LIFE PINNARCA

LIFE NAT/ES/001265

DELIVERABLE DA.1

PLANNING CORRECTION MEASURES: ITALY

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UNINA

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1 EXECUTIVE SUMMARY

The bivalve pen shell *Pinna nobilis* (Linnaeus, 1758) is an endemic Mediterranean species and among the largest bivalves worldwide playing an important ecological role for communities and contributing to the increase in local biodiversity. This species can grow on soft-bottom in coastal areas inhabits seagrass meadows (like *Posidonia oceanica* and *Cymodocea nodosa*), or can live on unvegetated bottoms. The species has a key ecological role, filtering water and retaining large amounts of organic matter from suspended detritus. The animal shell provides a hard-surface easily colonized by many different benthic species, and at high densities can create high-diversity biogenic reefs (Giacobbe, 2002; Zavodnik et al., 1990). MMEs affecting noble *P. nobilis* were first described in late 2016 in the Mediterranean Sea in Spain (Darriba, 2017; Vázquez-Luis, 2017). Initial pathological changes observed in dying specimens were associated with a new parasite species belong to the Phylum *Haplosporidium* and classified as *Haplosporidium pinnae* (Darriba, 2018; Catanese et al., 2018). The MMEs have been observed throughout Mediterranean basin, along the coasts of France, Greece, Cyprus, Italy, and northern Africa. In several *H. pinnae* negative cases observed in Italy, Greece and Spain, pathological changes were associated with bacteria of the genus *Mycobacterium* (Carella et al., 2019; Carella et al., 2020; Lattos 2020, 2021; Saric 2020; Scarpa et al., 2020). Recent studies confirmed co-occurrence of several parasitic and bacterial pathogens in the diseased animals, with *Mycobacterium* sp, *H. pinnae*, and in a few cases, *Vibrio* spp. and *Perkinsus* sp., suggesting that exposure to multiple pathogens could increase complexity of disease pathogenesis (Carella et al., 2020; Lattos 2020, 2021; Saric 2020; Scarpa et al., 2020). By the end of 2018, multiple MMEs with high (close to 100%) mortality rates caused complete disappearance of noble pen shell populations in many areas across Mediterranean. Since then, the bivalve is catalogued in critical danger of extinction (BOE 10/17/2018) and included in the Spanish Catalog of Threatened Species (CEEA) (Order TEC / 596 / 2019, of April 8) and in the IUCN red book of threatened species within the category of 'Critically Endangered'.

After the expanding of the MME, protected lagoons have become the main refuge for the fan mussel populations. At present, In Italy reports of population located in lagoons Venice lagoon, Lake Faro, and few scattered individuals on the open coast.

2 LIST OF ITALIAN STAKEHOLDERS

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Piero Barani	Specialist Tecnical Scientific Advisor – Italian Ministry of Environment, Land and Sea	barani.pietro@mite.gov.it	+(39) 06/5722.8311	Processing the permissions of activity on P. nobilis for the Italian Ecological Transition Ministry.
Sandro Bucalo	Head of the Nucleo carabinieri subacquei-Local police diving	ccnasubcdo@carabinieri.it	(+39) 081/261004	Support of diving operation in restricted areas for checking P. nobilis individuals
Antonino Miccio	Director of AMP (Marine Protected Area) Regno di Nettuno- Ischia and Procida island	direttore@nettunoamp.it www.nettunoamp.it	(+39) 338/4077563	responsible of environmental protection and enhancement of the sea surrounding the islands of Ischia Procida and Vivara, in the Gulf of Naples
Maria Cristina Palumbo	Director of AMP (Marine Protected Area) Punta Campanella	direttore@puntacampanella.org	(+39) 081 808 98 77	responsible of environmental protection and enhancement of the sea of the AMP areas in Campania region

Francesco Maisto	Regional Park of Campi Flegrei	parcocampiflegrei@libero.it	(+39) 081. 5231736	Director responsible of environmental protection of Miseno, Fusaro and other lagoons in the Regional Campi Flegrei Park
Emanuela Spadoni	General direction for the naturalistic Patrimony (PNA)- Section Sea and Coast – Italian Ministry of Environment	spadoni.emanuela@mite.gov.it https://www.mite.gov.it/pagina/direzione-generale-il-mare-e-le-coste-mac	(+39) 06/57225761	• Requirements related to the establishment and management of national Marine Protected Areas and submerged parks; • Supervision of national and international marine protected areas; • Activities related to the management of the marine and coastal sites of the Natura 2000 Network in coordination with the PNA Management; • Relations with the Marine Environmental Department of the Port Authority Corps - Coast Guard and with the Port Authorities in relation to surveillance activities on protected areas and sites; • Coordination for the establishment of new marine protected areas in the Mediterranean on a cross-border or transnational basis;

