Life Pinna

LIFE20 NAT/IT/001122

LIFE PINNA

Conservation and re-stocking of the Pinna nobilis in the western Mediterranean and Adriatic Sea

D_E.2.1: Protocols for replication

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Sommario

1. Introduction

1.1 Background and context

The Mediterranean fan mussel *Pinna nobilis* has undergone an unprecedented mass mortality event since 2016, leading to the near-collapse of populations across the entire Mediterranean basin. This dramatic decline, driven primarily by pathogenic agents such as *Haplosporidium pinnae* and compounded by environmental stressors, has placed the species at imminent risk of extinction.

In response to this conservation emergency, the LIFE PINNA project developed and tested a set of innovative, science-based approaches aimed at preserving the remaining genetic diversity of the species, mitigating disease risks, and exploring feasible pathways for population recovery. The project operated in a context of high uncertainty, ethical constraints, and rapidly changing ecological conditions, requiring adaptive management and close collaboration between scientists, managing authorities, and policy-makers.

One of the most significant outcomes of LIFE PINNA is the development of operational knowledge that extends beyond the specific pilot sites and can be transferred to other Mediterranean contexts facing similar challenges.

1.2 Purpose and scope of the document

This document presents a structured compendium of five operational protocols developed within the LIFE PINNA project. Its primary purpose is to facilitate the transfer and replication of validated methodologies for the conservation and potential restocking of *Pinna nobilis* in other geographical areas.

The protocols are designed to:

- translate scientific research into practical guidance;
- support decision-making by managing authorities and practitioners;
- reduce risks associated with translocation, disease spread, and genetic erosion;
- promote harmonisation of conservation practices across Mediterranean regions.

While the protocols are grounded in the LIFE PINNA experience, they are presented in a flexible format that allows adaptation to different ecological, institutional, and logistical contexts.

1.3 Target audience and users

The document is addressed primarily to:

- managers and technical staff of Marine Protected Areas;
- national and regional environmental authorities;
- research institutions and marine laboratories;
- conservation practitioners and NGOs involved in marine biodiversity protection.

The text adopts a technical yet accessible style, aiming to be understandable to professionals from diverse backgrounds while maintaining scientific rigour. The protocols are intended not as rigid prescriptions, but as structured frameworks to support informed and responsible action.

1.4 Methodological approach and guiding principles

The elaboration of the protocols followed a collegial and interdisciplinary approach, involving marine biologists, geneticists, pathologists, conservation managers, and legal experts. Each protocol integrates ecological, sanitary, genetic, and administrative dimensions, reflecting the complex nature of *Pinna nobilis* conservation.

The guiding principles underpinning the document include:

- precaution and risk minimisation;
- ethical responsibility towards a critically endangered species;
- scientific transparency and reproducibility;
- adaptability to evolving knowledge and environmental conditions.

1.5 Structure of the document

The document is organised into five main protocol chapters, each corresponding to a specific operational domain addressed by the LIFE PINNA project. Introductory sections provide guidance on how to use the protocols and how they relate to each other, while cross-cutting chapters address overarching issues such as health surveillance, genetic integrity, and replicability.

Together, these elements form an integrated toolkit intended to support current and future conservation efforts for *Pinna nobilis* and to contribute to a coordinated Mediterranean response to one of the most severe biodiversity crises of recent decades.

2. How to Use This Protocol Compendium

2.1 Purpose and Scope of the Compendium

This protocol compendium has been developed to support the transfer and replication of the methodologies tested within the LIFE PINNA project, providing practical guidance to institutions and practitioners involved in the conservation of *Pinna nobilis*. Its primary purpose is to facilitate the uptake of project results beyond the original implementation sites, enabling other Marine Protected Areas, Natura 2000 site managers, research institutions and competent authorities to apply the lessons learned in their own territorial contexts.

The document is designed to bridge the gap between scientific research and operational conservation practice. While it is grounded in robust scientific evidence and validated methodologies, it is intentionally written in an accessible and applied manner. The compendium does not aim to replace scientific publications or legal texts, but rather to translate complex procedures into operational guidance that can be realistically implemented by multidisciplinary teams working in the field.

The scope of the compendium includes the five core protocols developed under Action E.2, covering the full chain of conservation actions, from site selection to restocking, captive reproduction, governance arrangements and specimen tracking. Together, these protocols provide a coherent framework for planning and implementing conservation interventions for *Pinna nobilis* under current Mediterranean conditions.

2.2 Integrated and Modular Use of the Protocols

The protocols included in this compendium are intended to be used as part of an integrated conservation strategy. Although each protocol addresses a specific aspect of the intervention process, their effectiveness depends on their coherent and coordinated application. For example, restocking actions should only be planned following a rigorous assessment of site suitability and disease risk, while specimen transport and installation must be linked to long-term monitoring and traceability mechanisms.

At the same time, the compendium has been conceived as a modular tool. Users may decide to apply only selected protocols, depending on their institutional mandate, available resources and local priorities. In some contexts, the focus may be primarily on environmental and sanitary monitoring, while in others on larval recruitment or governance coordination among protected areas. The document explicitly recognises that not all entities will have the capacity or the mandate to implement all protocols simultaneously.

For this reason, the compendium provides sufficient contextual information to allow users to understand how individual protocols fit within the broader conservation framework. Cross-references between protocols are highlighted to support informed decision-making and to avoid fragmented or ineffective interventions.

2.3 Adaptation to Local Contexts and Precautionary Approach

Mediterranean coastal and marine environments are characterised by high ecological, climatic and institutional heterogeneity. Consequently, the protocols described in this compendium are not intended to be applied mechanically, but rather adapted to local conditions. Users are encouraged to conduct a preliminary assessment of environmental characteristics, logistical feasibility, institutional responsibilities and regulatory requirements before implementing any action.

The compendium clearly distinguishes between core methodological elements, which should be maintained to ensure scientific validity and animal welfare, and flexible components that may be adjusted according to site-specific constraints. Particular attention is given to biosecurity measures and ethical considerations, given the critically endangered status of *Pinna nobilis* and the ongoing risk of pathogen transmission.

In line with the experience gained during the LIFE PINNA project, the compendium promotes a strong precautionary approach. In contexts where uncertainty is high, donor populations are extremely limited, or disease risks cannot be adequately controlled, users are advised to prioritise monitoring, data collection and

capacity building over direct manipulation or translocation of specimens. This approach reflects the lessons learned from the project and aims to avoid actions that could inadvertently exacerbate population decline.

2.4 From Operational Guidance to Strategic Planning

Beyond its immediate operational value, this protocol compendium is also intended to support strategic planning and policy-oriented decision-making. The methodologies described can inform management plans for Marine Protected Areas and Natura 2000 sites, contribute to the definition of conservation objectives and measures, and support the prioritisation of actions at regional or national level.

The document encourages users to document implementation processes, results and challenges, and to share this information within national and international conservation networks. Such feedback is essential to continuously refine methodologies and to build a shared knowledge base for *Pinna nobilis* conservation at Mediterranean scale.

Finally, while the compendium is specifically focused on *Pinna nobilis*, several of the approaches described may be relevant for other endangered bivalve species or sessile marine organisms facing similar threats. Any transfer to other species should, however, be undertaken with caution and in close collaboration with species-specific experts.

3. Protocol 1 – Selection of Restocking Sites

3.1 Objectives of the protocol

The selection of suitable restocking sites represents one of the most critical steps in any conservation or restoration strategy for *Pinna nobilis*. Experience gained during the LIFE PINNA project has clearly demonstrated that the success or failure of restocking actions is strongly dependent on the environmental, sanitary and ecological characteristics of the receiving sites. For this reason, site selection cannot be based on opportunistic choices, but must follow a rigorous, integrated and precautionary approach.

This protocol has been developed on the basis of activities carried out within Actions A1 and C1 of the LIFE PINNA project and aims to provide a transferable methodology for identifying sites with the highest potential to support the survival and long-term persistence of restocked individuals. It is intended to support Marine Protected Area managers, Natura 2000 site authorities, researchers and competent institutions in planning evidence-based restocking interventions, while minimizing ecological risks and ethical concerns.

The protocol promotes a holistic vision of site suitability, combining physical–chemical, sanitary, geomorphological and ecological criteria with historical and biogeographical considerations. By adopting this approach, restocking efforts can be focused on areas where environmental conditions are compatible with the species' ecological requirements and where the likelihood of long-term success is maximised.

3.2 Physical–Chemical Environmental Conditions

From a physical–chemical perspective, candidate restocking sites must present environmental parameters compatible with the physiological tolerance and ecological niche of *Pinna nobilis*. Water temperature, salinity, oxygen concentration and nutrient levels should fall within ranges known to support the growth, filtration activity and reproductive potential of the species.

