

axis minimizes the area of triangles formed by vertical and horizontal distances of the residuals, as well as the fitted line. It is important to consider that when the correlation coefficient (r) approaches 1, the difference between major axis and reduced major axis becomes smaller and can be negligible .

Nowadays two approximations are used to calculate Ht from the dimensions of unburied shell parts. One estimates Ht from its relationship with W, whereas the other is used to calculate the length of the buried shell (h), which is added to UL.

The advantage of using W is that Ht can be estimated only with one measurement. However, the posterior shell margins of the shell are easily eroded and if W measurement is made before shell reconstruction, important underestimations on Ht occur. Also, W is more sensible to differences in shell morphology and introduces more variance to the data. Using w to estimate h is more reliable, but more measurements must be made. Also, *Pinna nobilis* umbo is more or less abraded throughout the live of the individuals and initially this would change the proportions between w and h. Nevertheless, *P. nobilis* individuals reconstruct the anterior border to keep the triangular shape of the shell (García -March and Márquez-Aliaga, 2006a), maintaining the proportions of w and h more or less constant. The importance of polymorphism, shell erosion and “reshaping” for the development of equations to estimate Ht has not yet been accurately estimated, and equations should be considered as “good tools” provided they estimate real Ht under “acceptable ” error limits. One recommendation is using both approximations, since the comparison between them can shed light on the process of shell growth and erosion. For example, using both approximations , the individuals that have suffered important erosion on W are easily detected. Also at this step data on shell shape is important to discern between outlayers produced by postero-dorsal shell erosion or variability owed to the species polymorphism.

Three equations , based on these two approximations can be found in recent bibliography. They can be checked with Ht of empty shells collected during field surveys and compared to other approximations. It is recommended to use the equation showing the smallest residuals with our real Ht data.

Equation by de Gaulejac and Vicente (1990) for the Population of Diana lagoon (Corsica)

$$\begin{aligned} \text{Ht} &= 2.186W + 1.6508 & (3) \\ R &= 0.98 \end{aligned}$$

Equation By García -March and Ferrer (1995) for the population of Moraira (Spain)

$$\begin{aligned} h &= 1.79w + 0.5 & (4) \\ R &= 0.99 \\ \text{Ht} &= h + \text{UL} \end{aligned}$$

Equation By García -March (2006) for the population of Moraira (Spain)

$$\begin{aligned} \text{Ht} &= 1.29W^{1.24} & (5) \\ R &= 0.98 \end{aligned}$$

The latter equation relies in the observation that the relationship between H_t and W is allometric, and exponential regression produces a considerable improvement in the accuracy of the fit to data , reducing residuals and unexplained variance .

3.4.4. Study of population structure

The study of population structure shows an instantaneous image and provides a quantitative approximation of the stock of the population. It is separated from population dynamics herein, which provides data about the change of the population with time . In the following sections, the methodology to study density of individuals and spatial distribution is described.

3.4.4.1. Estimation of the stock: density of individuals

3.4.4.1.1. Strip transect sampling

Some remarks on transect sampling were done in section 3.2 , and the methodology to establish the coordinates of the strips is similar. The main difference between transect and strip sampling is that in the latter, the precision of the exploration is much increased. The explored surface area must be known, and we assume that c. all individuals located in the explored area are found . This is also important because it states the biggest difference with other sampling methods of distance sampling such as line transect sampling (LTS). Remarks, advantages and problematic of distance sampling to study *Pinna nobilis* populations will be treated in more detail in section 3.4.4.1.3 .

Strip sampling can be carried out either by two or more SCUBA divers at the same time. When it is made on bare sediment devoid of seagrass cover, each diver can explore up to one meter on each side (two meters per diver). One methodology to delimit the boundaries of the strip is using a rope of two meters tied to two bars of one meter on each side. Divers hold the bars in the point of attachment to the rope, leaving the bar on the external side. The bars delimit the external boundaries whereas the rope, marked in its centre , delimits the internal surface of the strip to be explored by each diver (Fig 23). With this method large areas can be explored and all individuals found counted and measured in a reasonable time. This method has been successfully used to explore Pinnid populations in Columbretes islands Marine Reserve (García - March and Kersting, 2006).

