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LIFE PINNA

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

Best practices protocol for manipulation, captation and growth of juveniles of *Pinna nobilis*

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Protocols for replication

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Sommario

Introduction

The fan mussel *Pinna nobilis* (Linnaeus, 1758) is the largest endemic bivalve in the Mediterranean Sea. This species occurs in soft-bottom habitats, particularly seagrass meadows of *Posidonia oceanica* and *Cymodocea nodosa*, in transitional water ecosystems and marine coastal zones at depths between 0.5 and 60 metres. It is a long-lived species, with some specimens reaching over 20 years of age, and an important benthic filter feeder that contributes to water clarity. It is recognised as an iconic species, playing the roles of flagship, key and umbrella species. Due to its ecological relevance, *P. nobilis* has recently been proposed as a reliable bioindicator of benthic coastal ecosystems in accordance with the EU Marine Strategy Framework Directive (2008/56/EC). In addition, the fan mussel is a host to two crustacean symbionts, *Pontonia pinnophylax* and *Nepinnotheres pinnotheres*, and is preyed upon by other species such as *Octopus vulgaris*. It plays a key role in the trophic web. *Pinna nobilis* has undergone a significant decline over the last three decades due to multiple factors, which have nearly caused its extinction. Historically, it has been exploited for its byssus, which is used to produce high-quality fabrics, although the impact on the species is not well understood. More recently, severe damage has been inflicted by recreational and commercial fishing for consumption, as well as the collection of large shells for ornamental purposes. Like *P. oceanica*, *P. nobilis* also faces significant impacts from trawling, dredging and uncontrolled anchoring. Consequently, *P. nobilis* is now a protected species under Annex IV of the EU Habitats Directive 92/43/EEC, Annex II of the SPAMI Protocol of the Barcelona Convention and national legislation in most Mediterranean countries. Within a few decades, this comprehensive protection regime led to the species' complete recovery throughout the Mediterranean, as evidenced by molecular analyses examining mitochondrial DNA markers. Since 2016, its populations have been subject to an additional severe negative impact. The protozoan *Haplosporidium pinnae* has caused mass mortality in the south-western Mediterranean. Within a year from the arrival of these pathogens, 90% of *P. nobilis* populations in Spain had disappeared, followed shortly by those in Italy, France, Türkiye, and Tunisia. This microorganism affects the digestive system of the mollusc, progressively reducing its feeding and ultimately causing death. *H. pinnae* appear to exhibit high specificity for *P. nobilis*, as mass mortality did not affect the closely related species *Pinna rudis*. However, it has recently been discovered that this pathogen is not specific to *P. nobilis*, as initially hypothesised by Catanese et al. (2018), and that it had been present in other bivalves in the Mediterranean basin since 2014 (Scarpa et al., 2020). Several bacterial species have also been identified as potential pathogens involved in the mass mortality of this species (Scarpa et al., 2020), suggesting that the underlying causes of this phenomenon are not fully understood and that multifactorial disease is the most probable cause. Currently, surviving and resistant specimens are scarce and scattered throughout the Mediterranean.

Consequently, *P. nobilis* is now listed as "critically endangered" on the IUCN Red List of Threatened Species. Based on the experience gained from the conservation and restoration of *P. nobilis* in the MERCES project (Restoring European Seas), RESTORFAN project (Miramare Marine Protected Area, Italy), the LIFE PINNARCA project (France, Greece, Italy and Spain) and the "Conservation of *Pinna nobilis* in the Adriatic Sea" (Croatian national project), a standardised and effective protocol for the restoration of juveniles of *P. nobilis* has been developed by the LIFE PINNA project "Conservation and re-stocking of *Pinna nobilis* in western Mediterranean and Adriatic sea". The following sections detail the most efficacious restoration techniques and protocols for larval recruitment, transport, the controlled growth in the laboratory and the installation of the juveniles of *P. nobilis* into Marine Protected Areas.