Particular attention should be paid to temperature regimes, as thermal anomalies and prolonged exposure to elevated temperatures have been associated with increased stress and susceptibility to disease. Sites characterised by strong seasonal stability and limited exposure to extreme thermal fluctuations are therefore preferable.

Nutrient concentrations and water transparency are also key parameters. Areas affected by eutrophication, high turbidity or chronic pollution should be excluded, as these conditions can negatively affect filtration efficiency, increase metabolic stress and favour the proliferation of pathogens. Where possible, long-term environmental datasets and existing monitoring programmes should be used to assess the temporal stability of these parameters rather than relying solely on short-term measurements.

3.3 Sanitary Assessment and Pathogen-Free Status

Sanitary conditions are a fundamental prerequisite for any restocking action involving *Pinna nobilis*. The dramatic decline of the species across the Mediterranean has been strongly associated with the spread of pathogenic agents, particularly the protozoan *Haplosporidium pinnae*. Consequently, the identification of pathogen-free sites is essential to avoid introducing individuals into environments that would rapidly compromise their survival.

This protocol recommends the systematic use of sentinel organisms as bioindicators of sanitary status. Sentinel species, typically filter-feeding bivalves such as mussels or oysters, can be collected from or deployed at candidate sites and subsequently analysed in the laboratory to detect the presence of pathogens known to affect *Pinna nobilis*. This approach allows for indirect but reliable assessment of disease risk without exposing the target species itself to potential infection (Figure 1).

Only sites that show no evidence of pathogenic contamination should be considered suitable for receiving restocked individuals. In cases where results are ambiguous or where pathogen presence cannot be excluded with sufficient confidence, a precautionary approach should be adopted, postponing restocking actions and prioritising further monitoring. Sanitary assessments should not be regarded as a one-off requirement but as part of a continuous surveillance strategy, especially in areas characterised by dynamic environmental conditions.

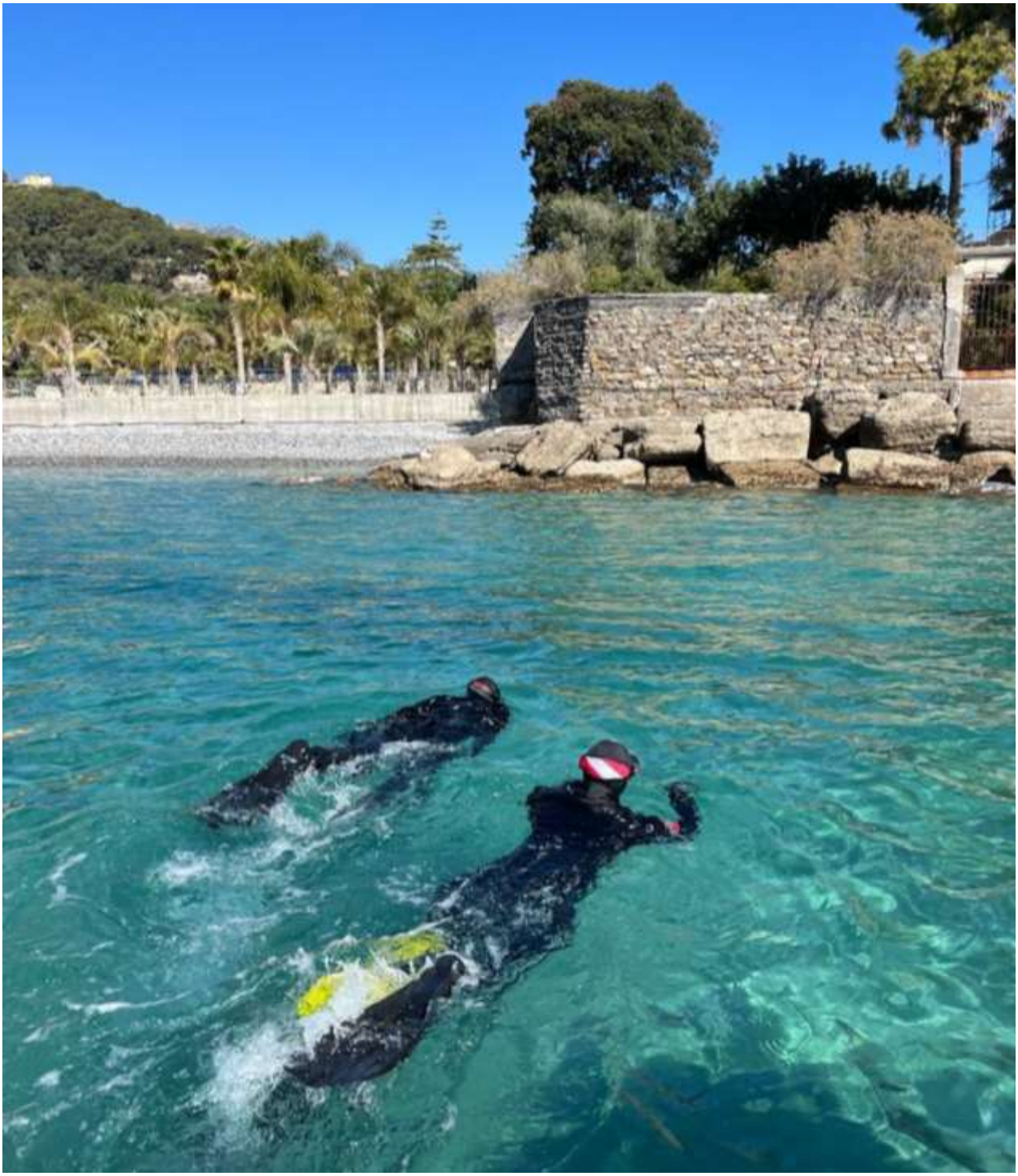

Figure 1. Collection of sentinel species from Capo Mortola Marine Protected Area

3.4 Geomorphological and Geographic Compatibility

Geomorphological and geographic characteristics play a key role in determining habitat suitability for *Pinna nobilis*. Candidate sites should be evaluated in terms of depth range, seabed morphology, sediment composition and exposure to hydrodynamic forces. The species typically inhabits shallow to mid-depth waters, and restocking sites should fall within depth ranges comparable to those of donor populations or historically occupied areas.

Sediment characteristics are particularly important, as *Pinna nobilis* anchors itself to the substrate through byssal threads. Substrates that are too compact, unstable or heavily disturbed may prevent proper anchoring and increase the risk of dislodgement, especially during storm events. Conversely, excessively fine sediments may lead to burial or reduced water circulation around the shell.

Geographic coherence between donor and recipient areas should also be considered. Sites located at similar latitudes and sharing comparable climatic patterns and seasonal variability are generally more suitable, as they reduce the risk of exposing individuals to unfamiliar environmental regimes. While long-distance transfers may sometimes be unavoidable, this protocol encourages prioritising regional or sub-regional coherence whenever possible to enhance acclimation success.

3.5 Ecological Status and Habitat Typology

The ecological status of candidate restocking sites is central to their suitability. Priority should be given to habitats known to support healthy or historically abundant *Pinna nobilis* populations. In the western Mediterranean, this typically corresponds to *Posidonia oceanica* meadows (Figure 2), which provide structural complexity, stable substrates and favourable hydrodynamic conditions for the species.

Figure 2. Healthy *Posidonia oceanica* meadow.

In the northern Adriatic Sea, suitable conditions may also be found in habitats structurally different from *Posidonia* meadows, such as coarse sandy bottoms or seagrass meadows dominated by *Cymodocea nodosa* or *Zostera* species (Figure 3). Although these habitats differ in terms of architecture and sediment dynamics, they have been shown to support *Pinna nobilis* populations under certain environmental conditions.

Ecological assessments should therefore be site-specific and based on direct surveys, habitat mapping and existing ecological data. Sites affected by intense anthropogenic pressures, such as anchoring, dredging or

coastal construction, should be carefully evaluated, as these activities may compromise habitat integrity and increase mortality risk.

Figure 3. *Pinna nobilis* in a Zostera meadow.

3.6 Historical Presence and Preliminary Field Surveys

Historical presence of *Pinna nobilis* is a key criterion for site selection. Restocking actions should preferentially target areas where the species is known to have occurred in the past, based on historical records, scientific literature, monitoring data or documented observations (Figure 4). Prior presence indicates that the environmental conditions were once suitable for the species and increases the likelihood that the site can again support viable populations, provided that current conditions meet the required criteria.

Figure 4. A dead *P. nobilis* in a *P. oceanica* meadow, indicating the species' historical presence in the area.

As part of the site selection process, targeted field surveys should be conducted to detect any surviving individuals. These surveys may involve scuba diving, remotely operated vehicles (ROVs) or other suitable monitoring techniques, depending on depth and visibility conditions. The involvement of citizen science initiatives can significantly enhance detection capacity, particularly in shallow coastal areas and lagoons.