3 RECENT ACTIONS IN ITALY

In 2018 A total of 13 moribund animals of the pen shell *P. nobilis* and 1 specimen of *P. rudis* were collected from two different regions of the Tyrrhenum: Campania and Sicily. At the time of the sampling, in all of the areas, SCUBA divers reported mortality episodes ranging between 80% and 95%, depending on the season and location, and represented by empty valves, as observed in many other part of the Italian Tyrrhenum. On laboratory examination, on the external valves, the animals presented with attached epibionts of different types. In 9 of the exanimated specimens (69%), gross examination of the bivalves revealed diffuse tissue oedema, mainly visible at the level of the gill and mantle. In 1 of three examined bivalves from Cilento (south of Campania) in 2018, a brownish/ black cyst-like area was visible at the level of the digestive apparatus. Attempts to acquire a cytological smear from the cyst revealed a liquid content, sticky and brownish/yellow in appearance. Once examined, the animal showed an atrophic yellowish/orange digestive gland. On light microscopy, all of the specimens showed inflammatory lesions of variable degree; depending on the animals and disease extent, infection seemed primarily present at the connective tissue circumscribing both the gonad and the digestive gland and then in other tissues, such as the mantle and gills. In two cases, necrosis of digestive tubules and gonadal follicles were also visible. Regarding the inflammatory response, the immune cells involved were of two distinct types resembling those described by as both hyalinocytes and granulocytes. The inflammatory condition was characterized by large nodular aggregates of the above immune cells, which were filled with long, slightly shaped, acid-fast positive bacteria. These bacteria-filled immune cells were distributed mainly at the level of connective tissue surrounding the gonads, mantle and digestive tissue, as well as in proximity to the haemolymph vessels forming aggregate-rich regions coupled with Brown cells. On the other side, infiltrative-type inflammation was instead visible at the digestive tubule level with visible haemocytes filled by mycobacteria around haemolymph vessels. In the sample from Cilento that showed a liquid cyst, both cytology and histopathology showed the presence of different phases of development of a haplosporidian parasite in the digestive tissue. Large numbers of multinucleate stages were disseminated in the digestive tubule epithelia. Sporogonic stages (sporocysts 20–50 µm in diameter) and acid-fast spores (3.13 ± 0.26 µm of length) were located at the epithelium of the digestive tubules. Sporocyst rupture was seen in the nearby digestive epithelial cells. In the connective tissue, few uni-nucleate stages (2.5 ± 032 µm of length)

dispersed with central or slightly eccentric dense nuclei were also observed. PCR amplification using the primer pair specific for the 16S ribosomal gene of mycobacteria yielded an amplicon of ~1000 bp in all of the samples of P. nobilis examined apart from the non-moribund specimens from south of Campania. BLAST analysis revealed that the nucleotide sequences of these fragments were homologues of the 16S subunit of *Mycobacterium* sp. The *Mycobacterium* sequences isolated from P. nobilis were very similar, (p-distance ranging from 0 to 0.0069). The sequences of the *Mycobacterium* infecting *P. nobilis* were grouped together with Mycobacterium sherrisii and appeared close to the group including *M. shigaense*, *M. lentiflavum* and *M. simiae*. Later in 2018 a survey of moribund P. nobilis specimens was also conducted in other Italian regions (Tuscany, Sardinia, and Apulia). Histopathological and molecular examination of 27 specimens of P. nobils revealed different types of pathogens associated with tissue lesions, morbidity and mortality. Presence of *Mycobacterium*, *Vibrio* species, *Haplosporidium pinnae* and *Perkinsus* sp. were detected, differently distributed into the areas. The *Mycobacterium* sp., previously reported in Campania and Sicily samples, was observed in all the analyzed areas and individuals, associated to systemic inflammatory lesions. Molecular study using hsp65 genes and Internal Transcriber Spacer ITS support that a new species of Mycobacteria is infecting *P. nobilis*, close to *M. triplex* and belonging to the group of *M. simiae* complex with *M. sherrisi*. Presence of *Perkinsus* spp. Resembling P. mediterraneus was observed in 2 out of 13 Italian individuals whose presence should be addressed as potential risk for shellfish aquaculture of the area. Vibrio spp. were also detected in some case. The preliminary results of this study suggest that *Mycobacterium* sp., *Vibrio spp., H. pinnae and Perkinsus* sp. cooperate to disease pathogenesis, being Mycobacterium and Haplosporidium most of the time involved. Vigilant inspection of those areas where MME is now starting, along with continuous systematic surveys, are crucial to define the spatiotemporal progress of mortality and the role of every single pathogen in the disease outcome.