1. Larval captation: methods and structures

Larvae of *P. nobilis* are collected using artificial collectors. This is one of the most effective and widely used methods for restoration projects. Larval collectors should be installed between August and November, or one month earlier, to coincide with the time when *P. nobilis* reaches gonadal maturity and releases gametes into the water column. For the western Mediterranean, the main breeding period is from May to August and settlement from July to the end of September. Installation in June and removal in October or November is recommended. In addition to the use of larval collectors to enhance recruitment, juveniles of *P. nobilis* can be found on longlines at mussel farms. Although harvesting operations are similar, they require greater attention due to the presence of other organisms, such as mussels, sponges, and ascidians. Larger specimens that stand out among the other organisms are easier to collect. The more structured byssus of larger specimens requires a more delicate cut. Again, the juveniles should be placed immediately in a box containing seawater and taken to the laboratory. The larval collector device consists of a main rope deployed vertically in the water column onto which a certain number of "collector bags" are attached in succession. Each collector bag is made up of an outer net bag that encloses a filler material. The structure consists of a rope with a maximum length of 8 metres, kept vertical with buoys and bags for collecting larvae positioned at 3 different depths: -2 metres, -4 metres and -6 metres. Outer bags for larval recruitment can be made from onion or potato bags. These bags have proven particularly effective for larval settlement. The filler inside the bags can be made of different materials (tangled nylon filaments, additional plastic bags, jute). This material provides a surface for the larvae to settle on and protects them from predators while allowing them access. As *P. nobilis* larvae are transported by currents, collectors should preferably be positioned in areas exposed to open water. However, they can also be installed in semi-enclosed sites, such as lagoons. Larval collectors can be anchored to a ballast fixed to the seabed and kept vertically with a floating buoy or attached to the long line of the mussel farm.

Three types of collector bags can used:

- **NIB approach** (National Institute of Biology, Slovenia). The adopted outer bag is made of a rigid plastic (PVC) net with hexagonal mesh of 2 x 2cm, and measures approx. 70 x 50 cm. This net is folded to obtain a bag of 35 x 50 cm. One side of the bag is secured with a rope, while the two opposite ends are fastened with cable ties. After filling, the ties are tightened to enhance the three-dimensional structure of the bag (Figure 1). The filler is made of jute fabric (approx. 50 x 85 cm, mesh size at least 1 mm), folded onto itself to create volume and allow water circulation. The jute was chosen to substitute part of the plastic used with natural material. The plastic potato bags previously tested in Slovenia were hard to clean and therefore difficult to reuse, as well as release a lot of plastic fragments during sorting operations. The use of hard plastic nets serves a two-fold purpose: to reduce the release of plastic fragments into the sea due to their higher durability, and to minimize plastic waste. In fact, once sorted and cleaned, the outer bags, thanks to the design and durability of the material, can be cleaned and reused.

Figure 1 . Collector bags from NIB (photo by Edoardo Batistini).

- **CNR-ISMAR approach** (Venice, Italy). Outer bag: 30 x 50 cm HDPE monofilament bag with 4 mm square mesh. Filler: a 7 m long PP/HDPE tubular net for mussel farming with 10 cm mesh (measured when fully stretched). This material can be obtained from old fishing nets and reused (Figure 2).

Figure 2. Collector bags from CNR-ISMAR (photo by Edoardo Batistini).

- **Croatian approach** (Natura Histrica, Croatia). Outer bag: 40 x 60 cm polyethylene potato or onion bag with 20 mm square mesh. Filler: it consists of three bags of the same size as the outer bag, for a total of four bags per collection bag (Figure 3).

Figure 3. Croatian collector bags fixed to the long line of mussel farms (photo by Saul Ciriaco).