The identification of remnant populations provides valuable information not only for confirming habitat suitability but also for refining restocking strategies. In some cases, the presence of surviving individuals may suggest the existence of local resistance factors or micro-environmental refugia, which should be prioritised in conservation planning.

3.7 Summary and Operational Implications

In summary, suitable sites for *Pinna nobilis* restocking are those that combine favourable and stable physical–chemical conditions, verified pathogen-free sanitary status, compatible geomorphological and habitat characteristics, and a good or high ecological status. Historical presence of the species and the potential presence of surviving individuals further strengthen site suitability and should guide prioritisation.

Adhering to this protocol helps ensure that restocking actions are implemented under conditions that maximise the likelihood of survival and long-term recovery, while minimising ecological and ethical risks. Moreover, the structured and transparent approach described here enhances the replicability and transferability of conservation practices developed within the LIFE framework, supporting coordinated efforts for *Pinna nobilis* conservation across the Mediterranean.

4. Protocol 2 – Restocking Techniques and Translocation Procedures

4.1 Objectives and rationale

The primary objective of this protocol is to implement active conservation strategies to counteract the dramatic collapse of *Pinna nobilis* populations caused by Mass Mortality Events (MME). Given that traditional passive protection is no longer sufficient, the rationale focuses on identifying pathogen-resistant individuals and establishing a transferable methodology for restocking affected areas with healthy specimens. This approach integrates advanced monitoring, molecular characterization, and experimental breeding to safeguard the genetic variability necessary for long-term species resilience.

4.2 Capture of juveniles and early life stages

It is extremely important to enhance larval recruitment and verify if larvae are reaching the impacted areas, thus potentially contributing to eventual recoveries. Capture strategies for larval interception rely on artificial collectors designed to intercept pelagic larvae before they settle in areas of high mortality. The basic design, a customized version of the one described by D. K. Kersting and Hendriks in the "Short Guidance for the Construction, Installation and Removal of *Pinna Nobilis* Larval Collectors," has been tested extensively.

Guidance for Construction of Larval Collector

Structure of larval collector bags:

- **Substratum:** The core of the collector consists of entangled nylon filament, onion bags, or similar durable, fine-filament materials that serve as the settlement substratum for the larvae.
- **Protection:** This material is placed inside a protective polyethylene (or similar plastic) mesh bag. This outer mesh bag shields the larvae from predators while still allowing access to the inner filaments.
- **Sealing and Anchoring:** The outer mesh bag must be securely closed using cord or nylon cable ties. The same cord used for closing can also be used to anchor the bag to the main rope.

Material Sourcing:

- **Entangled Nylon:** This material can be sustainably sourced by recycling old or broken trammel nets from fishermen. It is reusable multiple times if rinsed and dried after each deployment.
- **Mesh Bags:** Onion or vegetable nets/bags can be recycled or purchased from gardening/agriculture or online shops.

Deployment System:

- **Main Structure:** The collector bags are attached to a main rope.
- **Mooring Deployment:** The entire system is secured at the bottom with a small concrete mooring (or similar heavy ballast) to prevent dislocation from waves and currents. A submerged buoy (at a depth of >$ meters) keeps the rope vertical. This submersion prevents the system from being visible from the surface and reduces the risk of entanglement with boats.
- **Alternative Deployment:** The system can also be suspended from existing structures, such as mussel farms, using a light ballast to maintain the rope's vertical orientation.

Guidelines for **installation of collectors for *Pinna nobilis* Larvae**

Placement and Depth:

- **Exposure:** Collectors should ideally be positioned in locations exposed to open waters, as *P. nobilis*

larvae are transported by currents. However, installation in other sites, such as semi-enclosed lagoons, is possible if monitoring potential recruitment in those areas is needed.

- **Presence of Adults:** The installation location does not require the presence of adult *P. nobilis* populations. Larvae can travel long distances via currents, meaning recruits may originate from distant areas, even if the species is absent locally or affected by mass mortality.
- **Depth Range:** *P. nobilis* larvae are known to settle across a wide depth range, allowing for both deeper (e.g., 15 m) and shallower (e.g., 5 m) installations, depending on local conditions.
    - For deeper sites, bags can be attached to the rope at approximately 1.5 m intervals to cover a wider depth range.
    - For shallower sites, bags can be attached at a single point.

Timing:

- **Reproduction and Settlement:** The main reproduction period for *P. nobilis* is typically May to August, with peak settlement estimated between July and September in the Western Mediterranean. These periods can vary based on regional environmental conditions (e.g., water temperature).
- **Installation:** It is suggested to install collectors in late May or early June. Installing later reduces the possibility of covering the entire main larval settlement period.
- **Removal:** Collectors should be removed between October/November and January. Later removal increases exposure to storms in some regions and risks the juveniles running out of space to grow between the filaments.

Removal and Handling:

- **Careful Removal:** Collectors must be removed carefully, avoiding crushing the bags.
- **Juvenile Extraction:** The bags should preferably remain underwater until the juveniles are removed.
    - Juveniles must be extracted with care to prevent breaking their fragile valves or damaging their byssus
    - Extracted juveniles must be immediately placed in seawater.

4.3 Controlled growth and conditioning

Juveniles can be managed in two ways.

Option 1: Field Rearing and Subsequent Re-implantation

Place the collected juveniles in protective devices (lanternets or cages) in the field to continue their growth. Once a suggested size (e.g., greater than 15 cm) is achieved, they can be re-implanted onto a suitable bottom.

Option 2: Controlled Tank Rearing

Alternatively, specimens can be transferred to controlled tanks for rearing, following a specific suggested protocol. The tanks are cleaned three times a week by siphoning the bottom of the tank to remove pseudofeces and any food residue. The volume of water removed is subsequently replaced with clean water. The water change is partial (1/3 of the total volume) to avoid leaving the specimens dry. The water used to fill the tanks is filtered and sterilized: it filters through a sand filter, then a biological filter, two 10- and 1-micron cartridge filters, and finally through three 36W UV lamps. All tanks with porous stones connected to a compressor for aeration. The photoperiod is regulated using an LED lamp, set to increase the hours of light according to the natural photoperiod. Based on the size of the specimens and the density of the algae culture,

the specimens have to be fed daily with a mixture of two live microalgae (Isochrysis galbana: culture density 8 *106 cells/ml, Chaetoceros calcitrans: culture density 7 *106 cells/ml); optional is an aliquot of Easy Reef® artificial feed (8 ml/day/tank, following the product instructions) and a small amount of decapsulated Artemia salina cysts (Gold Pearls®, the dose is at least 0.25 g), to increase the protein intake in the diet. The microalgae dose can be administered to the specimens using peristaltic pumps (ie Jebao Doser 3.4®) every hour for 19 hours a day. Avoid administering food during tank cleaning and water changes. The different strains of microalgae can be maintained and grown in the laboratory in 40-litre bioreactors in order to produce microalgae in large quantities. I. galbana, D. lutheri and T. suecica are maintained at a salinity between 31.5 and 32 practical salinity units (PSU) and C. calcitrans at 25 PSU (Helm et al., 2006). The density of the cultivated algal cells is calculated using a Bürker haemocytometer. The water used for microalgae is filtered and sterilized in the same way as that used for *P. nobilis* individuals, but was also chemically sterilized (0.4 ml/L sodium hypochlorite with aeration for 24 hours and then 0.024 g/L sodium thiosulphate to remove excess chlorine). The culture medium used for microalgae growth is Guillard F/2 (Cell-Hi-F2P; Varicon aqua solution®).

4.4 Transport methodologies and stress minimisation

Building upon the experience gained from transporting adult *Atrina fragilis* in tanks to the Camogli laboratory, the same transport and maintenance methodology was successfully adapted for *Pinna nobilis* specimens (adult and young).

The initial transport, involving eleven *P. nobilis* specimens, took place on 19 June 2023, covering the six-hour journey from the Venetian lagoon to the Camogli laboratory. This protocol has been consistently applied in subsequent years for transports from the Venetian lagoon to the Ligurian sea.