In the **Lake of Faro** animals are still present and monitored constantly by the University of Messina. As regards the recruitment, in addition to the search for juveniles in the natural substrate, special larval collectors have been placed, 5 in number for the basin plus 4 in the sea, the latter in a position in front of the mouths of the connecting channels. These collectors, already previously tested in the lake and in the sea, will be removed and repositioned on a quarterly basis, considering the problem of competition for space determined by the speed of colonization of the local epibiontic fauna (especially ascidians and porifers). Any recruits

captured by the collectors are positioned in different sectors already identified in the lake, able to guarantee the best chance of survival. Furthermore, considering the ongoing process of colonization of the basin by the congener species *P. rudis* and the invasive alien oyster *Pinctada imbricata*, the dynamics of these two species will also be used and monitored.

2018-2020 monitoring for the coastal waters of Veneto relating to the populations of *Pinna nobilis* of the coasts of Veneto. The activities relating to Pinna nobilis in progress also include the citizen science **Project Mappa La Pinna** (https://cutt.ly/pinna) for the mapping of the species in the Venice Lagoon, and monitoring activities in the same lagoon, which have led at the first report of a massive mortality event and, in collaboration with the Specialized Fish Center of the Experimental Zooprophylactic Institute of the Venezie (IZSVe), of the presence of the pathogen *Haplosporidium pinnae* (http://www.ismar.cnr.it/eventi- e-news / news / pinna-nobilis-worsens-the emergency) and recently of Mycobacterium.

4 MONITORED AREAS AND CANDIDATE ZONES

The evaluated areas correspond with those still hosting *Pinna nobilis* or in which individuals have been observed in the recent past, and therefore could be suitable for the survival of the species. In Italy, in Campania region, the first description of MMEs was reported by Carella et al. (2019) in the Tyrrhenian Sea, in the region of Campania, following a Monitoring activity in the context of the Italian Marine Strategy in 2017 in the Marine Protected Areas of Punta Campanella, the AMP Regno di Nettuno in the islands of Ischia, the island of Procida and Santa Maria di Castellabate where few moribund individuals were sampled (Figure 1).

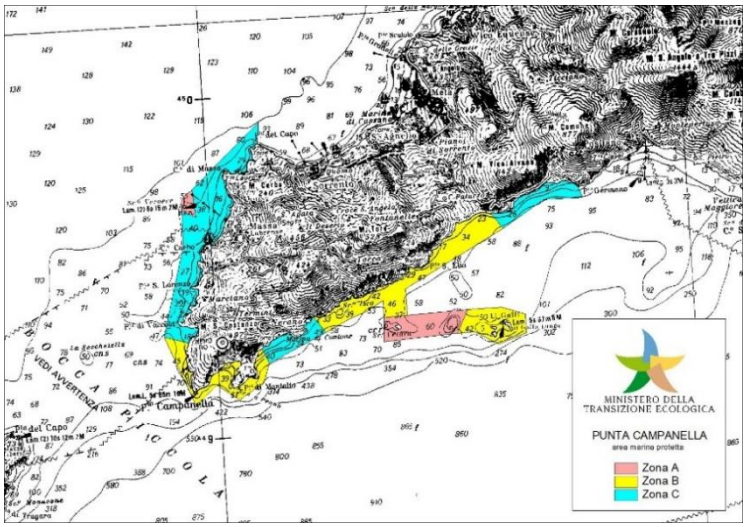

Figure 1. Map of Marine Protected Area (AMP) of Punta Campanella and sampling activity in the AMP in 2018.

