seemed in past years to be mussel farms. Recent experiences, however, at least in the Northern Adriatic, show a very high recruitment almost exclusively of Pinna fragilis and no longer as was the case until a few years ago of Pinna nobilis.
2. **Collectors**
    - Recruitment and collection of fan mussel juveniles using artificial devices following the methodologies from Kersting & Hendriks (2019).
3. **Transport and rearing**
    - Once juvenile individuals have been collected, they have to be immediately placed in a box filled with seawater in order to be conducted, in the safest way, to the location prepared for their growing and rearing. Before moving juveniles in tanks, operators will check the integrity of the shell and byssus. Whether byssus can regrow, big damages on the shell will affect P. nobilis ability to close itself. This is important in the next phases, where fan mussel specimens will have to be transferred to other sites and they'll need to close their valves in order to avoid stress and the loss of inner water. Checked P. nobilis individuals will be set in aquarium tanks, where they will spend the initial period of growth. Due to the stressful condition individuals may be in, they will be kept under observation for a first period (about 1 month). This is necessary to restore organism optimal conditions and to rebuild the byssus. It is necessary to proceed very carefully during the insertion of the juvenile specimens in the aquarium, paying attention to the physical and chemical conditions of the water in which specimens will be placed (acclimation phase). According to dimension, individuals can be placed directly in the soft bottom or in small support such as Petri dishes filled with coarse sediment or on small, open jute bags. Once ready, the organisms can be placed in baskets attached to the mussel farm's longline and will thus remain suspended in the aquatic medium for a period necessary for the growth and rear of fan mussel specimens. Operators will conduct periodic monitoring (twice a month) to check the state of health of individuals. Also the correct location of the lantern-nets will be checked, since some extreme marine phenomena could affect the right attachment of the basket to the longline rope. At the end, P. nobilis specimens will be transported to the restocking sites, after having reached the escape size (6, 12 and 18 months).

4. **Identification of receiving sites**
    - Receiving sites will be identified after a careful analysis of the environmental characteristics of the receiving areas that display suitable environmental conditions for the survival of restocked individuals and where the pressure regime (both natural and human-induced) is as low as possible. The receiving pilot sites must be selected, where possible, in the habitat of *Posidonia oceanica* seagrass meadows or

Cymodocea nodosa/Zostera spp. beds. To assess the best sites where fan mussels can be restocked within the seagrass meadows or on coarse sandy bottoms, field activities through underwater scuba diving have to be performed by scientific divers. The best areas of the meadows, or of the sandy bottom, that will be likely to support a successful restoration will be chosen according to the occurrence of a matte substrate or a proper substrate, according to the ecological condition of the meadow, which must show high ecological quality (assessed through the adoption of ecological indices as requested by the D.Lgs. 152/2006 that has received the European Directive 2000/60/EC), high coverage of the bottom and high shoot density. According to MERCES results the presence of seagrass meadows and density of *Pinna nobilis* specimens will cooperate in best results. The sites need to meet the characteristics of safety from physical damage (anchoring etc) and absence of pathogens. Therefore, sites such as protected areas that guarantee through their prohibitions the highest degree of safety at least for mechanical hazards will be preferred. An action of monitoring the presence of pathogens also will have to be conducted through a molecular characterization of etiological agents in that area, obtained by the survey on bivalves sentinel species: at least 100 specimens of the species *Mytilus galloprovincialis* or other bivalves per site. These specimens will be used as bio-indicators, able to provide a cognitive framework about the presence of etiological agents that could invalidate the restocking initiatives of juveniles. Acquiring data on the occurrence of specific etiological agents in sentinel species will test the magnitude of pathogens concentration in the column water in the sites. The utility of sentinel species for this purpose has been tested by Scarpa & Sanna et al. (2020). Tests can be performed by using the Real-time reverse transcription polymerase chain reaction (RT-PCR, also known as quantitative PCR or qPCR), which represents the most sensitive and specific test for quantification and sorting of different etiological agents (Kralik and Ricchi, 2017). Considering that, to date, the causes of the mass mortality of Pinna nobilis are far to be fully understood, and the scenario of a multifactorial disease is the most plausible (see Scarpa & Sanna et al., 2020), it is very important to perform a wide recognition of the etiological agents probably involved in the mass mortality. Accordingly, RT-PCR could be used to identify both protozoans and bacteria, which have been already detected in several populations of dying Pinna nobilis (see e.g. Panarese et al., 2019; Carella et al., 2019; Prado et al., 2019). In addition, considering that viruses have been reported to be often the cause of the mortality in installation of breeding bivalves, RT-PCR should be carried out with specific primers for the most pathogenic viruses so far known for bivalves.