When the leaves of seagrass cover the seabed, surveying only one meter per diver is recommended. Some researchers (e.g. Nardo Vicente and col.) use a bar of one metre with two folded extensions and slowly “comb the site” with it, pulling gently the leaves of *Posidonia oceanica* to uncover the individuals. This method slows down the speed of the survey, but is the only way to ensure that c. all individuals are found.

Strip sampling is a good tool for stratified random sampling, mainly when the boundaries are depth levels. It permits to follow the stratum along its length, minimizing the intersection with the depth gradient. If the purpose of the survey is tagging individuals, then other methods allowing the accurate positioning of

the individuals are more appropriate. These methods are described in the following section.



Fig 23. Picture of two divers exploring the seabed with strip sampling

3.4.4.1.2. Circle sampling

Circle sampling, as strip sampling, also relies on the assumption that all individuals located inside the explored area are found. Again, this is the main difference with point sampling, which would be, in turn, analogous to line sampling.

This method improves the precision of individuals' location. The area surveyed is clearly delimited by the radius of the circle, divers do not have to go far from the starting point and to get the coordinates of the sampling station, only one GPS coordinate of the centre of the circle is required. Also, a long line is not necessary, nor to use time for its deployment previous to the survey. Depending on meadow cover, depth and density of individuals, the surface area explored can be increased or reduced (Table 2), maximizing the sampling time. If many divers are available, this proves to be a very quick and efficient method. Circle sampling can be easily adapted to position the individuals for spatial distribution or population dynamics studies. Finally, the sampling units are easily allocable in a chart for posterior location. The chart can be subdivided in a population of squares of a determined surface area (e.g. 100 m^2) and the centre of the squares used as centre of circles of 100 m^2 . For statistical calculations, the results are nearly identical, given that the same surface is explored. Therefore, this method will be usually recommended for the evaluation of populations, especially within *Posidonia oceanica* meadows.

Table 2. Advantages and disadvantages of circles of different radius, according to depth, *Posidonia* leaves cover and shoot density, number of individuals and bottom structure.

Radius	Advantages	Disadvantages
5.6-8 m	Units easily sampled. Appropriate for deep samples and/or for meadows with high shoot density or populations with high number of individuals. Surface area between 100-200 m ² .	Small surfaces with low density of individuals. If number of individuals is high, the 8 m radius could be excessive
10 m	A large area is surveyed, and individuals may be tagged, in reasonable time for a SCUBA dive.	Area is too large with high shoot density and in deep locations (>15 m), independently of the number of individuals.
15-20 m	Large areas are explored (700- 1256 m ²) in a reasonable time. Advisable for 3-4 divers.	Feasible with low shoot density, low depth (<15 m) and when individuals are neither tagged nor measured. It is difficult to keep the line marking the radius straight. The points of origin and turn of the circles must be signalled at different distances from the centre. Rock outcrops and irregular seabed topography difficult the work.

To mark the centre of the circles a hollow stainless still rod is driven in the seabed with a hammer. The length of the rod depends on the composition of the bottom and the purpose of the circle, and the diameter recommended is between 1 and 2 cm. In hard substrate, the rods should be short, i.e. 50 cm, whereas for soft sediments they should be longer, i.e. 100 cm. It is important to taper one end, to facilitate the burial with the hammer. Also a small hole of 0.5 cm at c. 5 cm from the top should be made on the opposite side of the tapered end to attach an identifying plate and a buoy. The latter should float c. 30 cm above the meadow canopy to facilitate future localisation. To delimit the circles it is recommended to use a tape between 10-20 m long, attached to the rod.