In the AMP of Punta Campanella were observed in total 148 individuals, of which only 10 alive and 1 damaged. Mortality stands on the 92.57%, with peaks of 100% in 1 of the monitored stations. In Ischia Island, at the AMP Regno di Nettuno, 51 individuals of *P. nobilis*, of which only 4 are alive. Mortality recorded is 92.16% with peaks 100% in 2 stations. In the south of the same region, In Santa Maria di Castellabate, 81 individuals of P. *nobilis* were found, of which only 20 alive and 1 with broken shell. Mortality was of 74.01%, showing a lower value than the other MPAs. In the same, in Campania, moving from north to south, the areas of Sorrento, Positano, Vico Equense showed a scattered mortality situation, ranging between 63% and 90%. Later, in March 2018, the populations of Sorrento, Ischia and Procida islands totally disappeared. The monitoring activity in the coastal waters of the municipality of Pollica, in Cilento, began in 2018 in collaboration with the Marenostrum Association which was entrusted with the management of the Pioppi Museum of the Sea. Continued until in the Marine Protected Area "La Punta", with the aim of assessing the population density (**Figure 2**).

Figure 2: Activities in Torre La Punta (Cilento) in 2018.

Recently, at the end of 2021 few young individuals were observed in the south of the coast (Cilento), in the island of Ischia at high depth (30m) and in the internal marine lake Miseno, in the Northern area of the region. In the Lake of Miseno the presence of the penshell is historically reported. The lake extends over 40 hectares and has a perimeter of about 2,800 meters while the average depth is 2.25 m and the maximum is 4m. It is separated from the sea by a sandy barrier about 200 meters wide but relates to it through two foci: the first located near the town of Miliscola and the second located near the bay of Miseno. Lake is a lake situated in the province of Naples, in the territory of the community of Bacoli. It is about 0.5 miles (0.80 km) from Baia, and about 1 mile (1.6 km) from the acropolis of Cumae, separated from the sea by a narrow coastal strip. It is a very unusual ecosystem of great interest, characterized by a variety of vegetation which is specific to the region and interested partially by bivalve farms.

Figure 3. Lake of Fusaro (Campi Flegrei).

Venice lagoon is an enclosed bay of the Adriatic Sea, in northern Italy with a surface area of around 550 square kilometres. The presence of *Pinna nobilis* in the Venice Lagoon is documented at least since the end of the 18th century. More recently, Coen (1938), Cesari (1992; 1994), Russo (2012; 2017) and Franceschini et al have reported their presence (2017). Russo (2012; 2017) qualitatively describes the state of health of the population of a population near Santa Maria del Mare (Figure 4), kept under observation over a period of 8 years estimating a density of 12-16 specimens/m2. The persistence of this community is probably due to the absence of fishing activities with dredges and the fact that the shellfish are not edible.

Figure 4. *P. nobilis* population in S. Maria del Mare (Pellestrina), in low tide.

Figure 5. *P. nobilis* in S. Maria del Mare (ISPRA 2021)

Description of the population in the lagoon have been observed in different areas and covering different meters square (Table 2):

Area (observed in low tide)	Area	Data made by counting, drones, photos
S. Maria del Mare (emerged)	50.000 m^2 circa	ISPRA
Caroman (submerged)	13.000 m^2 circa	ISPRA
Velma di fronte in front of the harbour (emerged)	1 Km^2 circa	ISPRA
Fossa S. Felice (emerged)	40.000 m^2 circa	ISPRA
Velma Isola Saloni (emerged)	0.15 Km^2 circa	ISPRA

Table 2 Areas were population of *P. nobilis* are described in the Venice lagoon.

In the lagoon the sediment varies between muddy sand and sandy mud. Fan mussel individuals are dispersed among seagrasses, macroalgae or in bare patches. Shells are both colonized and not by epibionts, mainly brown algae such as *Sargassum muticum* and *Cystoseira barbata*. Data from all stations and all years indicate that, based on yearly averages, the lagoon may be divided along its major (long) axis into three areas: 1) a northern, freshwater impacted area (S ¼ < 28 PSU) of high, tidally caused, variability, 2) a southern, marine, zone of S > 32 PSU of low, tidally-caused, variability, and 3) an intermediate zone. Salinity changes are closely associated with rainfall events, and the incoming freshwater is consistently distributed throughout the lagoon by tidal action. Much variability is simply a result of the forward and backward motion of the tides and is not caused by a salinity change in the water itself. The consistency of the 2000–2009 data and the historical (to 1961) watershed record support the hypothesis that the Venice Lagoon has been and is currently at steady state with respect to its salinity distribution. As such, it is conducive to the development of (at least) three separate ecosystems. Mortality was reported in the last three years and presence of both *Haplosporidium* and *Mycobacterium* is documented.