5. **Transplantation of juveniles**
    - Once at the destination sites, P. nobilis specimens will be placed into the marine environment. The most critical phase, after the transport is the transplantation in the aquatic environment characterized by different water values of salinity and temperature, respect of those one occurs in the transport (and even earlier, with respect to biophysical and chemical parameters in the growth and reproduction tanks). Particular attention must be paid to the handling of specimens. It's very important to not damage the byssus and to not break the shell of the specimens. In fact, P. nobilis needs byssus to anchor itself to the seabed, while the intact shell permits the tightly closing of the organism and preserves the internal water, held between the valves, during the installation operations. Before any transplanting operations, between the transport and the installation, there will be an intermediate phase, in order to avoid as much stress as possible to the organisms and to facilitate their acclimation to the new site. This adaptive step involves the storage of the organisms in specific tanks that reproduce the chemical and biophysical conditions of the transplant site. With the aim of transplanting as many juveniles as possible and keeping them alive during installation operations, the group of juveniles to transplant

will be splitted in different sub-groups. In this way, different sessions of acclimation will be carried out. Therefore, it is of fundamental importance to be able to transplant as many juveniles as possible in at least one protected area, to support the division of the group to be transplanted into different sub-groups and then into different receiving sites. After the acclimation phase, the organisms will be placed by experienced dive operators in the receiving sites placing them in the different types of substrate, either Posidonia matte, Cymodocea meadows or coarse sand. The specimens of P. nobilis will be placed at a certain distance from each other, to avoid external criticalities that could ruin the transplantation experiment, such as abusive nets, emergency anchoring, presence of pelagic large animals etc. This distance will not be too large to ensure genetic exchange between organisms during the reproductive period. Each transplanted organism will be tagged in order to ensure monitoring operations and the geographical location (geographic coordinates) will be recorded via GPS.

Target TWO - Adults

The search for adults is aimed at finding spawners and verifying their health conditions to ensure that they are not in potentially dangerous and pathogen-free locations. Mapping and a geographic analysis of the data may also provide insight into whether or not they should be transplanted to a single location at a distance that facilitates fertilization. The actions to be pursued will therefore be aimed at finding and protecting live individuals and assessing their health conditions. This will require:

1. **Search for adults**
    - Extensive action to search for live adults; The research activities of adult individuals conducted in many places in recent years have proven how effective citizen science actions are that succeed in guaranteeing a large number of observers who, if properly trained, can provide very precise indications, greatly reducing the effort of researchers engaged therefore in the sole actions of verification and monitoring of health conditions.
2. **Molecular characterization of surviving individuals of Pinna nobilis**
    - The first task of the action is aimed to carry out the molecular characterization focused to acquire the proper knowledge on the genetic make-up of the specimens of P. nobilis sampled; the second one is aimed to perform diagnostic analyses, in order to search for (possible) etiological agents in the fun mussels analyzed. Indeed, the analyses of the genetic make-up of the still-alive specimens can provide some hints on their resistance capability to the disease responsible for the mass mortality of the fan mussel. In addition, based on these data it could be possible to carry out a comparison with the pre-epidemic genetic status of fun mussels comparing them with data reported in Sanna et al., 2013, one of the most complete surveys on genetic variability of Pinna nobilis performed a few years before its mass mortality. The second analysis has to be focused to detect the occurrence of etiological agents. This step represents a crucial point, since the introduction of “pathogens-free” recruited specimens is the critical condition that allows to increase the chances of success for restocking activities.
3. **Mapping of surviving individuals of Pinna nobilis**
    - Mapping is a crucial aspect in order to be able to properly assess the appropriateness of moving the specimens; a comparative analysis of the distances between individuals, possible risks from mechanical damage, and the main oceanographic features of the sites will in fact be able to provide the best guidance on how to proceed. If the condition of the individuals is sufficiently safe and the site conditions good one can simply mark the individuals and maintain their monitoring over time. If, on the other hand, it is appropriate to move the individuals, it will be necessary to proceed with the steps of receiving site identification and transplantation.