2. Manipulation and transport of the juveniles

The collector bags must be removed carefully to avoid damage or breaks. The bags must be stored in safety containers once collected and transported safely to the laboratory (Figure 4 and 5). It is essential to collect temperature and salinity data on site when collecting collector bags. Keep the bags in water until the juveniles have been removed. Juvenile pinnids found on the bags range in size from approximately 0.2 to 6 cm in anterior-posterior length. They can be seen with the naked eye, either inside or outside the bags. The juveniles must be removed gently to avoid damaging the fragile valves and byssus (Figure 6 and 7). Juveniles are placed in aquariums (with either an open or closed water system) as soon as possible. An acclimatization period may be necessary to prevent thermal shock. The acclimatisation phase can last 30-40 minutes. Seawater in laboratory tanks can warm quickly, creating a significant temperature difference with the water in the aquarium. In such cases, water from the destination tank should be added gradually to the container holding the juveniles until the temperature difference is no higher than a few degrees. It is extremely difficult to distinguish *P. nobilis* from other pinnids found in larval collectors, such as *Atrina fragilis* and *P. rudis*, especially when they are very small. Since pinnids found in larval collectors range in size from 0.2 cm to 6 cm, environmental DNA tests can be carried out to verify whether an individual is actually *P. nobilis*. Alternatively, specimens can be waited for to reach a size of 10/12 cm, at which point the species can be distinguished from one another.

Figure 4. Retrieval of CNR-ISMAR larval collectors in mussel farms in concession to Shoreline (photo by Eleonora Camastra).

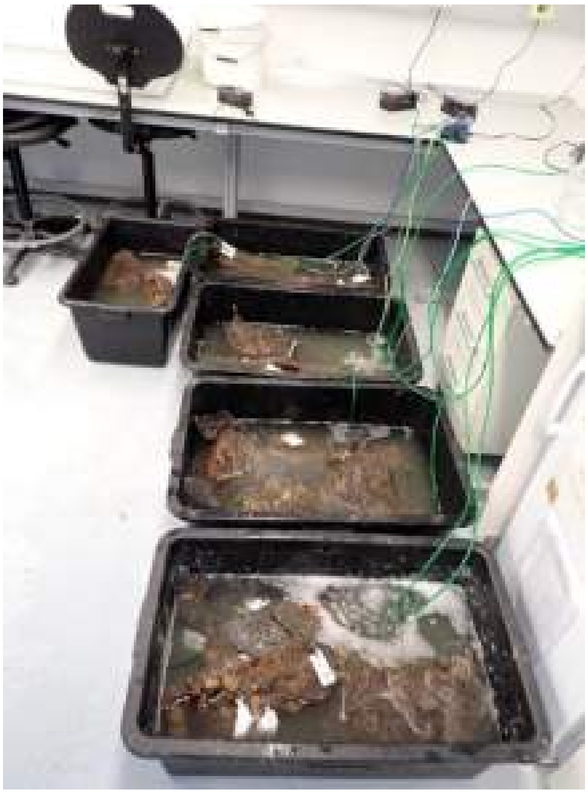

Figure 5. Collector bags inside the containers with air pumps in NIB laboratory (photo by Valentina Pitacco).

Figure 6. Croatian collector bag from Shoreline mussel farms (photo by Edoardo Batistini).

Figure 7. Juvenile of pinnids from NIB laboratory (photo by Valentina Pitacco).

3. Controlled growth in laboratory and in lanter-net

Once in the laboratory, the juvenile must be carefully placed in the aquariums. At the biginning it is crucial to ensure that the physical and chemical conditions in the aquarium reflect those conditions in the collection area. After the acclimatisation phase, the juveniles are kept at a temperature of 18°C, with a salinity of 36–37 PSU and a pH of 8–8.3. Depending on the capacity and dimensions of the aquarium, juveniles can be placed directly on the bottom or in supports such as Petri dishes filled with coarse sediment, useful for very small specimens (Figure 7). For medium-sized specimens, small open jute bags can be placed in the sediment, while rigid containers that allow the specimen to remain vertical are ideal for bigger specimens (Figure 8). This creates a transplant unit that can be replanted without causing further damage to the byssus. Cutting the byssus tissue stresses the specimens, weakening them and reducing their chances of survival. Maintain optimal chemical and physical conditions, avoiding large temperature fluctuations. The photoperiod should be adjusted according to the harvest season and changed gradually. For juveniles and adults specimens in the laboratory the diet consists of a mixture of three strains of algae: 60% *Isochrysis galbana* (~ $8 * 10^6$ cell/mL); 30% *Chaetoceros calcitrans* (~ $7 * 10^6$ cell/mL)/*Phaeodactylum tricornutum*; 10% *Tetraselmis suecica* (~ $7 * 10^6$ cell/mL). It is administered three times a week using a dosing pump. For their diet, it is also possible to administer artificial food, such as Easy Reef and decapsulated *Artemia salina* cysts.