In Sicily, in the Lake Faro, a small population of *Pinna nobilis* survives in a biologically stressful environment, in a precarious balance with human activities. Lake Faro (38◦16′07′′ N, 15◦38′13′′ E, NE Sicily, central Mediterranean) belongs to the Capo Peloro coastal brackish system, a Special Protection Area under the Directive 79/409/EEC in the Water Project of 1972; it is a Site of Community Importance under the Directive 92/43/ EEC and was declared a Natural Reserve by the Sicilian Region in 2001. Lake Faro covers an area of just 263,600 m2, it is the deepest coastal basin in Italy and reaches 29 m depth in the eastern area, and up to 3.5 m depth in the western area (Figure 6). The lake is connected to the Strait of Messina by the “Faro Canal” (4000 m2 surface, 1.2 m average depth), which, despite Lake Faro’s microtidal regime, is affected by strong tidal currents. Lake Faro is connected to the Tyrrhenian Sea only in summer, when a connection is established by dredging the “degli Inglesi” Canal (800 m2 surface, 0.8 m average depth). A third canal, “Margi”, connects the Lake Faro to the most brackish Lake Ganzirri locally altering the Lake Faro termo-haline regime.

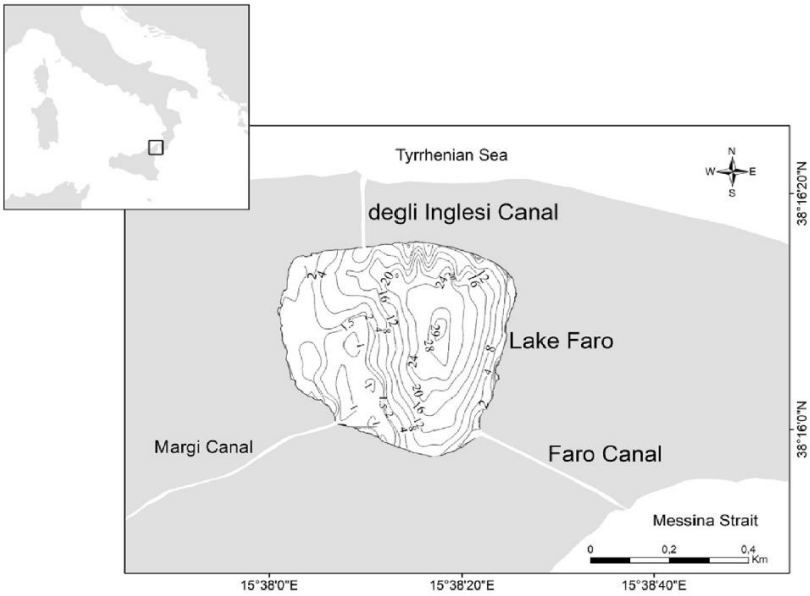

Figure 6. Map of the study area showing the bathymetry of Lake Faro (from Donato 2021).

In October 2020, the settlement of *Pinna rudis* and *Pinctada imbricata radiata* populations in the Faro Canal, suggested their inclusion in the monitoring protocol. After a preliminary survey, reporting only the GPS position of each specimen, a second monitoring of *P. rudis* was performed in December 2020. In the 2010 monitoring, a total of 452 living individuals were recorded, with a population density of 0.28 ind./100 m2. The lake hosted 202 living individuals (0.13 ind./100 m2) and 82 dead specimens. In 2018, a total of 170 living specimens were recorded (0.10 ind./ 100 m2); 69 living specimens in the lake (0.04 ind./100 m2), and 39 dead specimens (ratio: 1.77 to 1). In the Faro Canal 101 living specimens were found (2.53 ind./100 m2) and there were no recognizable dead specimens. Survey was found in 2018, except for a dozen of large-sized empty shells. Finally, in the 2020 survey, only 36 living *P. nobilis* were detected (0.02 ind./100 m2), 22 of which had been taken from "degli Inglesi" Canal in 2019; there were 94 dead specimens (ratio: 0.38 to 1) (Donato et al., 2021). The existence of a residual population was highlighted, which recent observations indicate as a possible core of repopulation.