The starting point of the exploration should be marked with a buoy floating over the meadow canopy. For two divers it is advisable to make concentric rings of increased length. Care should be taken to divide the surface to be explored by each diver proportionally. For a first ring of 5 m of radius, the diver closer to the centre should explore from 0-3.5 m, and for a second ring comprising the interval between 5 to 10 m, from 5-8 m. The second diver would cover the areas from 3.5 to 5 m and from 8 to 10 m respectively. The diver holding the line to mark the radius should be careful to keep it as straight as

possible. The line of the tape can be deposited on the seafloor and the area carefully explored in small portions (Fig 24 and 25).

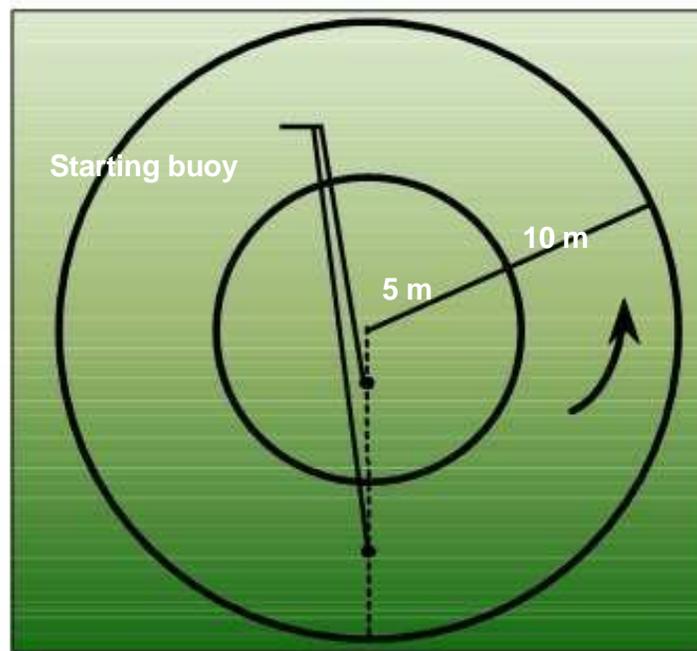


Fig 24. Schematic representation of a circle of 10 m radius with two rings .



Fig 25. Picture of three divers exploring the meadow of *Posidonia oceanica* with circle sampling in Rđum il-Majjiesa to Ras ir-Raheb Marine Protected Area (Malta).

3.4.4.1.3. Line and point sampling

In line transect sampling researchers would follow a straight line, under which the probability of finding individuals is maximum. In point sampling, the researcher is in the centre of the circle, where probability of location of individuals is also maximum. In both cases the probability decreases with the distance normal to the line or radial to the centre of the circle, following a distribution that can be fitted to a detection function according to different models. Akaike's information criterion (AIC) provides a quantitative method to choose the best model. Finding the parameters of the model provides the function that estimates the specimens missed (Buckland et al, 2001).

Although a priori, line transect sampling should provide an important improvement for *Pinna nobilis* population density estimations, unfortunately, the special conditions of SCUBA diving sampling, density of individuals as well as the typical concealment of Pinnids under the leaves of seagrasses, constraint the applicability of this methodology to this species.

Under certain conditions, line sampling can be more efficient than strip or circle sampling. For example, if there are enough sampling stations to ensure the location of sufficient individuals of different sizes on and close to the line (e.g. 50 stations), the estimation of *Pinna nobilis* population structure with line sampling can be improved with respect to other methodologies. If sample size is reduced, the success of line sampling requires the presence of clear water, Pinnids in bare sediment and a low number of individuals (c. 1 individual/100 m²). Divers should follow a line. After a sight, one diver should record the perpendicular distance to the line or point and take the measurements. With high number of individuals, this would either increase the probability of detecting other individuals, or slow down the work of the rest of divers, which should stay waiting until all the individuals were positioned and measured. With low number of individuals, the sighting of one Pinnid usually would not condition the sighting of others, little time would be dedicated to measurements and the displacements could be accelerated, only exploring carefully the area over the line. With turbid water, dense seagrass cover or with many individuals, the benefits of line sampling are counteracted by the difficulty of observing individuals into the meadows, even on the line and the inefficiency of one diver having to stay alone when the others are positioning and measuring the individuals.