4. **Identification of receiving sites**
    - Receiving sites will be identified after a careful analysis of the environmental characteristics of the receiving areas that display suitable environmental conditions for the survival of restocked individuals and where the pressure regime (both natural and human-induced) is as low as possible. The receiving pilot sites must be selected, where possible, in the habitat of *Posidonia oceanica* seagrass meadows or *Cymodocea nodosa/Zostera* spp. beds. To assess the best sites where fan mussels can be restocked within the seagrass meadows or on coarse sandy bottoms, field activities through underwater scuba diving have to be performed by scientific divers. The best areas of the meadows, or of the sandy bottom, that will be likely to support a successful restoration will be chosen according to the occurrence of a matte substrate or a proper substrate, according to the ecological condition of the meadow, which must show high ecological quality (assessed through the adoption of ecological indices as requested by the D.Lgs. 152/2006 that has received the European Directive 2000/60/EC), high coverage of the bottom and high shoot density. According to MERCES results the presence of Seagrass meadows and density of Pinna nobilis specimens will cooperate in best results. The sites need to meet the characteristics of safety from physical damage (anchoring etc) and absence of pathogens. Therefore, sites such as protected areas that guarantee through their prohibitions the highest degree of safety at least for mechanical hazards will be preferred. An action of monitoring the presence of pathogens also will have to be conducted through a molecular characterization of etiological agents in that area, obtained by the survey on bivalves sentinel species: at least 100 specimens of the species *Mytilus galloprovincialis* or other bivalves per site. These specimens will be used as bio-indicators, able to provide a cognitive framework about the presence of etiological agents that could invalidate the restocking initiatives of juveniles. Acquiring data on the occurrence of specific etiological agents in sentinel species will test the magnitude of pathogens concentration in the column water in the sites.
5. **Transport and transplantation of adults**
    - Collected individuals have to be immediately placed in a box filled with seawater in order to be conducted, in the safest way, to the destination site. Before moving, operators will check the integrity of the shell and byssus. Any storage phase between adult collection and transfer should be of short duration and carried out in such a way as not to expose the animals to stressful conditions and should be carried out by keeping the removed organisms in a water environment with sufficient exchange of water. Replanting should take place within two days of harvesting the animals and in the shortest possible time. Once at the destination sites, *P. nobilis* specimens will be placed into the marine environment. The most critical phase, after the transport, is the transplantation in the aquatic environment characterized by different water values of salinity and temperature, respect of those that occur in the transport (and even earlier, with respect to biophysical and chemical parameters in the growth and reproduction tanks). Particular attention must be paid to the handling of specimens. It's very important to not damage the byssus and to not break the shell of the specimens. In fact, *P. nobilis* needs byssus to anchor itself to the seabed, while the intact shell permits the tightly closing of the organism and preserves the internal water, held between the valves, during the installation operations. Before any transplanting operations, between the transport and the installation, there will be an intermediate phase, in order to avoid as much stress as possible to the organisms and to facilitate their acclimation to the new site. This adaptive step involves the storage of the organisms in specific tanks that reproduce the chemical and biophysical conditions of the transplant site. With the aim of transplanting as many juveniles as possible and keeping them alive during installation operations, the group of juveniles to transplant will be splitted in different sub-groups. In this way, different sessions of acclimation will be carried out. Therefore, it is of fundamental importance to be able to transplant

as many juveniles as possible in at least one protected area, to support the division of the group to be transplanted into different sub-groups and then into different receiving sites. After the acclimation phase, the organisms will be placed by experienced dive operators in the receiving sites placing them in the different types of substrate, either Posidonia matte, Cymodocea meadows or coarse sand. The specimens of *P. nobilis* will be placed according to MERCES outcomes with density from 1 to 5 indd/m2. Each transplanted organism will be tagged in order to ensure monitoring operations and the geographical location (geographic coordinates) will be recorded via GPS.

Work programme and timetable for 2023-2028

Action	Deadline	To be implemented by

Bibliography

CARELLA, F., et al. A mycobacterial disease is associated with the silent mass mortality of the pen shell Pinna nobilis along the Tyrrhenian coastline of Italy. *Scientific reports*, 2019, 9.1: 1-12.

CATANESE, Gaetano, et al. Haplosporidium pinnae sp. nov., a haplosporidan parasite associated with mass mortalities of the fan mussel, Pinna nobilis, in the Western Mediterranean Sea. *Journal of invertebrate pathology*, 2018, 157: 9-24.

COPPA, Stefania, et al. Density and distribution patterns of the endangered species Pinna nobilis within a Posidonia oceanica meadow in the Gulf of Oristano (Italy). *Journal of the Marine Biological Association of the United Kingdom*, 2010, 90.5: 885-894.

COPPA, Stefania, et al. The effect of hydrodynamics on shell orientation and population density of Pinna nobilis in the Gulf of Oristano (Sardinia, Italy). *Journal of Sea Research*, 2013, 76: 201-210.