Steps for Transport:

1. **Container Selection:** Food-grade plastic containers (30-40 L), commonly used for agricultural purposes (e.g., olive harvesting) and readily available in hardware or agricultural stores, are used. The container features a sealed cover with a 30 cm diameter opening (Fig. 1). The cover is perforated to accommodate small tubes for ventilation of the *P. nobilis* individuals. Depending on size, one container can hold a maximum of 3 individuals with a shell height between 20 and 30 cm.
2. **Cushioning Material:** A thick layer (15-20 cm) of soft material, specifically rock wool (often used in aquarium mechanical filters), is placed at the bottom. Suitable alternatives include jute sacks, potato bags, non-floating artificial foam, or a layer of natural bath sponges.
3. **Specimen Orientation and Immobilization:** Based on tests with *A. fragilis*, the vertical system was preferred. This system uses a circular plastic tube with an attached rubber net (the type used in trawling, available from fishing shops) to keep the individuals securely in place. This structure prevents movement without obstructing the opening of the specimens' shells.
4. **Aeration System Assembly:** The aeration system is constructed using a 1L plastic jar with the bottom removed. A small tube with a porous stone is inserted inside. A wad of rock wool is placed at the top to minimize the circulation of air bubbles in the water, preventing potential damage to the organisms from internal bubbles.
5. **Filling and Loading:** The container is filled with seawater. Organisms are transferred directly by immersing the container into the seawater to prevent the specimens from being exposed to air and drying out. Once inside the container, specimens are secured and wrapped with soft material (such as rags, cotton wool, or jute) placed between the container's nets. This operation is performed on the boat. Finally, the cover is screwed on after feeding the aeration tube through one of the two pre-drilled holes and connecting it to a bubbler device (e.g., AMTRA, 5V, 360 L/h).
6. **Monitoring During Transport:** The condition of the individuals, the containers, and the functionality of the bubblers are checked at least every 2 hours throughout the transport duration.

4.5 Installation techniques in recipient sites

Long-term survival of restocked *Pinna nobilis* individuals critically depends on their mechanical stability after reintroduction. Specialized techniques for the successful installation of juveniles and adults in their natural habitat have been developed, a consensus supported by current scientific findings, the LIFE PINNA project, and the RESTORFAN framework.

Micro-site Selection and Environmental Validation

Successful installation must occur within validated "safe zones" characterized by historical species presence and favorable environmental parameters.

Habitat Type: Optimal sites are located within healthy seagrass meadows (*Posidonia oceanica* or *Cymodocea nodosa*), which provide essential hydrodynamic protection and a stable substrate for byssus attachment.

***Depth and Hydrodynamics**:* Preferred installation occurs at depths between 5 and 20 meters in areas with low hydrodynamics to prevent the physical uprooting of the shells before they can regenerate their byssus.

Sanitary Conditions: we suggest a preliminary 3-month monitoring period using sentinel organisms (*Mytilus galloprovincialis*) to confirm the absence of *Haplosporidium pinnae* DNA in the local water column.

Pre-Installation Acclimatization

To minimize the risk of osmotic and thermal shock, specimens must undergo a gradual acclimatization phase directly in the field or in specialized tanks at the recipient site.

Procedure: Local seawater is added to the transport containers at a rate of 3 liters every 10–15 minutes for approximately one hour until parameters (T, pH, Salinity) are equalized.

Vitality Check: Divers must verify the reactivity of the organisms; only individuals showing active shell closure in response to external stimuli should be planted.

Core Planting Techniques

The physical burial of the specimen must mimic its natural life position while ensuring structural integrity.

Excavation: Divers scavenge a hole manually or using a lightweight sorbonne (air-lift pump) to reach the necessary depth without causing excessive sediment turbidity.

Burial Depth: The specimen must be interred for one-third to one-half of its total shell length. Shallow planting leads to uprooting by currents, while over-burial may interfere with valve opening and filter-feeding efficiency.

Orientation: The individual is placed in a natural vertical orientation with the umbo facing down. While they naturally tend to reorient themselves, initial placement "at a cut" (perpendicular) to the dominant current can reduce hydrodynamic drag.

Anchoring Systems and Structural Supports

Various methods are employed to provide immediate stability while the animal regenerates its byssus threads, a process that can take weeks/months.

MERCES Method: Employs U-shaped stainless-steel rods to fix the shell into the substrate. The hole is sometimes partially filled with pebbles to facilitate faster byssal anchoring.

Jute Bag Technique: Juveniles grown in jute baskets can be planted along with the entire bag into the sediment. The jute serves as an initial anchor and degrades naturally within weeks, allowing the roots to penetrate the substrate.

Natural Support: In the absence of a *Posidonia* root matrix, surrounding the shell base with small stones or fragments of dead matte is recommended to simulate a complex anchoring environment.

Predation Mitigation (Caging)

Restocked individuals are highly vulnerable to predators such as octopuses (*Octopus vulgaris*).

Exclusion Cages: The installation of rigid anti-predation cages is mandatory. These are typically constructed from plastic or coated metal mesh (1–2 cm mesh size).

Securing the Structure: Cages must be anchored with 20–30 cm stakes to prevent displacement by predators or extreme weather events.

Maintenance: Cages require regular cleaning to remove macro-biofouling (e.g., bryozoans, algae) that might otherwise restrict water flow and nutrient availability.

Traceability

Every individual is assigned a Unique ID (CUI) and tracked through technical data sheets documenting biometry, origin, health status etc. throughout the collection and translocation cycle.

Post-Restocking Monitoring Protocol

The installation cycle concludes with a rigorous monitoring schedule to evaluate the "escape size" success.

Temporal Resolution: Initial verification at 15 days, followed by monthly inspections for 12 months, and annual surveys thereafter.

Key Indicators: Survival rates, shell growth (Htot/Lt), stability in the sediment, and updated sanitary status via molecular diagnostics.

5. Protocol 3 – Reproduction of *Pinna nobilis* in Captivity

5.1 Objectives and conservation relevance

The objective of Protocol 3 is to establish a scientifically robust and ethically sound framework for the captive reproduction of *Pinna nobilis* as a conservation measure to support the survival of the species in the context of the widespread collapse of Mediterranean populations. Ex situ reproduction is conceived as a complementary action to in situ protection, to be adopted when natural recruitment is absent and donor populations are no longer able to sustain restoration efforts.

Furthermore, it is important to highlight the growing interest in the use of aquaculture for biodiversity conservation (Patterson, 2019), through a sustainable aquaculture approach, aimed not only at relieving pressure on wild populations, but also at promoting the resilience of aquatic ecosystems (Overton et al., 2024). Therefore, assuming that rearing techniques can also be transferred to species of great ecological interest or threatened species, aquaculture may be a valid tool to be implemented for the recovery of *P. nobilis*.In fact, the LIFE PINNA project demonstrated that it is possible to maintain adult individuals under laboratory conditions, induce spawning, achieve fertilisation and rear larvae up to advanced developmental stages, thereby filling a major knowledge gap in the reproductive biology of the species.

From a conservation perspective, captive reproduction allows the preservation of genetic diversity, the safeguarding of genetic lineages potentially associated with disease resistance, and the reduction of the risk of irreversible genetic loss through controlled broodstock management and fertilisation.

The protocol also addresses the need to minimise pressure on the remaining wild populations by reducing the collection of individuals from natural habitats and promoting conservation strategies based on knowledge and laboratory support. Although the production of juveniles suitable for reintroduction has not yet been achieved, reaching the umbonate stage represents a crucial advance and opens new perspectives for improving larval management and settlement success.

Finally, the protocol is intended as a dynamic and shared tool, with potential applicability to other related species, and as a scientific baseline for coordinated research and long-term conservation planning for *Pinna nobilis*.

5.2 Management of broodstock in the laboratory

During the Life Pinna project, we tried to maintain the adults inside the tank in three different positions: horizontal, oblique and vertical using plastic boxes, to facilitate their handling for spawning induction trials to achieve controlled reproduction. The specimens were maintained at an approximate temperature of 21°C, air conditioning the room or using coolers, and the photoperiod was regulated using an LED lamp (12 h light/12 h dark or according to natural photoperiod). The seawater was filtered using sand and cartridge filters (10 µm - 1 µm), then sterilized with three UV lamps (36W/each). The water was changed, and the tank was cleaned three times a week, as it is a semi-closed system.

In 2024, based on the size of the specimens and the density of the algae culture, the specimens were fed daily with a mixture of two live microalgae (*Isochrysis galbana*: culture density 8 *10^6 cells/ml, *Chaetoceros calcitrans*: culture density 7 *10^6 cells/ml), an aliquot of Easy Reef® artificial feed (8 ml/day/tank, following the product instructions) and a small amount of decapsulated *Artemia salina* cysts (Gold Pearls®, the dose was gradually increased from 0.25 g to 2.5 g/tank), to increase the protein intake in the diet. The microalgae dose was administered to the specimens using peristaltic pumps (Jebao Doser 3.4®) every hour for 19 hours a day, while routine tank cleaning and water changes (3 times a week) were carried out during the remaining 5 hours, during which no feed was administered. In this way, it was possible to administer approximately 79 ml/h/specimen of *I. galbana* and 53 ml/h/specimen of *C. calcitrans*. As a result, each specimen was fed a total of 2.5 L/day of microalgae mix (*I. galbana*: 1.5 L and *C. calcitrans*: 1 L per day).