On the other hand, it is difficult to apply distance sampling theory to estimate individuals missed during strip or circle sampling. There is no reason to suppose that the probability of finding an individual near the line and centre of a strip or a circle is higher than the probability of finding individuals at any point inside the delimited surface area. The searching implies giving all the points the same searching effort and the same probability of finding an individual. Therefore, there is no shoulder in the distribution of sights far from the line or circle centre, what is basic for the application of distance sampling theory.

The free software DISTANCE is available in the web, in the page <http://www.ruwpa.st-and.ac.uk/distance/>, and can be downloaded in case line or point sampling are the methods elected to study *Pinna nobilis* population structure.

3.4.4.2. Study of spatial distribution

The only difference between studying density of individuals with circle sampling and studying spatial distribution is that for the latter, the distances to the centre and the orientation of the tape from the centre of the circle to the individuals are recorded (Fig 26). This methodology is a fast-and -easy method to record the positions of individuals and allows the realisation of a high number of circles with reasonable effort. The technique has been applied successfully in Columbretes Islands Marine Reserve (Spain) (García -March and Kersting, 2006). Individuals may also be tagged for future monitoring.

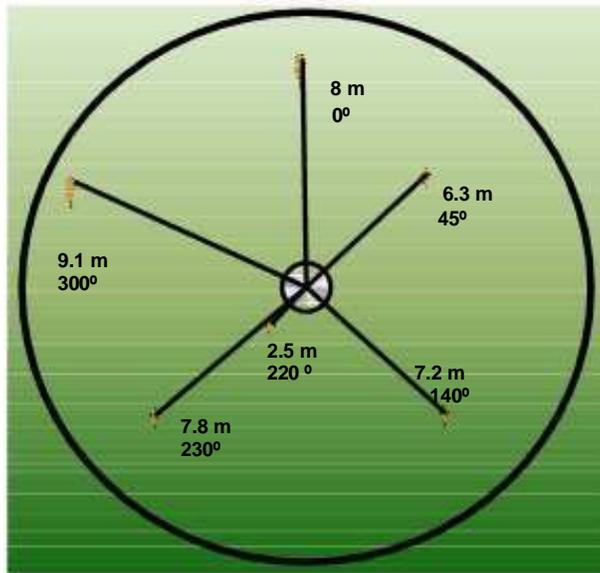


Fig 26. Example of positioning of individuals measuring the distance of each specimen to the centre of the circle and the orientation of the tape.

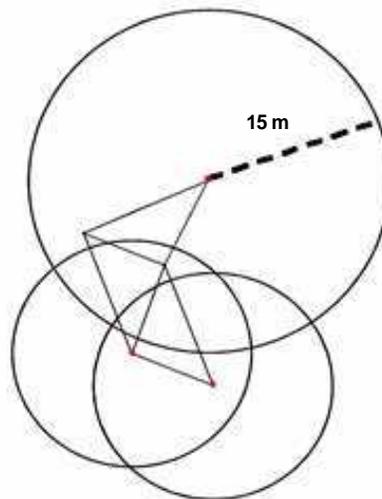


Fig 27. Map of 1000 m² built with different triangles to study population dynamics and capacity of displacement of *Pinna nobilis* in Moraira (Spain, Western Mediterranean). Modified from García-March, et al (2007b).

When the area to be surveyed is large, i.e. it is necessary to control a whole population, the more precise method of triangles can be used. Three stainless steel rods are buried in the seabed forming a triangle (e.g. 7.5x7.5x10.6 m). One of the rods is used as centre for the circle, and individuals are positioned with reference to the three rods. The area explored may be increased using more rods to build new triangles adjacent to the precedent, and using the new rods as centre for more circles (Fig 27).