GARCÍA-MARCH, José R., et al. Population structure, mortality and growth of Pinna nobilis Linnaeus, 1758 (Mollusca, Bivalvia) at different depths in Moraira bay (Alicante, Western Mediterranean). *Marine Biology*, 2007, 150.5: 861-871.

KATSANEVAKIS, Stelios. Population ecology of the endangered fan mussel Pinna nobilis in a marine lake. *Endangered species research*, 2005, 1: 51-59.

KRALIK, Petr; RICCHI, Matteo. A basic guide to real time PCR in microbial diagnostics: definitions, parameters, and everything. *Frontiers in microbiology*, 2017, 8: 108.

ORFANIDIS, Sotiris, et al. Benthic macrophyte communities as bioindicators of transitional and coastal waters: relevant approaches and tools. *Transitional Waters Bulletin*, 2007, 1.3: 45-49.

PANARESE, Rossella, et al. Haplosporidium pinnae associated with mass mortality in endangered Pinna nobilis (Linnaeus 1758) fan mussels. *Journal of invertebrate pathology*, 2019, 164: 32-37.

PRADO, Patricia; CAIOLA, Nuno; IBÁÑEZ, Carles. Habitat use by a large population of Pinna nobilis in shallow waters. *Scientia Marina*, 2014, 78.4: 555-565.

PRADO, Patricia, et al. Presence of Vibrio mediterranei associated to major mortality in stabled individuals of Pinna nobilis L. *Aquaculture*, 2020, 519: 734899.

RABAOUI, Lotfi; TLIG-ZOUARI, Sabiha; BEN HASSINE, Oum Kalthoum. Distribution and habitat of the fan mussel Pinna nobilis Linnaeus, 1758 (Mollusca: Bivalvia) along the northern and eastern Tunisian coasts. *Cahiers de Biologie Marine*, 2008, 49.1: 67.

RICHARDSON, C. A., et al. Age and growth of the fan mussel Pinna nobilis from south-east Spanish Mediterranean seagrass (Posidonia oceanica) meadows. *Marine Biology*, 1999, 133.2: 205-212.

SANNA, Daria, et al. Mitochondrial DNA reveals genetic structuring of Pinna nobilis across the Mediterranean Sea. *PLoS One*, 2013, 8.6: e67372.

SANNA, Daria, et al. New mitochondrial and nuclear primers for the Mediterranean marine bivalve Pinna nobilis. *Mediterranean Marine Science*, 2014, 15.2: 416-422.

SCARPA, Fabio, et al. Multiple non-species-specific pathogens possibly triggered the mass mortality in Pinna nobilis. *Life*, 2020, 10.10: 238.

VAZQUEZ-LUIS, Maite, et al. SOS Pinna nobilis: a mass mortality event in western Mediterranean Sea. *Frontiers in Marine Science*, 2017, 4: 220.

ZAVODNIK, Dusan; HRS-BRENKO, M.; LEGAC, Mirjana. Synopsis on the fan shell Pinna nobilis L. in the eastern Adriatic Sea. *Les espèces marines à protéger en Méditerranée*, 1991, 169-178.

ANNEX 1 – The RESTORFAN Protocol

Area Marina Protetta di Miramare
Viale Miramare 349
34014 Trieste
Tel 040 224147
Fax: 040 224636
e-mail: info@riservamarinamiramare.it
www.riservamarinamiramare.it

Pinna nobilis,
Protocols for manipulation, captation and restoration (2019)

1. Protocol for uptake
2. Protocol for collection and extraction from sediment
3. Protocol for the housing and growth of organisms
4. Protocol for the re-implantation of organisms

1. PROTOCOL FOR *PINNA NOBILIS* JUVENILE COLLECTION

The populations of *Pinna nobilis* in the Gulf of Trieste reach a gonadal maturity in the period between August and November. During this period it is possible to observe the fans emitting gametes into the water column.

The operations of captation must be conducted during this period.

We then proceed with the preparation of the captation structure (Figure 1) consisting of 1 ballast, a rope with a maximum length of 2 meters, a float and the collector. Among the 2 collection systems tested (vertical and horizontal) the horizontal system was preferred. A circular lanter-net (plastic devices used in ostrey maricoltures) is therefore used on which it is possible to fix various types of textile material to increase the efficiency of collection. Simplest method is put inside the lanternet some textile material like potato-bag, jute bag, ropes etc. This method help juveniles to attached helding larvas.