In 2025, the specimens were fed daily with a diet consisting of a mix of three algal strains (between $1.05*10^{10}$ and $2.1*10^{10}$ cells/sample): 60% *Isochrysis galbana*; 30% *Chaetoceros calcitrans*/*Phaeodactylum tricornutum*; 10% *Tetraselmis suecica*/*T. chuii*. In addition, zooplankton was administered in vivo (copepods, *Artemia salina* nauplii and rotifers) or as artificial feed (Bea zoo plus®; Gold pearl®). The different strains of microalgae are maintained and grown in the laboratory in 40-litre bioreactors in order to produce microalgae in large quantities.

5.3 Spawning induction techniques

Pinna nobilis specimens reacted to various spawning induction stimuli:

a) stress from collection and transport
b) thermal shock
c) gradual increase in the temperature of the water

The adult specimens, if mature, release gametes even after transport stress, so it is necessary to be prepared to handle the gametes and larvae even immediately after the arrival of the specimens in the laboratory.

As regards the thermal shock, the adult specimens were maintained at around 21°C and moved repeatedly (about every 30-50 minutes) from cold to warmer water tanks and *vice versa*, with a thermal gap of 10°C (±5 °C from the temperature of the adult specimen tanks). Temperatures were maintained through the use of two heaters (Tetra HT® 100 w) and a chiller (Teco TK500®). The stimulation process lasted 2-4 hours (Trigos et al., 2018; Hernandis et al., 2023; Ferranti et al., 2024), depending on the response observed in the specimens. At the end of the induction trials and after the possible release of gametes, the specimens were returned to their tanks.

Instead, the gradual increase in temperature was applied to induce gonadal maturation in some specimens of *P. nobilis*, collected in winter and matured in a controlled environment, bringing forward the release of gametes compared to the natural spawning period and increasing spawning events frequency. In this case, the specimens are maintained in tanks with different volumes, gradually increasing the temperature (from 11° to 25°C) and the photoperiod (from 14 h to 18 h of light), to simulate the seasonal variations in abiotic parameters.

5.4 Fertilisation and larval rearing

Following spawning (Fig. 1), the eggs released (already fertilised or not) were collected, filtered on two filters: 125 µm (or less, depending on the availability of filters) to retain faeces or aggregates of decomposing organic matter, and 45 µm on which the eggs were retained (egg diameter 55 µm). During this process, the eggs were kept submerged so as not to dry out on the filter. Then, the eggs were placed in a known volume, counted and measured. The number of eggs was counted by homogenizing the sample, taking a 1 mL subsample (at least 3 replicates), and counting in a Sedgwick Rafter Counting Chamber. Embryo development was monitored during egg release counts to determine fertilization rate. If unfertilized eggs were released, fertilization was achieved by adding a few mL of sperm. The eggs were then gently stirred several times to facilitate contact between the gametes, and after about half an hour the development of the embryos was observed. As the eggs settled to the bottom, it was important to fertilise them in a container (beaker or tank) with sufficient surface area to ensure the formation of a monolayer on the bottom. In this way, the eggs do not overlap and are more likely to be fertilised.

Fig. 1: Eggs spawning of *Pinna nobilis* specimen

The zygotes were then placed in several tanks of different volume and equipped in order to insufflate air through a small rigid tube, creating a chain of bubbles (about 1 bubble/second). The development of the embryo was monitored until the formation of the larvae (Fig. 2).

The larvae were reared at a water temperature of about 21°C (room temperature), according to Trigos et al. (2018), to avoid the rapid development due to the high temperature observed in Ferranti et al. (2024). The water used was filtered and sterilized in the same way as for adult specimens. The larvae were placed in tanks with different volumes, at the density of 2.5 larvae/mL and 5 larvae/mL.

The larvae were fed daily with a mix of 2 microalgae (*I. galbana, C. calcitrans*): the first day a full dose was provided, the second day only the amount consumed was added. *I. galbana* is a species characterized by nutritional profile rich in docosahexaenoic acid (DHA) (Helm et al., 2006; Martino et al., 2023), whereas *C. calcitrans* has nutritional profile rich in eicosapentaenoic acid (EPA). Essential fatty acids (EFAs), particularly the omega-3 fatty acids, EPA and DHA, are important for larval growth and development because they are the main components of cell membranes and play a key role in modulating membrane functions (Marshall et al., 2010). Consequently, the larvae were fed a total of 133.3 cells/µl of *I. galbana* and 150 cells/µl of *C. calcitrans*. This is because, although the polyunsaturated fatty acid content is highly dependent on the culture conditions of the microalgae (Fernandes et al., 2016), an equivalent biomass of *C. calcitrans* and *I. galbana* provides an EPA/DHA ratio close to 2/1 (2.25 cells/µl of *C. calcitrans*: 1 cell/µl of *I. galbana*; Helm et al., 2006). However, we preferred to test an EPA/DHA ratio of 1/1 and dosed an amount of *C. calcitrans* biomass equivalent to half the biomass of *I. galbana* (Tab. 1). In addition, once the larvae reached a size above 120 µm, the diet was supplemented with another microalga, *Tetraselmis suecica* ($4.4*10^6$ cell/mL), which is known to improve the survival of bivalve mollusc larvae, compared to a diet based on only two microalgae (Helm et al., 2006). Therefore, the microalgal concentrations were recalculated, supplying 88.86 cells/µL of *I. galbana*, 8.89 cells/µL of *T. suecica* (knowing that 1 cell of *I. galbana* = 0.1 cells of *T. suecica*) and 200 cells/µL of *C. calcitrans* (knowing that 1 cell of *I. galbana* = 2.25 cells of *C. calcitrans*), thus further enriching the diet in EPA.

No microalgae were fed to the larvae until they reached the D-larvae stage, as they only start feeding at this developmental stage. (Helm et al., 2006).

The water in the larval tanks was changed every two days using a 45 µm filter on which the larvae were collected, which was kept submerged inside the tank, so that the larvae were not left to dry on the filter. The larvae retained on this filter were then transferred to a known volume of water, counted in a Sedgwick Rafter Counting Chamber (1 mL subsample/3 replicates) and then were put back into the tanks filled with renewed water and fed, trying to maintain concentrations of approximately 1-2 larvae/mL, after the initial mortality. At the same time, larval survival and development was monitored.

In both 2023 and 2024, we observed that larvae produced by self-fertilization stopped their cycle at 9 days post fertilization (dpf), at the D-larva stage, with an average shell size of 86 µm, resulting in less viable larvae (Fig. 3Af). Whereas the larvae obtained from controlled fertilization developed better, surviving until 21 dpf (Fig. 3B), of these, 40% reached the umbonate larval stage at 16 dpf with a shell size of 150 µm (Fig. 3Ah), in accordance with Hoyos-Chairez & Chavez-Villalba (2023), who report that *Atrina maura* (Family Pinnidae) reaches this larval stage at the same size and after about 15 dpf. This larval size was never achieved before for *P. nobilis*

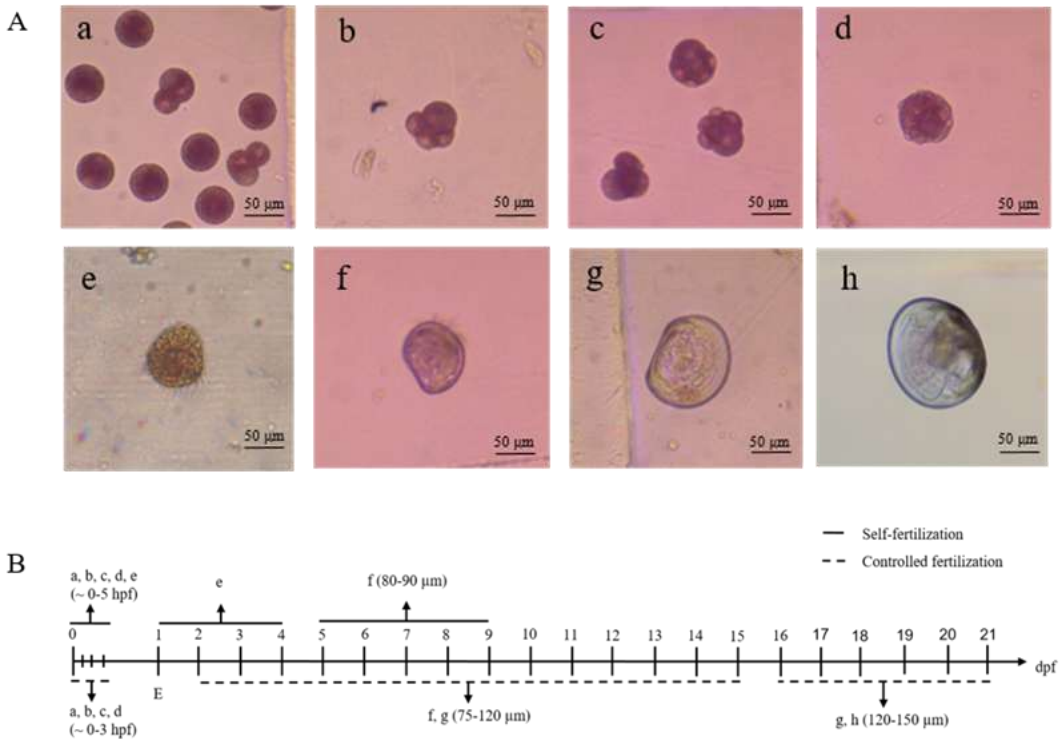

Fig. 2: A) Larval developmental stages of *Pinna nobilis*: a: Egg and 1st complete division; b: 4th division; c: End of cell division phase (>8 cells); d: Morula; e: Early trochophore; f: Early D-larvae (~80 µm); g: D-larvae (~120 µm); h: Umbonate larvae stage (~150 µm). Line bar: 50 µm. B) Timing of *Pinna nobilis* larvae development, comparison between self-fertilization (continuous line) and controlled fertilization (dotted line).