One other option is to place the young specimens in lanter-net that are either attached to longlines in mussel farms or anchored to the seabed and suspended with a buoy (Figure 9). This keeps the individuals suspended during their growth period. Suspended culture showed a positive contribution to specimen survival (survival rate of 78%), reducing predatory pressure compared to placing specimens directly on the seabed. Furthermore, many specimens kept in lanter-nat showed complete reformation of the byssus after a few months (Figure 10 and 11). Once the juveniles have grown to a sufficient size (approximately 10/12 cm in height, or "escape size", which is reached after 6, 12 or 18 months), they can be placed on the seabed inside Marine Protected Areas.

Figure 7. Young specimens in an aquarium at the Shoreline laboratory (photo by Edoardo Batistini).

Figure 8. The NIB laboratory is home to two small specimens, kept in small jute baskets (photo by Valentina Pitacco).

Figure 9. Juveniles kept in small cages suspended above a lanter-net in the Miramare MPA (photo by Marco Segarich).

Figure 10 and 11. Reformation of byssus in two juveniles kept in the lanter-net in the Miramare MPA (photo by Saul Ciriaco).

4. *Pinna nobilis* transplant

Pinna nobilis transplants are a key component of the strategy for protecting and restoring this species. This process involves several stages, from preparing the organisms to placing them in their final location. The main objective of transplantation is to ensure the survival of specimens found in dangerous situations (Table 1), young specimens found in larval collectors and specimens obtained following reproduction experiments (adults). For the transplantation of juveniles and adult organisms, it is necessary to proceed with the choice of a suitable site. Taking care that the turbulence is not excessive also in case of unpredictable sea storms, as it could undermine the newly planted organisms. The sites must have optimal environmental and hydrodynamic conditions, with the lowest possible pressure regime (both natural and anthropogenic). The optimal depth for implantation is usually 10-18 metres, offering a good compromise between light intensity and low hydrodynamics. Sites that receive *P. nobilis* individuals should have *P. oceanica* meadows and *Cymodocea nodosa*/*Zostera* spp. beds, as well as muddy or sandy substrates. Important indicators for a successful site include the presence of a suitable substrate (such as "matte," which consists of intertwined rhizomes and roots within the sediment), good ecological quality of the seagrass bed (if present), high bottom coverage, and high shoot density. For this reason, *P. nobilis* specimens are being transplanted to Marine Protected Areas and locations where they were previously found (table 1).

Table 1. List of characteristics of areas where specimens of *P. nobilis* are found and suitable locations for transplantation.

Anthropic pressure/solution	
Founding Site	**Receiving Site**
DOCKS-MUSSLEFARM-HARBOURS	MARINE PROTECTED AREAS
Anchorage	No anchor zone
Mooring chain / working areas	Managed area / restricted zone
Pollution	Natural site
Tourism	No entry zone

It is crucial to monitor pathogens at both donor and recipient sites and to conduct genetic characterisation. Once retrieved from the aquariums, the juveniles must be placed immediately in a container with seawater and air circulation to ensure they can be transported safely to their destination (Figure 12). Extreme caution must be exercised to avoid damaging the fragile valves, and in particular the byssus and shell. Jute blankets can be used to place the specimens inside the container (Figure 13). An intact shell allows the organism to close hermetically and preserve the internal water during installation operations. Before being transplanted into the sea, the organisms are acclimatised. This can take place either in special tanks in the laboratory, which reproduce the chemical and physical conditions of the transplantation site, or directly on the boat, to reduce stress. This phase lasts approximately 30/40 minutes. The specimens in the lanter-net are collected and placed in small, numbered containers before being transported directly to the transplant site (Figure 14).