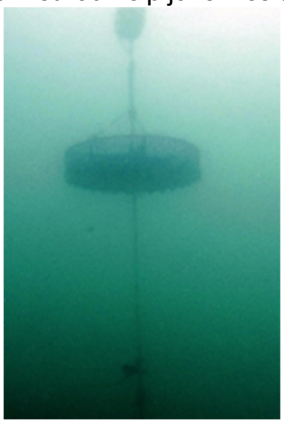

Figure 1Horizontal collector

2. PROTOCOL FOR THE COLLECTION OF JUVENILE OF *PINNA NOBILIS* ORGANISMS

The juvenile organism is harvested as soon as it reaches a height of 1-2 cm (Figure 2) as it is slightly more resistant during the diver's harvesting operations.

Once collected, the organism is transported in a box paying particular attention to not stress it.

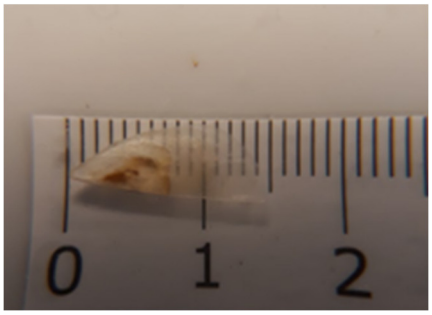

Figure 2 Juvenile Pinna nobilis

Harvesting operations are carried out in the same way on the longlines of mussel farms (Figure 3). After a careful analysis of the longline by the diver, once the individual is identified, the collection is carried out. Often the operation is not easy because the organisms are found among other specimens of *Mytilus galloprovincialis* or sponges and ascidians. In this case we try to remove first the organisms around the *Pinna nobilis* and then we try to cut the byssus without damaging the gland responsible for the production of byssus. Once collected the specimens should be placed in a closed rigid container (Figure 4) paying attention to not stress it.

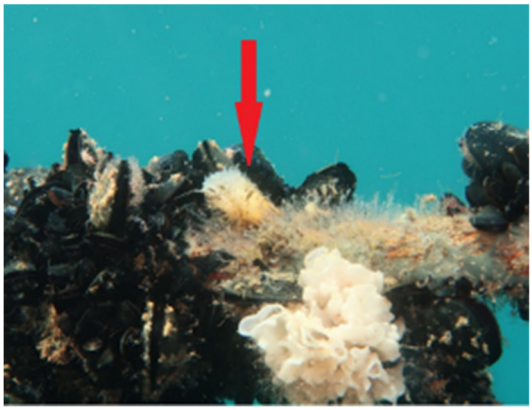

Figure 3 Pinna nobilis on longline

Figure 4 Plastic-box for collected organism

IMP: Temperature and salinity data must be collected on site in order to reproduce them in laboratory.

In case of extraction of organisms from the sediment, a small sorbonne is used (Figure 5), i.e. an instrument that is operated with air coming from a compressor or a scuba bottle allows to remove the sediment around the fin without damaging the organism. After removing most of the sediment around the organism you should see the byssus attached to the solid substrate. Usually, the fin sticks to a few little solid bodies, which can be a rock or a very large rock. In case the byssus is attached to a removable stone we proceed with the extraction of the fin with the whole stone. If the fin is attached to a rock, then proceed by cutting the byssus in the proximity of the rock without damaging the byssus gland.

Figure 5 Sorbonne

3. PROTOCOL FOR BREEDING AND GROWTH OF *PINNA NOBILIS*

Once reached the laboratory in the shortest possible time, we proceed with the insertion of juvenile organisms in the enclosures.

First of all it is important to verify that the chemical-physical properties of the tanks-enclosure are equal to the conditions of the sampling area. Good practice for the insertion of organisms in the tanks is however to proceed gradually, inserting small amounts of water from the aquariums into the boxes with the collected organisms. This operation can be completed within half an hour.

Once you have inserted the organisms in the tanks you can choose whether to insert them in the free sediment or put some gross sediment inside a petri dish and then insert the organism (it is valid for very small ones), otherwise you can also use small open bags made with jute, inserting first the sediment and then the organism (Figure 6).

It is good practice together with the sediment to insert also a stone on which the juvenile of Pinna nobilis is able to fix the byssus. This practice helps the Pinna nobilis in a subsequent transplant operation as it would avoid a second splitting of the byssus. It should be remembered that the cutting of the byssus cloth brings anyway a stress to the organisms, debilitating it and reducing the chances of survival.