In Sardinia the "2018 POA monitoring program - ARPAS" was carried out pursuing the Legislative Decree 190/2010, as part of the Marine Strategy within which monitoring actions of the *Pinna nobilis*. *Pinna nobilis*, despite being a common species throughout the Sinis coast, showed a

relevant density only in AMP (Coppa et al., 2018, 2019) and in the northern part of the Gulf of Oristano. Based on the results of the monitoring campaigns developed during the years 2007, 2009, 2014, 2016, 2018 it can be stated that the Gulf of Oristano was a particularly suitable area for the growth of this species and that the conservation status of the population present in those years it was good. Since June 2018, the investigated population has been in extremely critical conditions due to pathogen infection (Scarpa et al., 2020 and Carella et al., 2020). The percentage of specimens found dead in that year was 79.8% with evident variability between stations ranging from a maximum of 95 ± 3% (Torre Vecchia) to a minimum of 26.2 ± 3.8% (Sa Mardini).

5 EVALUATION OF RISK AREAS FOR PATHOGEN INFECTION

Previous (2018-2020) Histopathological and molecular examinations of specimens showed the presence of *Haplosporidium* sp. in only one specimen in one area sampled in Campania and Sicily. In all the surveyed moribund animals, strong inflammatory lesions at the level of connective tissue surrounding the digestive system and gonads and linked to the presence of intracellular Zhiel-Neelsen-positive bacteria were observed. Molecular analysis of all of the diseased specimens confirmed the presence of a *Mycobacterium* similar to the human mycobacterium *M. sherrisii* close to the group including *M. shigaense*, *M. lentiflavum* and *M. simiae*. Successive analysis, also including Sardinia, reported the same types of pathogens.

In Venice lagoon mortality episodes in different monitored areas are observed. Lastly, as from last autumn, the Venice unit of the Italian Marine Science Institute (Istituto di Scienze Marine [CNR-ISMAR]) has observed abnormal mortality levels also in the Venetian Lagoon and in the coastal waters of the Veneto Region, where the outbreak is affecting ever-increasing proportions of the lagoon population. Analyses carried out in collaboration with the IZSVe Reference Centre confirmed the presence of the protozoa *Haplosporidium pinnae* and *Mycobacterium simiae* involved in the mortality events in the Lagoon. The risk of pathogen infection is high because already found in all the areas.

6 EVALUATION OF OTHER LOCAL RISKS

Aside the risk of pathogen infection, pen shells populations in Italy are threatened by many factors of anthropogenic nature. In Campania, Sicily and sardinia Islands fishing activities, recreational boats and diving are important activities through Italian coastline. Animals in the past have died due to actions of vandalism and poaching with illegal extraction of healthy specimens, intentional or accidental removal of specimens due to malpractice by some fishermen or illegal harvesting for alimentary or decorative purposes and indirect threats, such as habitat regression and degradation. Scarce monitoring have been perfomed in some areas to evaluate the sate of the species.

7 CONCLUSIONS

MME the disease aetiology is complex, involving multiple pathogens causing co-infections, and strongly related to environmental conditions that are specifically predisposing *P. nobilis* to poor disease outcomes. Establishing targeted surveillance programs with the participation of multiple stakeholders is of high importance, especially due to the pan-Mediterranean distribution of the MMEs and the official IUCN status as a critically endangered species. Developing highly specific and sensitive non-invasive diagnostic methods, combined with standardized investigation protocols will be crucial in supporting national veterinary diagnostic laboratories, in fulfilling the requirements of the EU habitats directive and Barcelona Convention. In Italy the internal lakes represent the possibility to better understand the complex factors involved in the aetiopathogenesis and also to work for animal conservation. The Venice lagoon along with the lake of Faro in Sicily are area perfect for the study, even if the classification of the area as optimal for the survival of the species requires further valuation.

8 RECOMENDATIONS

- Continuous monitoring of the population, which will allow reacting to any indication of mortality in the population.
- Study to know the stock and the dynamics of the unaffected populations Identification of optimal areas for recruitment, development and translocation.
- Reduction of anthropic pressures suffered by existing populations.

- Definition of action protocols for translocation and/or rescue to ex situ maintenance centers in situations of environmental crisis.
- The rescue processes must include maintenance in adequate facilities (that meet the requirements to ensure the survival of the specimens) for the necessary time, probably years.
- Carry out detection tests for the possible identification of the pathogen
- Boost the captive breeding project

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