One method commonly used by ecologists to analyse the data of spatial distribution is the variance-to-mean ratio. This method is based on the fact that the dispersion of a population determines the relationships between variance (s^2) and arithmetic mean (X):

If $s^2/X = 1$ ($s^2 = X$), then the distribution is random

If $s^2/X > 1$ ($s^2 > X$), then the distribution is contagious

If $s^2/X < 1$ ($s^2 < X$), then the distribution is uniform

Some mathematical distributions are good suitable models for these three relationships between variance and mean:

Poisson if $s^2 = X$

Negative binomial if $s^2 > X$

Positive binomial $s^2 < X$

Here we will address the utilization of Poisson distribution and the assumptions required to apply the model to check randomness of spatial distribution. The use of the Poisson series involves the following conditions:

- 1) The probability of any given point in the sampling area being occupied by a particular individual is constant and very small.
- 2) The number of individuals per sampling unit must be well below the maximum possible number that could occur in the sampling unit.
- 3) The presence of an individual at a given point must not increase or decrease the probability of another individual occurring nearby.
- 4) The samples must be small relative to the population.

If sample size is large enough ($n > 31$), as usually will occur when researching *Pinna nobilis* spatial distribution, a frequency distribution can be built and the observed frequency distribution of the counts can be compared with the expected frequency from the mathematical model. The model is a good fit to the original counts when the observed and expected frequencies agree. This "goodness-of-fit" is tested by a χ^2 :

$$\chi^2 = \sum (\text{observed} - \text{expected})^2 / \text{expected} \quad (6)$$

It is usually recommended that some frequencies are recombined so that no expected values are less than 5, although some authors suggest that no expected value should be less than 1. The total χ^2 for the whole frequency distribution is referred to tables of χ^2 with $v = \text{number of frequency classes} - 2$ degrees of freedom.

Given the typical density of individuals of *Pinna nobilis*, dividing the area sampled in each circle in 3 x3 m or 5x5 quadrates is a good option. With this sample size, assumptions 1 and 2 are met even with high numbers of individuals (e.g. >10 individuals/100m²).

The procedure to be followed for the evaluation of a population can be summarized in the following diagram (Fig 28).