Figure 6 Juta bag and Petri dish

For stabling and growth operations, attention must be given above all to maintaining the optimal chemical-physical conditions. Although the Pinna nobilis is a very resistant and adaptable bivalve mollusc (it survives even for short periods out of the water) we try not to produce large fluctuations in the tanks during normal maintenance operations. The photoperiod should be adjusted according to the seasonality of collection and gradually varied according to the progress of the seasons. As far as the growth is concerned, it is possible to proceed with the insertion of nutrients or, if the tank already has a started ecosystem (at least 5 cm of sediment, different stones, vegetable and animal organisms present) then it is also possible not to insert nutrients for the fans. If the tanks instead are only filled with water without any kind of ecosystem started, then it is recommended to insert once a week a microalgal culture concentrate in the tank.

To choose the most suitable algal culture for feeding *P.nobilis* you can proceed with monocultures (i.e *Dunaliella tertiolecta*) or mix of algae monocultures available on the market. Usually available algae cultures are used because they are selected and free of other organisms. It is also possible to proceed with the culture starting from a sampling in seawater in the juvenile organisms sampling area, but this method does not guarantee the purity of the final result. Inside the taken water there are many predatory organisms of the seaweed and maybe even pathogenic organism for the fin, which in culture could even increase their population.

4. PROTOCOL FOR THE RESTORATION OF THE *PINNA NOBILIS*

The organisms, once they reach 10 cm in size inside the tanks, can be re-implanted in the final site. For the re-implantation of both juveniles and transplanted adult organism, it is sufficient to proceed with the choice of a suitable site for the transplantation of the organisms. In particular, it is important to make sure that the turbulence is not excessive in case of sea storms, as it could undermine the newly planted organisms.

We proceed with the excavation of a hole in the sediment either with sorbonne or by hand that is at least 1/3 of the total length of the organism. If, on the other hand, the organism has passed the "growth phase" in a yute bag, you can proceed with the insertion of the whole bag in the sediment. Within a few weeks the yute degrades.

5. PROTOCOL FOR THE COLLECTION OF MATERIAL FOR THE GENETIC ANALYSIS

This kind of protocol is intended for the detection of *Haplosporidium pinnae* infection. The material detected for genetic analysis is the faeces and pseudofaeces of the organisms. A diver dives into the site where the organisms to be monitored are located, equipped with 60 mL syringes and tubes for the collected material (10 mL tubes are sufficient) (Figure 7). The diver moves slowly to the living organism so as not to provoke a reaction in the body and thus

miss the opportunity to collect the material. Once the syringe and tube are prepared, the syringe can be brought closer to the body and the pseudo-faeces present on the edge of the valve opening opposite the hinge can be aspirated. At that point the gills secret this mucus which serves as protection against excessive sedimentation. If you want to take the fecal pellets you will have to pay attention to the exit of the cloacal channel of the organism that is more or less near them. If the organism does not emit, you can try knocking on a valve, in this way the organism will close and emit fecal pellets. After sampling, biological material are conserved in alcool (90°) and put in freezer at -80°C, ready for the genetic analysis.

Figure 7 Underwater operations

ANNEX 2 – The IUCN Short guidance for the construction, installation and removal of Pinna nobilis larval collectors

SHORT GUIDANCE FOR THE CONSTRUCTION, INSTALATION AND REMOVAL OF *PINNA NOBILIS* LARVAL COLLECTORS

D. K. Kersting[1,2], I. E. Hendriks[3]

[1] Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals, Facultat de Biologia, Institut de Recerca de la Biodiversitat (IRBIO), Universitat de Barcelona, Spain.

[2] Working Group on Geobiology and Anthropocene Research, Institute of Geological Sciences, Freie Universität Berlin, Germany.

[3] Global Change Research Group, Mediterranean Institute for Advanced Studies (IMEDEA, CSIC-UIB), Esporles, Spain

CONTEXT

An unprecedented mass mortality event is impacting *Pinna nobilis* populations throughout the Mediterranean Sea (https://www.iucn.org/news/mediterranean/201907/mediterranean-noble-pen-shell-crisis-pinna-nobilis-june-2019-update; Vázquez-Luis et al. 2017). The eventual recovery of impacted populations will depend mainly on the existence of unimpacted populations, resistant individuals and recruitment. Therefore, it is extremely important to assess larval recruitment to evaluate if larvae coming from unaffected sites or resistant individuals are reaching the impacted areas, thus potentially contributing to eventual recoveries.

Larval collectors have been successfully used to assess *P. nobilis* recruitment in different contexts and areas (Cabanellas-Reboredo et al. 2009, Kersting & García-March 2017, Wesselmann et al. 2018). Additionally, if needed, this methodology might eventually be used to provide juveniles to restock populations (Kersting & García-March 2017).

Here we describe how to construct, install and remove larval collectors in order to assess larval settlement in *P. nobilis*.