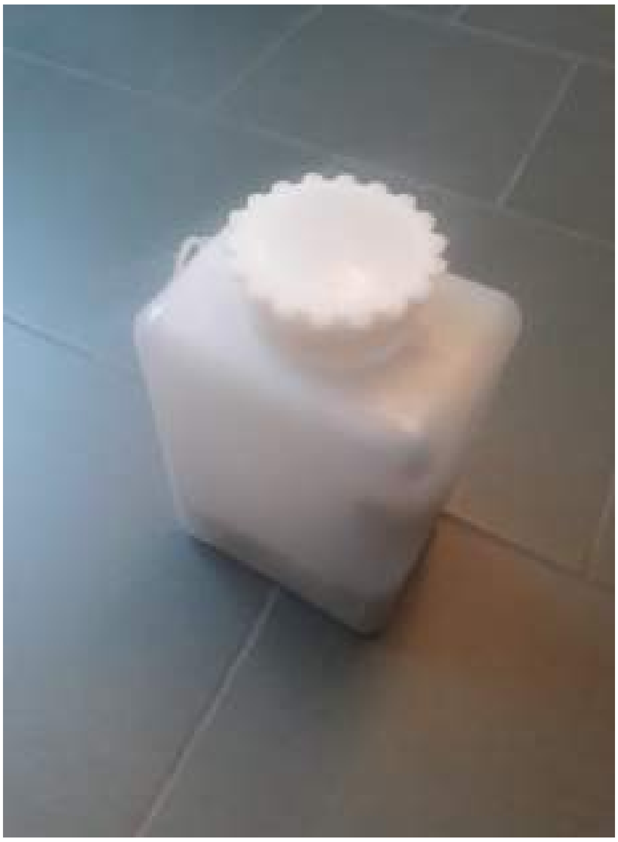

Figure 12. The container for the transport of the specimens (photo by Marco Segarich).

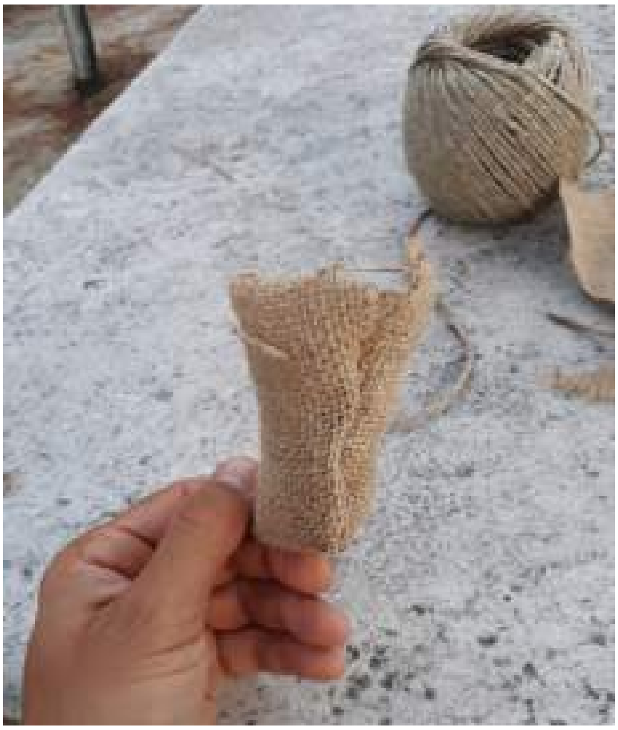

Figure 13. Jute blankets used to place the specimens inside the container (photo by Marco Segarich).

Figure 14. Small juvenile collected from a lanter-net (photo by Saul Ciriaco).

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 (Figure 15). The individual should be inserted at least halfway into the substrate and positioned vertically. For small specimens, it is useful to manage and transfer them in Petri dishes filled with coarse sediment or in small open jute bags. If the organism has grown in a jute bag, the entire bag can be placed in the sediment; the jute will degrade within a few weeks. Good practice is to place a small stone in the sediment next to the individual, allowing the juveniles to anchor its byssus around it. This creates a transplant unit ready for the seabed without damaging the byssus gland. Cutting the byssus causes stress and reduces the chances of survival. It is important to cover implanted juveniles with appropriately sized netting or cage to protect them from predators. Cages can also help to anchor the plants after they have been moved (Figure 16). For adults, the maximum recommended density is one individual per square metre. Even when *P. nobilis* adults are large, it is useful to place a rock on top of the specimen after inserting 1/3 of it into the sediment to ensure it remains upright.