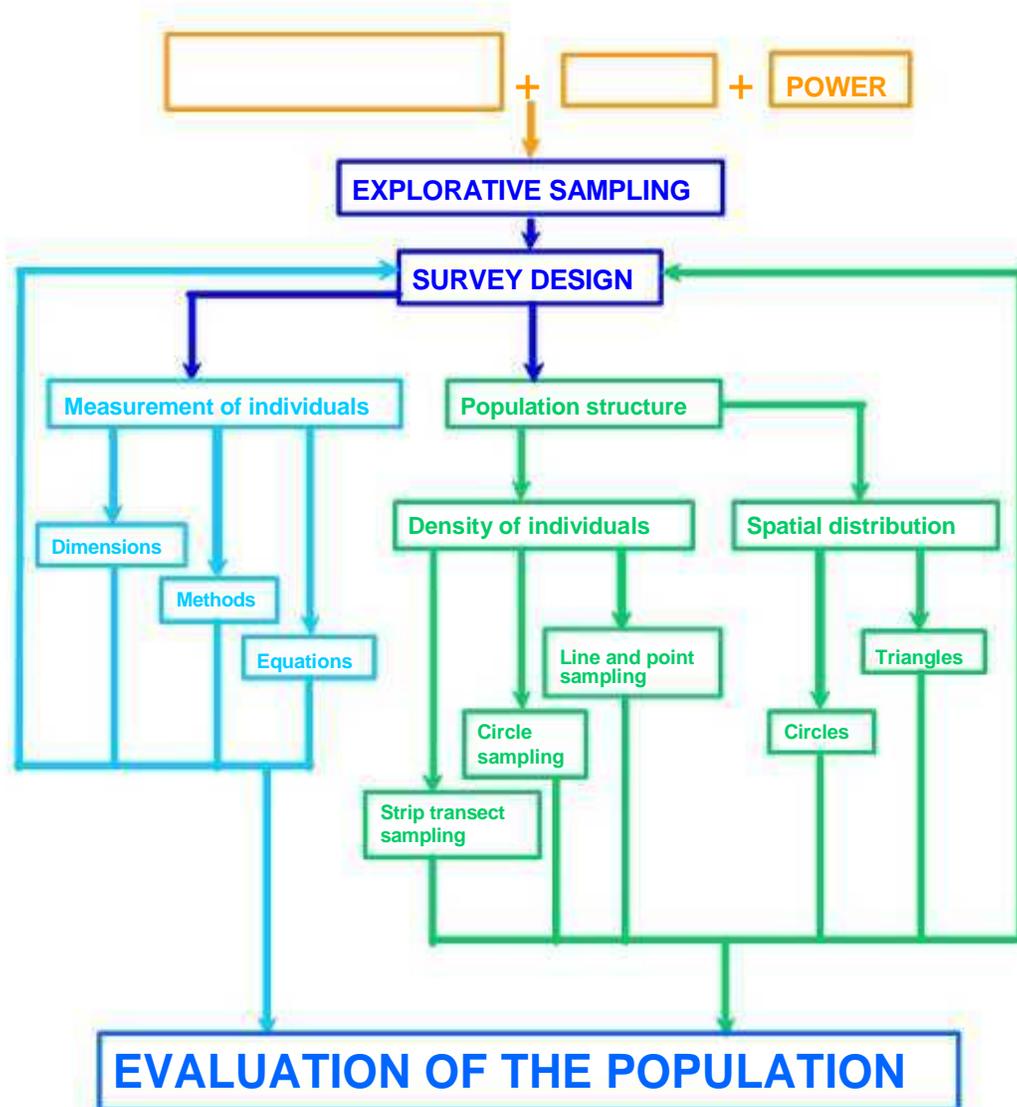


Fig 28. Schematic summary of the steps and methods proposed for the evaluation of *Pinna nobilis* populations .

3.4.5. Population dynamics

For bivalve molluscs, much information about recruitment, growth and age structure can be obtained from size frequency histograms. However, in the case of *Pinna nobilis*, an important effort is required to locate and measure enough Pinnids to build these histograms. Each specimen must be located and measured underwater, and usually densities of individuals are too low to reach a sufficient sample size, compared with other bivalves. Proportionally investing the same or smaller effort, maps of individuals can be constructed tagging and positioning specimens using circle sampling. These specimens can be monitored thereafter, and cohorts followed calculating also accurate mortality rates per age classes. Although with some restrictions, owed to the lesser number of individuals, some information can also be obtained from frequency distributions. For this reason, it is advisable to monitor positioned individuals for population dynamics studies. Some remarks will also be given about the possibilities of using frequency distributions in the corresponding sections.

From the methodological point of view, little more questions have to be addressed for population dynamics studies, and only some remarks will be done in the following sections. Recruitment will deserve some more attention, for artificial techniques for seed collection will also be considered. However, the most important aspect to be treated herein are the statistics and models to be applied to calculate growth, mortality and recruitment. For the development of population dynamics studies it is necessary to tag the individuals for future identification.

3.4.5.1. Tagging

Tagging is used to identify individuals to be monitored in the future. Tags must be lasting but innocuous to the individuals. One method that has demonstrated its utility for long periods of time is the utilisation of plastic plates codified with wholes (Fig 29) and fixed with slackly fastened bridles around the burial perimeter of the individuals. Prof. Nardo and col. fix numbered brass plates with a thin cord (pers. com.).