CONSTRUCTION

Collector bags

The collector bags consist of entangled nylon filament, onion bags or any similar material composed of fine filaments that endure underwater, placed inside polyethylene (or similar plastic) mesh bags (Fig. 1). Different designs can be applied here, the important thing is to have entangled filaments (settlement substratum for larvae) and a plastic mesh bag containing that substratum that acts as a protection against predators (but allows larvae to access the inner filaments). The outer plastic mesh bag must be securely closed using cord or nylon cable ties. At one of the ends the same cord used to close the bag can be used to anchor the bag to the main rope (see next step).

Entangled nylon can be obtained by recycling old trammel nets (or similar); usually fishermen throw them away when old or broken. This material can be reused many times if rinsed in water and dried after each use as larval collector. Onion or vegetable nets/bags can be obtained by recycling used ones or can be bought in gardening or agriculture shops (as well in internet shops).

Fig. 1. Two different bag designs. Left. Entangled nylon (trammel net) inside plastic mesh bags. Right. A similar outer plastic bag but using onion nets as substrata inside. Photographs: D. K. Kersting, I. Hendriks.

Main rope

The bags are attached to a main rope (Fig. 2). The whole system is fixed to a small concrete mooring (or similar, but it must be heavy enough to prevent dislocation by waves and currents) and the rope is kept vertical by a submerged buoy. Submerged buoys (depth > 3m) prevent the whole system to be seen from the surface and potential entanglements with boats.

Fig. 2. Collectors' bags attached to the main rope and buoy ready to be deployed. Photograph: D. K. Kersting.

There are several ways to distribute the bags along the rope. In deeper sites the bags can be attached in approx. 1,5 m intervals throughout the rope (Fig. 3), thus covering a wider depth range. In shallow sites the bags can be attached in a single point (Fig. 3). It has been observed that *P. nobilis* larvae settle in collectors in a wide depth range, so both deeper (for example 15 m) and shallower (for example 5 m) collector installations are possible.

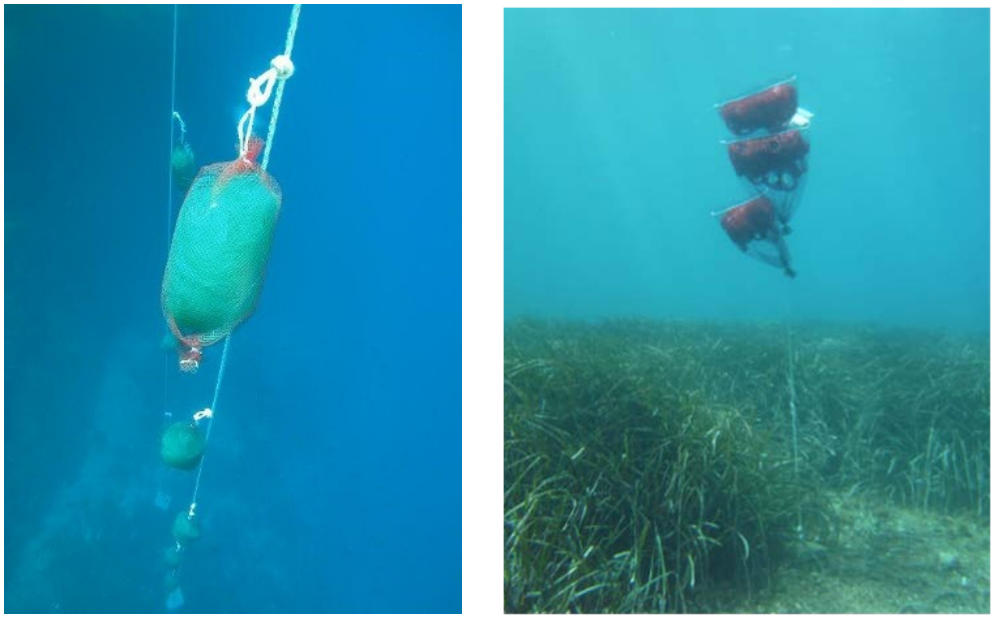

Fig. 3. Larval collector bags attached in 1,5 m intervals in a deep site (left) and a shallow site installation (righty). Photographs: D. K. Kersting, I. E. Hendriks.

INSTALLATION AND REMOVAL

Where?

The collectors should be preferably placed in a location exposed to open-waters, as *P. nobilis* larvae are transported by currents. Of course, they can be installed as well in other sites if needed, for example to check for potential recruitment in semi-enclosed lagoons.