Figure 15. A scientific diver is digging a hole to house a medium-size specimen inside Miramare MPA (photo by Saul Ciriaco).

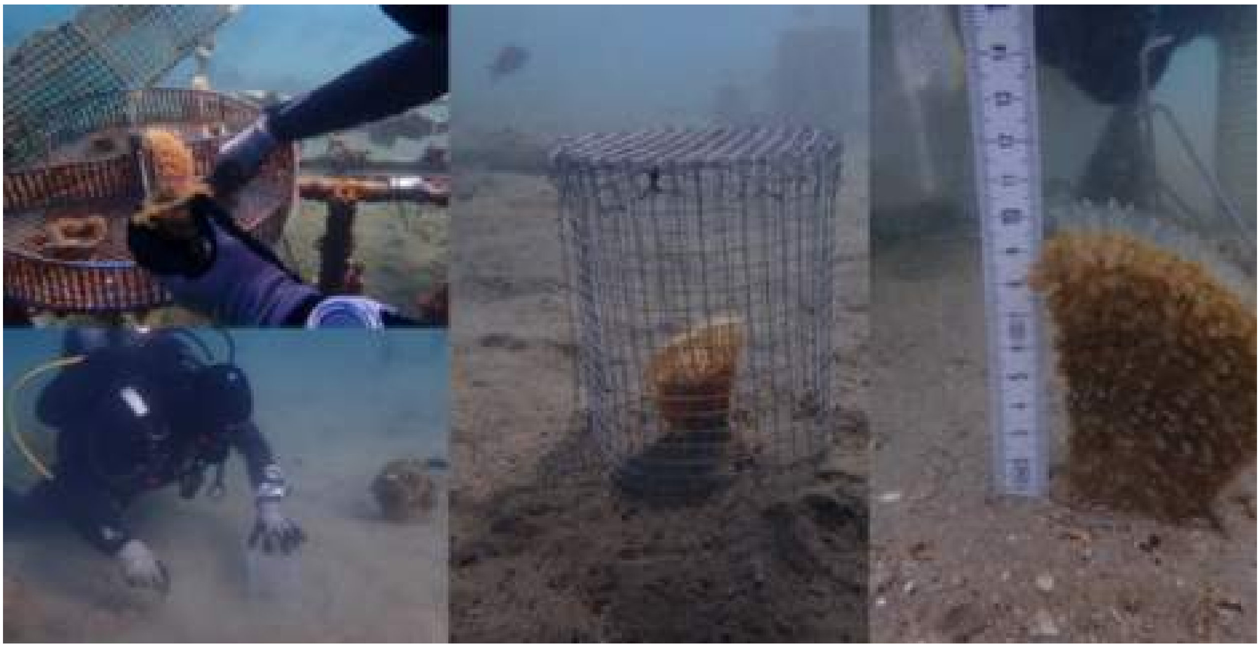

Figure 16. Planting of a 10 cm specimen protected by a small cage in Slovenia (photos by Ana Fortič and Simone Spinelli).