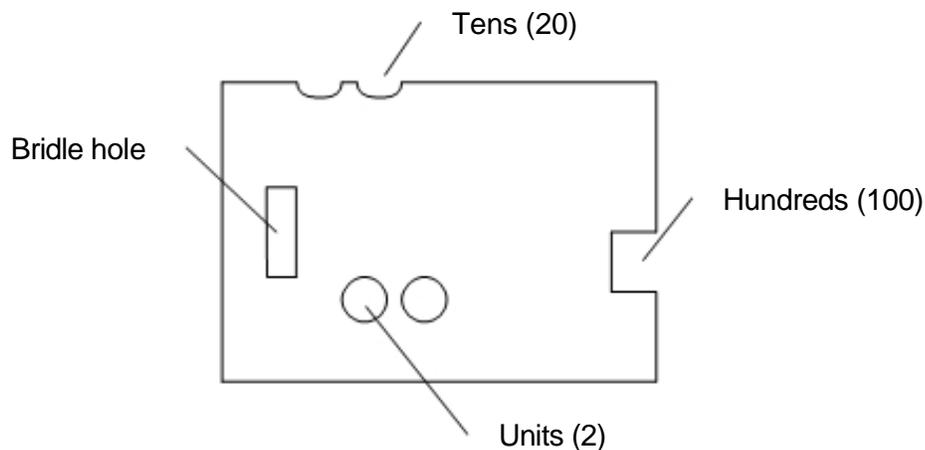


Figure 29. Example of a plastic plate codified with the number 122.

Bridles and cords must be checked periodically (each one or two years), especially those of young individuals, for *Pinna nobilis* specimens tend to burrow in the sediment and the shell can be constrained by the bridles.

3.4.5.2. Growth

Size-at age data is not easily available for *Pinna nobilis*, since although several authors have used posterior adductor muscle scars to estimate *P. nobilis* age (Richardson et al 1999; 2004; Kennedy et al, 2001) the utilisation of empty shells to estimate population growth parameters is not straightforward, and these techniques must be handled with care. The recent discovery of a clear internal register of growth lines in the shell of *P. nobilis* has introduced some light on these aspect (García -March and Márquez-Aliaga, 2006b) but still more research is necessary to define a precise methodology overcoming the problems produced by shell polymorphism. These techniques, as well as some aspects of the internal register, will be treated more deeply in section 5 . Until techniques to estimate population growth parameters of *Pinna nobilis* from empty shells are depurated, growth will still have to be studied from measurements of individuals *in situ* .

The methodology recommended herein is based on tag-recapture methods. Individuals should be tagged, measured and their positions accurately recorded with the techniques exposed in previous sections . Repeated measurements should be done thereafter to estimate the change in Ht with time. The periodicity of measurements is not as important as the time lapse. However, the higher the number of measurements, the higher the precision. At least one measurement/year is recommended, although if several measurements are performed during a year (e.g. 2 -3/year), the influence of seasonality on growth may also be estimated .

As explained in section 2 , *Pinna nobilis* population growth parameters are strongly influenced by hydrodynamics. For these reason, data from individuals inhabiting different depths and sheltered/exposed locations should not be mixed for the calculations. This is an important question since great bias can be introduced in growth and age estimations if it is not taken into account (Fig 30). Besides, this introduces the necessity of doing stratified random sampling rather than simple random sampling, when growth estimations are considered as part of the research objectives.

Pinna nobilis growth data is usually fitted to a Von Bertalanffy (VB) equation of the form:

$$Ht = H_{t_{max}}(1 - e^{-kt}) \quad (7)$$

where Ht (cm) is size, $H_{t_{max}}$ (cm) is the asymptotic maximum size reached by the individuals of the population, k (y^{-1}) is the growth coefficient (the speed at which the asymptotic size is reached) and t (y) the time or age of the individual. This equation implies a fast growth during the first years of life and a sharp break in growth speed afterwards, until the asymptotic size is reached (Fig 30).

Different models are used to fit VB equation to tag-recapture data . With Fabens (1965) method the parameters of VB equation can be obtained using the Newton-Raphson algorithm to obtain the “ceros” from the equations calculated by least squares (LS) method. However, Fabens’ method is biased

when individual variability is high (Ratkowsky, 1985; James, 1991), underestimating K and overestimating Ht_{max} . A similar but unbiased method was proposed by James (1991). The latter method corrects the biases produced by the variability in Ht_{max} among individuals assuming that the individual asymptotes of VB curves are random. Wang (1999) generalised the work by James (1991) considering the growth models with covariates dependent of time and stochastic components. This permitted obtaining functions to calculate the parameters of VB equation with seasonal and tagging effects included.