The presence of adult *P. nobilis* populations is not a prerequisite to install the collectors. They can be installed in locations where the species is not present or in areas where the ongoing mass mortality event has killed all individuals. *Pinna nobilis* larvae can travel long distances transported by currents, therefore the larvae arriving to a certain site may come from distant areas.

When?

The main reproduction period of *P. nobilis* is from May to August and the main settlement period is estimated to occur between July and September (in the W Mediterranean). These periods could change depending on environmental conditions (for example water temperature) in the different Mediterranean regions. We suggest to install the collectors in June and remove them in October-November. While this would be the ideal installation and removal period, later installations and removals are possible. It must be taken into account that later installations will lower the possibility of covering the whole main larval settlement period. While the main problem of a later removal of the collectors is a higher exposure to storms in some regions and the fact that at some point juveniles might not have enough room between the filaments to keep growing.

How to remove settled juveniles?

The collectors should be carefully removed, avoiding crushing the bags. The bags should be preferably maintained underwater until the removal of the juveniles.

At the end of the installation period juveniles' sizes (antero-posterior length) may range approx. from 0,5 – 9 cm. In general, they can be seen by the naked eye inside the tangled fibers (Fig. 4). They have to be removed carefully in order not to break the fragile valves. Juveniles should be immediately placed in seawater after their extraction from the collector bag (Fig. 4).

Fig. 4. *Pinna nobilis* juveniles settled inside the collectors. Notice different morphologies and sizes. Juveniles have to be kept in seawater immediately after extraction from the bags. Photographs: D. K. Kersting.

What to do with the juveniles?

Juveniles can be placed in protection cages in the field where they will continue growing, giving the possibility of re-implanting them in suitable substrata when a certain size is reached (Fig. 5). See Kersting & García-March (2017) for further information.

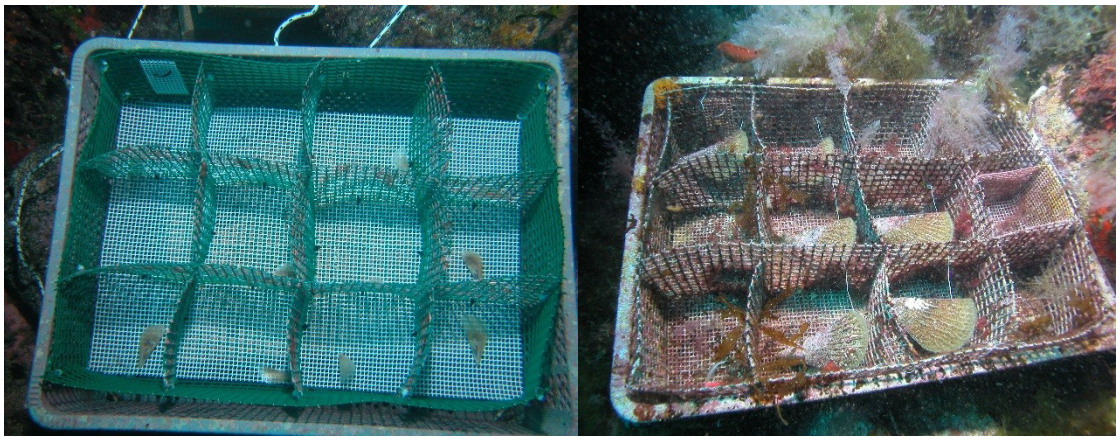

Fig. 5. Left. Juveniles just extracted from the collectors and placed in the protection cage (in the field). Right. *Pinna nobilis* individuals of approx. 2-3 years of age in the protection cage. Notice the photographs have been taken without the mesh protection covering the cages. Photographs: D. K. Kersting.

Bibliography

Vázquez-Luis et al. (2017) S.O.S. *Pinna nobilis*: A mass mortality event in Western Mediterranean Sea. Frontiers in Marine Science doi: 10.3389/fmars.2017.00220.

Kersting DK, García-March JR (2017) Long-term assessment of recruitment, early stages and population dynamics of the endangered Mediterranean fan mussel *Pinna nobilis* in the Columbretes Islands (NW Mediterranean). Marine Environmental Research 130:282-292.

Cabanellas-Reboredo et al. (2009). Recruitment of *Pinna nobilis* (Mollusca: Bivalvia) on artificial structures. Marine Biodiversity Records 2:1e5.

Wesselmann et al. (2018) Genetic and oceanographic tools reveal high population connectivity and diversity in the endangered pen shell *Pinna nobilis*. Scientific Reports 8:4770.

Citation: Kersting D. K., Hendriks I. E. (2019) Short guidance for the construction, installation and removal of *Pinna nobilis* larval collectors. IUCN. 6pp.