Figure 3 shows the trend in larval survival for both types of fertilizations: for self-fertilization, the first count was only at 5 dpf, with a survival rate of 16% (D-larva stage). On the other hand, in controlled fertilization, the survival rate reached 37.2% at 1 dpf (trochophore stage) and remained around this percentage until 5 dpf with the development of the D-larva. At 7 dpf there was a further marked decrease with a survival rate of 5%.

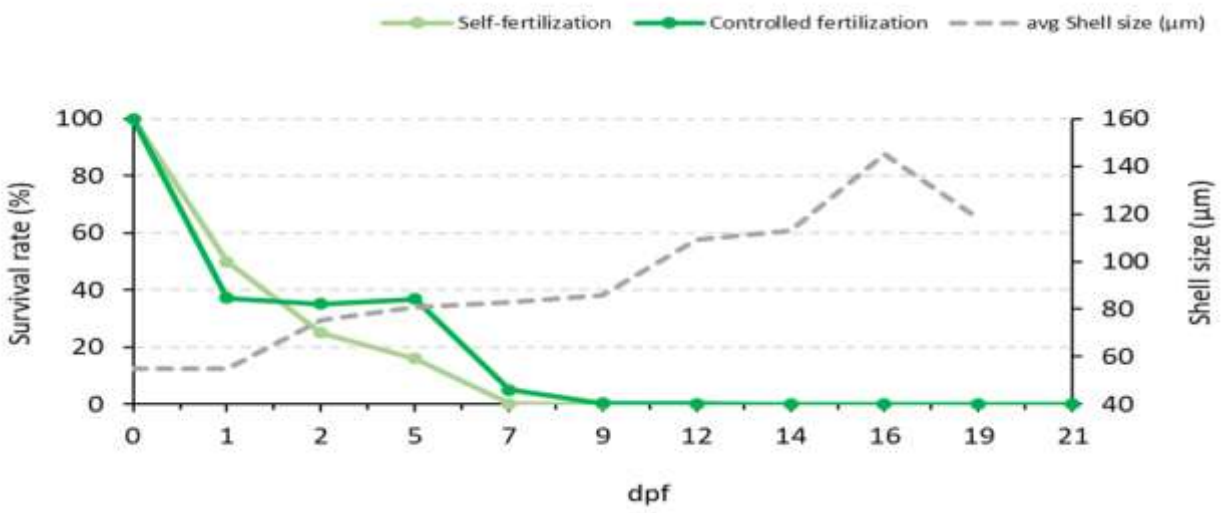

Fig. 3: Larval survival rate (self-fertilization and controlled fertilization) and average shell size of larvae of *Pinna nobilis*

Unfortunately, the pediveliger stage and settlement were not reached. However, it was hypothesised that these would be achieved within a few days, based on information reported for other species of Pinnidae. In fact, Hoyos-Chairez & Chavez-Villalba (2023) report that settlement starts at 29 dpf for *A. maura*; Hashimoto et al. (2023) at 24 dpf for *A. lischkeana.* Consequently, the previous assumption that the stage of settlement of *P. nobilis* larvae is between 5 and 10 days (De Gaulejac, 1989; Basso et al., 2015), contrasts with the information reported in the recent literature and with what was observed in the present study. Although the diet used resulted in good larval development and survival up to 21 dpf, it is hypothesized that in order to achieve settlement it will be necessary to further develop techniques for larval management of *P. nobilis*, focusing efforts on improving the nutritional profile of the diet provided and also on water quality management. In this regard, it could be useful to work with larger volume rearing systems, which can guarantee a greater stability of water parameters (i.e. nutrients) for a longer period of time, as proposed by Hashimoto et al. (2023), facilitating cleaning and water changes without significant manipulation of the larvae.

5.5 Applicability in Mediterranean research facilities

The maintenance of adult specimens of *P. nobilis*, as well as the controlled reproduction and larval development phase, can also be applied and replicated at other research facilities in the Mediterranean. Further research on the maintenance of larvae is needed to reach the settlement phase and consequently the development of juveniles, but this could be achieved by sharing experiences among experts.

5.6 Critical constraints and ethical boundaries

The critical constraints relating to the captive breeding of *P. nobilis* are linked to the limited knowledge available on the larval development of this species, rather than on methods for maintaining and stimulating adult specimens. Despite all efforts, no juveniles suitable for reintroduction to the sea have yet been obtained in captivity. Furthermore, from an ethical point of view, the drastic reduction in natural populations must be considered. Therefore, before removing any specimens for captive breeding purposes, it is necessary to carefully assess the availability of specimens in good health, in order to avoid further damaging the last remaining specimens in the wild.

6. Protocol 4 – Agreement Scheme for Juvenile Capture among MPAs

Legal and Bureaucratic Framework for *Pinna nobilis* Translocation

The active conservation of the "Critically Endangered" *Pinna nobilis*—listed in Annex IV of the EU Habitats Directive (92/43/EEC) and Annex II of the Barcelona Convention—necessitates integrating strict scientific protocols with complex legal and administrative requirements. Consequently, the translocation of this species is not merely a biological task but a demanding legal exercise.

Challenges in Translocation

Moving *P. nobilis* individuals from donor populations (e.g., the Venice Lagoon) to high-mortality recipient sites (e.g., Ligurian MPAs) faces significant legal and bureaucratic obstacles:

- **National Derogation Requirements:** The manipulation, detachment, or transport of this strictly protected species is strictly prohibited under Article 16 of the Habitats Directive unless a formal derogation is granted. In Italy, this requires approval from national authorities (e.g., MASE) following a positive technical opinion from specialized bodies (e.g., ISPRA). Bureaucratically, proponents must demonstrate the "no satisfactory alternative" principle and ensure the action will not compromise the conservation status of the donor populations.
- **Transboundary Coordination:** International transfers (e.g., between Italy and Slovenia) demand harmonized bureaucratic efforts. This coordination is essential to synchronize quarantine protocols and prevent fatal delays at customs, which pose a high risk to organisms sensitive to transport stress.
- **Sanitary Clearance Protocol:** To mitigate the unintended spread of pathogens like *Haplosporidium pinnae* or *Mycobacterium spp.*, a mandatory sanitary clearance protocol must be bureaucratically enforced. This protocol may involve deploying sentinel organisms (e.g., *Mytilus galloprovincialis*) at the recipient site for a minimum of three months to confirm the absence of pathogenic DNA.

This protocol serves as the administrative blueprint for inter-institutional cooperation, essential for managing fragmented surviving populations.

6.1 Rationale for inter-institutional agreements

Individual Marine Protected Areas (MPAs) frequently operate under significant constraints that compromise their ability to independently secure the recovery and long-term sustainability of vulnerable marine species. A primary limiting factor is often the insufficient spatial scale, leading to a restricted gene pool and a deficit in the biological resources—such as viable larval supply or sufficient adult broodstock—necessary for effective population replenishment. This isolation and lack of connectivity can render species within a single MPA susceptible to localized extinction events and hamper their resilience to environmental perturbations, such as climate change impacts or disease outbreaks.

To overcome these inherent limitations and foster regional ecological resilience, collaborative strategies are implemented through formal, inter-institutional agreements. These agreements serve as the foundational mechanism for standardizing critical technical methodologies across a network of participating sites. A prime example is the establishment of a standardized "Transport System" protocol, which outlines the precise, safe, and effective procedures for the collection, handling, transfer, and subsequent re-establishment of biological material—including, but not limited to, larvae, juvenile organisms, or adult colonies—between MPAs.