5. Genetic analysis and monitoring activities

This protocol is designed to detect *Haplosporidium pinnae* and other pathogen infections in living specimens. The material collected for genetic analysis is the faeces and pseudo faeces of the specimens. A diver dives to the transplant site, equipped with 60 ml syringes and tubes for the collected material (suggested tube size is 10 ml). The diver must move slowly towards the living specimens so as not to provoke a reaction in the body and thus lose the opportunity to collect the material. Once the syringe and test tube are ready, the syringe can be moved closer to the body, and the pseudo faeces present on the edge of the valve opening in front of the hinge can be aspirated (Figure 17). It is precisely in that position that the gills secrete this mucus, which serves as protection against excessive sedimentation. More precise results were obtained from faecal pellets. When sampling faecal pellets, attention should be paid to the exit of the body's cloacal canal. Patience is needed while waiting for the output. After a while, if the specimen does not emit, you can try knocking on a valve, this way the specimen closes and emits faecal pellets. After sampling, the biological material is stored in alcohol (80°) and placed in a freezer at -80°C, ready for genetic analysis. Another technique for collecting genetic material requires the use of cotton buds to take swabs. A diver equipped with 10 ml test tubes and cotton buds goes down to take the swabs. The swab must be inserted between the specimen's two valves to swab as many parts as possible. Great care must be taken not to damage the valves or cause the specimen too much stress. It should be noted, however, that this is a non-invasive type of sampling (Figure 18). Once the swab has been taken, it is placed in a 10 ml test tube and brought to the surface. The tube must then be emptied of seawater and filled with 80°C alcohol. All samples must then be placed in the freezer before being sent for analysis.

Figure 17. A scientific diver collects faeces from a young specimen in the Miramare MPA (photo by Saul Ciriaco).

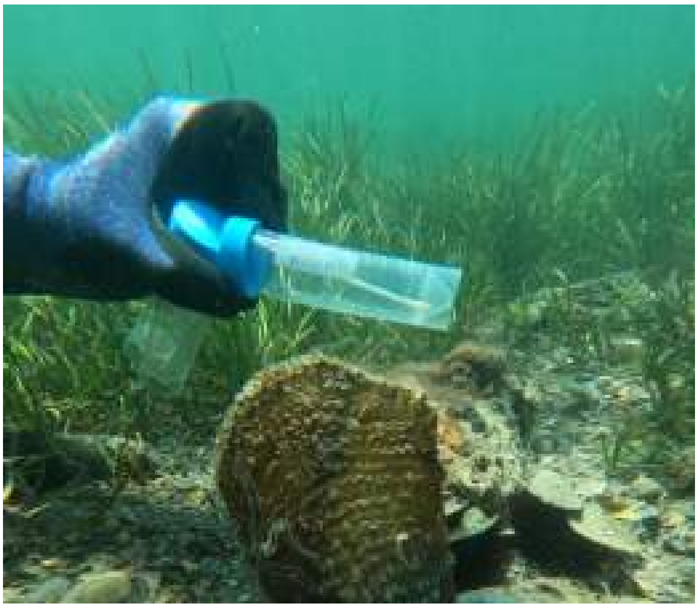

Figure 18. Swab test performed on a specimen in the Venice lagoon (photo by Eleonora Camastra).

For monitoring purposes, each transplanted specimen must be tagged and its geographical location recorded via GPS. To assess their survival, growth and health status, young and adult specimens of *P. nobilis* placed in the natural environment must be monitored regularly. Depending on marine weather conditions, monitoring activities must be conducted at monthly intervals. All monitoring activities are carried out through underwater dives performed by scientific divers. Monitoring must include the measurement of the following indicators:

- visual census of all the restocked specimens of *P. nobilis* (by distinguishing them between living and dead). If the body of the deceased is found, a sample can be collected for genetic analysis.
- size structure assessment of restocked specimens using a 0.1 cm tape (i.e., height of the unburied shell, maximum shell width, and minimum shell width at the bottom).
- state of health of restocked specimens.
- census of potentially self-recruited juveniles of *P. nobilis*, if present.
- census of broken/unbroken shells or dead specimens.
- assessment of the environmental status of the sites (e.g., status of seagrass meadows, occurrence of litter and of other signs of natural or human-induced pressures).

It is also recommended that videos and photos are taken during each monitoring. The following indicators can be calculated using the data collected in the field during the monitoring period:

- survival rate: n° of living specimens/total n° of stocked specimens.
- mortality rate: n° of dead specimens/total n° of stocked specimens.
- growth rate: average increase in the length of specimens per month (in mm)
- recruitment rate: n° of new recruits (when present) per month.

Figure 19. A scientific diver is measuring a specimen inside a lanter-net suspended in the Miramare MPA (photo by Saul Ciriaco).

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