Crucially, the entire framework is predicated on strict adherence to a "precautionary approach." This principle mandates that all transfer activities are executed only after rigorous risk assessment, ensuring minimal ecological disturbance, preventing the spread of non-native or invasive species, and safeguarding the genetic integrity of the recipient populations. The cooperative schemes formalize the pooling of resources and expertise, facilitating the centralization of highly specialized knowledge, technological

infrastructure, and laboratory capabilities in one or a few core centers. Concurrently, this model enables the effective decentralization of essential field operations, allowing for the consistent and timely execution of monitoring, restoration, and transplantation activities across a geographically dispersed network of designated "safe zones." These "safe zones" are carefully selected MPAs or conservation sites that offer optimal conditions for the survival and growth of the transferred material, thereby maximizing the success rate of the regional conservation efforts. This coordinated, network-based approach ultimately amplifies the conservation impact far beyond what any single MPA could achieve alone.

6.2 Legal and administrative framework

Authorizations for Handling and Transporting *Pinna nobilis*

The handling, collection, or transport of live *Pinna nobilis* specimens requires specific ministerial derogations due to the species' strict protection status under Annex IV of the Habitats Directive (92/43/EEC) and Annex II of the Barcelona Convention.

Required National Authorisation (Italy)

The primary requirement is the **Ministerial Exemption** issued by the Ministry of the Environment and Energy Security (MASE). In Italy, MASE grants this exemption *only* after receiving a favourable opinion from ISPRA.

Requests for derogations must be supported by a robust technical dossier demonstrating three essential requirements:

1. **Absence of Alternatives:** Proof is required that options like translocation or captive breeding are the *only* viable methods for saving individuals or the local population (e.g., in areas with high mortality or unmitigable human impacts).
2. **Absence of Prejudice to Conservation:** The proposed activity must not negatively impact the health of the remaining donor populations.
3. **Scientific or Repopulation Purposes:** The actions must be integrated into nationally or regionally approved research projects or recovery plans.

Required National Authorisation (Slovenia)

The primary requirement is the **Ministerial Exemption** issued by the Ministry of Natural Resources and Spatial Planning (MNVP).

Requests for derogations must be supported by a robust technical dossier including the following main requirements:

1. species, subspecies, or higher taxonomic group, and the maximum estimated number of animals for which reintroduction or translocation is planned
2. reason for the reintroduction or introduction of the animal species, with justification
3. evidence showing that animals genetically most suitable for the existing population are being introduced
4. expected time period for reintroduction or restocking, and the expected geographical area of reintroduction or restocking.

Schemes for Specimen Exchange

For the exchange of specimens between different Marine Protected Areas (MPAs) or regions, an **Agreement Scheme** must be drafted. This scheme defines legal responsibilities and biosecurity protocols.

- **National Context (Italy):** These agreements are regulated by Article 15 of Law 241/90, which permits public administrations to collaborate on activities of common interest.
- **International Framework:** The basis for international agreements is provided by the Barcelona Convention's SPA/BD Protocol and Decision IG.26/5, which relates to the *Pinna nobilis* restoration program.

Cross-border Authorisations

International transfers (e.g., between Italy and Slovenia) necessitate **bilateral agreements** to harmonise permits and quarantine requirements between the participating countries.

6.3 Roles and responsibilities of involved authorities

Once authorized, each operational phase must be thoroughly documented to ensure full legal compliance. This documentation includes:

1. Tracking Technical Data Sheet:

This document must accompany each specimen and record its unique ID (often a coloured tag), biometric data, and current health status. It also details the roles of the authorities and partners involved:

- **Donor Marine Protected Area (MPA) Authority:** Responsible for identifying and georeferencing the juvenile specimens.
- **Scientific Partners:** Responsible for the technical execution of capture (using larval collectors) and managing the nursery phase, including diet optimization.
- **Recipient MPA Authority:** Responsible for validating the new site, long-term monitoring, and related tasks.

2. Health Certification:

Prior to any movement, an accredited laboratory must issue a certificate confirming the specimen is negative for pathogens, specifically *Haplosporidium pinnae*.

3. Georeferencing:

Precise GPS coordinates must be recorded for all individuals that are handled or reimplanted. This is mandatory for future monitoring efforts.

6.4 Operational workflow for authorisation and coordination

1. Site Identification: Analysis of historical presence and seagrass suitability (Posidonia oceanica).
2. Sanitary Screening: Deployment of sentinel mussels for 12 months to validate the site as a "safe zone".
3. Authorization Request: Submission of the technical dossier to the Ministry for a capture/transport derogation.
4. Logistical Execution: Implementation of Transport in food-grade containers (30-40 L) with continuous aeration and thermal control (18–21°C).
5. Acclimatization and Replanting: Gradual parameter equalization (1 hour) followed by manual burial (1/3 of shell length).

6.5 Risk management and liability considerations

The comprehensive nature of the agreement requires explicit and detailed provisions concerning all aspects of the research, particularly the delicate matter of accidental mortality. To ensure clarity and accountability, the agreement stipulates that research personnel will be completely absolved of any liability related to the unexpected loss of specimens, contingent upon their strict and demonstrable adherence to the approved project protocols. This adherence includes, but is not limited to, critical steps such as the meticulous maintenance of the cold chain for biological samples and the rigorous conducting of valvular reactivity checks to monitor specimen viability. Furthermore, recognizing the unpredictable nature of external events, the agreement clearly specifies the precise Force Majeure conditions. These conditions define the extraordinary circumstances—such as natural disasters, unforeseen regulatory changes, or significant civil unrest—under which the project's operations may be temporarily or indefinitely suspended. Crucially, any such suspension enacted under a valid Force Majeure event will occur without prejudice or financial penalty to either the research institution or the funding body, ensuring an equitable distribution of risk. A final, but paramount, requirement of the agreement is the mandatory inclusion of a robust and environmentally sound disposal protocol. This protocol is designed specifically for the handling and final disposition of any specimens confirmed to be infected. The core objective of this mandate is the prevention of further environmental contamination, requiring methodologies such as high-temperature incineration or chemical sterilization to neutralize all biohazardous material before disposal. Regular audits of the disposal logs will be required to ensure compliance with both the agreement and relevant public health and environmental regulations.

7. Protocol 5 – Tracking and Identification of Translocated Specimens

7.1 Objectives of specimen tracking

The primary objective of specimen tracking is to establish a detailed biological and clinical history for every reintroduced individual. This enables researchers to correlate long-term survival and growth rates with the environmental conditions of the donor site and the specific genetic lineage of the animal. Furthermore, rigorous tracking is essential to manage the risk of disease spread, ensuring that no individual is moved without a verified sanitary status.

7.2 Identification and labelling methods

Each specimen is assigned a Unique ID (CUI) to ensure absolute traceability. Physical identification is achieved through external tags made of non-corrosive plastic or stainless steel, which are attached to the shell using non-toxic epoxy resins. Color-coding systems (e.g., Tag Green-18, Tag Blue-20) are frequently employed to facilitate rapid visual verification by divers during underwater monitoring. For young specimens on which it is difficult to apply physical markings, tags can be used in the immediate vicinity until they reach a size sufficient for individual marking.

7.3 Data collection and management

Standardized Technical Data Sheets must be initialized at the moment of collection. These sheets record the following mandatory parameters:

- Event Metadata: Date of collection, coordinates (GPS), depth, and habitat description (e.g., Posidonia meadow).
- Biometric Data: Height from the bottom (Hs), maximum width (W), width at sediment level (wc), and estimated total height (Htot).
- Health and if possible Genetic Status: Results of PCR/qPCR diagnostic tests for Haplosporidium pinnae and if possible DNA barcoding markers (e.g., COI gene) for species identification and lineage mapping.

7.4 Chain-of-custody during transport

The chain-of-custody ensures that every specimen is accounted for from the donor site to the receiving site. The transport log must include departure and arrival times, the name of the responsible officer, and regular monitoring of environmental parameters (Temperature, O2, pH) every two hours during transit. Any physiological anomalies observed, such as unexpected gamete release or valve closure failure, must be documented in the "Anomalies" section of the tracking sheet.

7.5 Integration with monitoring activities

Specimen tracking is directly linked to post-restocking monitoring protocols. Replanted individuals are checked at 15 days, monthly for one year, and annually thereafter. The unique ID allows for the measurement of individual growth increments and the assessment of mechanical stability in the sediment. Integration with Sentinel Monitoring is also mandatory; restocking sites are validated only if local filter-feeding bivalves (e.g., Mytilus galloprovincialis) remain negative for pathogens for at least three consecutive months.

7.6 Data sharing and interoperability

To facilitate Mediterranean-wide conservation, data management must prioritize interoperability between regional and international databases. Biometric datasets are shared through common platforms and "Agreement Schemes" between institutional partners. This cooperative approach supports the creation of a standardized repository of best practices, ensuring that lessons learned in one region can be effectively replicated in another.

8. Cross-Cutting Considerations

8.1 Health surveillance and disease prevention

Health surveillance represents a transversal pillar underpinning all protocols described in this compendium and constitutes a non-negotiable prerequisite for any manipulation, translocation or restocking activity involving *Pinna nobilis*. As demonstrated throughout LIFE PINNA and reflected consistently in Protocols 1, 2, 4 and 7, the persistence of pathogenic agents—particularly *Haplosporidium pinnae*—remains the most critical limiting factor for the recovery of the species at Mediterranean scale.

Disease prevention cannot be confined to a single operational phase, but must be embedded throughout the entire conservation chain, from site selection and juvenile capture to transport, installation and long-term monitoring. The use of sentinel organisms, as formalised in Protocols 1 and 6, constitutes the cornerstone of this preventive strategy, allowing for indirect but reliable assessment of pathogen presence without exposing *P. nobilis* individuals themselves to unnecessary risk. This approach is fully coherent with the precautionary framework adopted in the After LIFE Plan, which prioritises continuous surveillance over short-term restoration targets.

Health surveillance must also be temporally continuous. Sanitary clearance prior to translocation, while essential, is insufficient if not followed by post-restocking monitoring integrated with specimen tracking systems (Protocol 7). The linkage between individual Unique IDs (CUIs), molecular diagnostic results and site-level sanitary status enables early detection of emerging risks and supports rapid management responses, including the suspension of further translocations if warning signals arise.

Importantly, the compendium adopts a conservative stance regarding uncertainty: where diagnostic results are inconclusive, or where surveillance capacity cannot be guaranteed over time, the protocols consistently recommend refraining from active manipulation and focusing instead on monitoring, data collection and capacity building. This approach ensures alignment with both EU legal obligations and ethical responsibilities towards a critically endangered species.

8.2 Genetic integrity and long-term resilience

The preservation of genetic integrity emerges as a cross-cutting objective that informs site selection, translocation decisions, captive reproduction and long-term management strategies. As highlighted in Protocols 2, 5 and 7, LIFE PINNA shifted the conservation paradigm for *Pinna nobilis* from numerical restoration to genetic safeguarding, recognising that population recovery is meaningless without maintaining adaptive potential.

Translocation and restocking actions are therefore designed not as mass-release interventions, but as carefully controlled operations aimed at conserving distinct genetic lineages and, where possible, identifying traits potentially associated with disease tolerance. The avoidance of indiscriminate mixing of specimens from distant biogeographical regions, consistently recommended across Protocols 1, 4 and 6, reflects this principle and is fully coherent with the After LIFE Plan's emphasis on regional coherence and network-based management.

Captive reproduction (Protocol 5), while still experimental in terms of producing juveniles suitable for reintroduction, plays a strategic role in genetic conservation. The documentation of controlled fertilisation outcomes, larval performance and developmental bottlenecks contributes to a growing body of knowledge that will be essential for future recovery strategies. Crucially, the protocols stress that any scaling-up of ex situ reproduction must be preceded by clear genetic objectives and safeguards, avoiding the risk of genetic homogenisation or maladaptive selection.

Genetic considerations are also operationalised through traceability mechanisms. The individual-based tracking system described in Protocol 7 allows genetic data, where available, to be linked to survival and growth performance, thereby informing adaptive management decisions and future protocol refinement.

8.3 Monitoring, feedback and adaptive management

Monitoring is not treated as a standalone activity, but as an integrative process that connects all protocols into a coherent adaptive management cycle. As formalised in Protocols 2 and 7, post-restocking monitoring serves multiple functions: assessing individual survival and growth, verifying mechanical stability and habitat suitability, and continuously validating sanitary conditions at recipient sites.

The compendium emphasises that monitoring data should actively feed back into decision-making processes rather than merely fulfilling reporting obligations. This feedback loop is central to the adaptive philosophy that characterised LIFE PINNA and that is explicitly continued in the After LIFE Plan. For instance, evidence of repeated failures at specific sites should lead to a reassessment of site selection criteria, while unexpected survival success may identify previously unrecognised refugia or resistance factors.

Standardisation of monitoring protocols and indicators across MPAs, as promoted through the agreement schemes in Protocol 6, is essential to ensure comparability and cumulative learning. At the same time, the document recognises the need for flexibility in monitoring intensity and methods, allowing adaptation to local capacities while maintaining core data requirements.

Importantly, adaptive management also implies the willingness to suspend or modify actions. The protocols consistently acknowledge that, under certain conditions, the most responsible management choice may be to pause active interventions, reinforcing the credibility and scientific robustness of the overall conservation strategy.

8.4 Transferability to other species and contexts

While this compendium is explicitly tailored to *Pinna nobilis*, several cross-cutting principles and operational approaches have broader relevance. The integrated use of health surveillance, genetic safeguards, inter-institutional agreements and specimen-level traceability can inform conservation actions for other endangered sessile marine species affected by disease, habitat degradation or climate-driven stressors.

However, the document clearly states that transferability should not be interpreted as direct replication. Any application to other species or regions must be preceded by species-specific ecological, pathological and legal assessments. This cautionary stance is consistent with the LIFE PINNA experience and prevents the inappropriate generalisation of methods beyond their validated scope.

9. Recommendations for Replication and Scaling Up

9.1 Minimum conditions for successful replication

Successful replication of the LIFE PINNA protocols requires the simultaneous fulfilment of ecological, sanitary, institutional and logistical conditions. At a minimum, recipient contexts must be able to ensure verified pathogen-free sites, access to appropriate donor material or larval sources, and the capacity to maintain long-term monitoring and traceability.

Replication should not be driven by urgency alone. As repeatedly emphasised across Protocols 1, 2 and 6, the absence of minimum conditions—particularly in terms of health surveillance—constitutes a clear contraindication to active intervention. In such cases, replication should focus on preparatory actions, including site assessment, capacity building and institutional coordination. The After LIFE Plan reinforces this staged approach, encouraging gradual expansion of the network of "safe zones" rather than rapid, large-scale restocking attempts.

9.2 Institutional and technical capacity requirements

Replication and scaling up depend heavily on institutional commitment and coordination. The agreement schemes described in Protocol 6 provide a tested governance model for overcoming fragmentation among MPAs and national authorities. This model, based on formalised roles, shared protocols and legal clarity, should be considered a prerequisite rather than an optional component of replication.

From a technical perspective, scaling up does not necessarily imply increasing the number of specimens handled, but rather enhancing the quality and consistency of operations. Investments in diagnostic capacity, data management systems and specialised training are more critical than infrastructure expansion alone.

The compendium stresses that centralised centres of expertise—such as laboratories capable of molecular diagnostics or controlled reproduction—should be complemented by decentralised field operations, ensuring both scientific rigour and territorial coverage.

9.3 Cost and resource considerations

The protocols implicitly reflect a cost structure dominated by personnel time, monitoring activities, laboratory analyses and coordination efforts rather than by material costs. Scaling up therefore requires sustained financial commitment over time rather than one-off investments.

The After LIFE Plan acknowledges that long-term conservation of *Pinna nobilis* cannot rely exclusively on project-based funding and calls for integration of protocol implementation into ordinary management budgets of MPAs and environmental authorities. This integration enhances financial sustainability and reduces dependency on external funding cycles. Cost-effectiveness can be improved through shared resources, harmonised monitoring and joint training initiatives across MPA networks.

9.4 Common pitfalls and mitigation strategies

Experience gained during LIFE PINNA highlights several recurrent risks: underestimating disease persistence, overestimating site suitability based on historical presence alone, and fragmenting responsibilities among institutions. The protocols address these pitfalls through explicit safeguards, including mandatory sanitary screening, multi-criteria site selection and formalised agreements.

Another critical risk is the pressure to demonstrate rapid results. The compendium consistently warns against equating success with short-term survival metrics and emphasises that meaningful outcomes for *Pinna nobilis* conservation can only be assessed over multi-year timescales.

10. Conclusions and Future Perspectives

This protocol compendium represents a consolidated synthesis of the operational knowledge generated by LIFE PINNA in response to one of the most severe marine biodiversity crises in the Mediterranean. Rather than offering definitive solutions, it provides a structured, precautionary and adaptive framework for action under conditions of uncertainty.

Cross-checking with Protocols 4–7 and the After LIFE Plan confirms a strong internal coherence: health surveillance, legal compliance, traceability and inter-institutional cooperation are consistently treated as foundational elements rather than ancillary components. This coherence is one of the principal strengths of the compendium and a key factor for its credibility and replicability.

Looking ahead, the future of *Pinna nobilis* conservation will depend less on technological breakthroughs than on sustained collaboration, knowledge sharing and institutional responsibility. Advances in captive reproduction, genetic analysis and disease ecology will undoubtedly refine existing protocols, but their effective application will continue to rely on governance frameworks and precautionary decision-making.

Ultimately, the LIFE PINNA experience demonstrates that even in scenarios of near-extinction, responsible conservation action remains possible—provided that it is grounded in science, humility and long-term commitment. This compendium is intended to support that commitment well beyond the lifetime of the project, contributing to a coordinated Mediterranean response to safeguard *Pinna nobilis* for future generations.