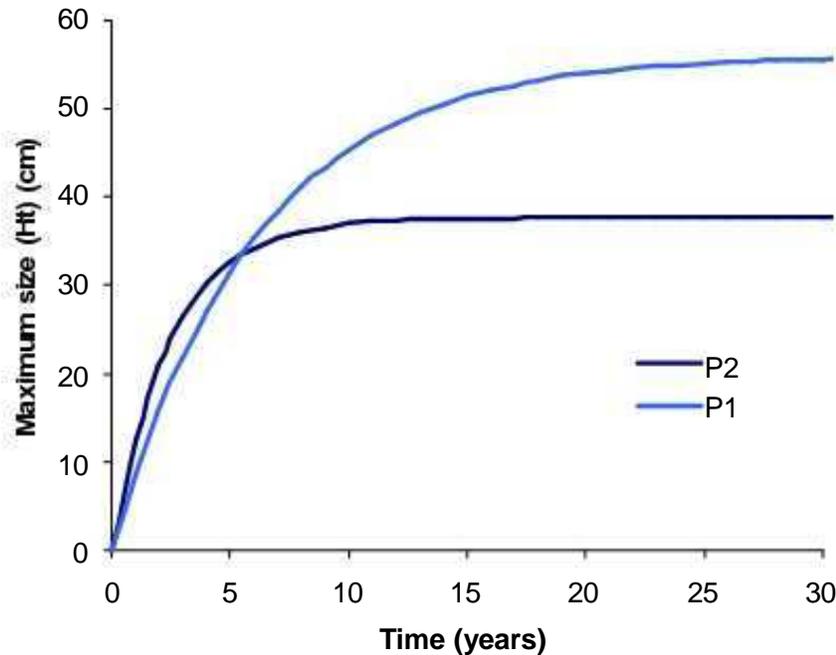


Fig 30. Example of two Von Bertalanffy growth equations fitted to two populations of *Pinna nobilis* inhabiting the same meadow but different depths. P1, population 1, located at -13 m. P2, Population 2, located at -6 m. Note the fast growth during the first 5 years of live and the sharp break in growth speed thereafter for P2. In P1 the speed approximation to the maximum size is slower but maximum size is larger.

However, all these methods are based in a single recapture, and are improperly used if more than one recapture is considered. Recently, Wang (2004) proposed a new method, based in the Generalised Estimating Equations (GEEs) (Liang and Zeger, 1986) that takes into account the effects of tagging and seasonality, as well as the effects of multiple recaptures with unknown ages for all the animals. The statistic used by Wang (2004) is recommended, and calculations can be done with appropriate statistical packages as the program NLIN (SAS inc.).

Some free software specialised for fisheries research (FISAT II, available at <http://www.fao.org/fi/statist/fisoft/fisat/index.htm>) allows the calculations of growth parameters with Fabens' method. If Fabens' method is used the

possibility of obtaining biased estimates owed to individual variability should be taken into account.

Gulland -Holt and Ford-walford diagrams are alternative methods for calculating VB equation parameters from successive measurement of the individuals (Ford, 1933; Walford, 1946; Gulland and Holt, 1959). However, again parameters are calculated using only one recapture, reducing the performance of repeated measuring.

Modal class progression analysis, e.g. Bhattacharya 's (1967) method is based on size frequency data. Although applying this methodology to calculate growth parameters of *Pinna nobilis* is usually constrained by sample size, it should not be ignored, for marked modes could be easily located in the graphics even with low sample sizes.

Growth using Gulland -Holt and Ford-Walford diagrams, as well as Bhattacharyas' method can also be calculated using the free software FISAT II.

3.4.5.3. Mortality

The knowledge of the mortality rate of a population is of great importance for population dynamics and demography. Also, the differences in mortality between endangered and healthy populations can be a quantitative measure of the impacts suffered by the former, and protection policies can be designed accordingly.

Once individuals are tagged and their positions accurately recorded, studying mortality is the easiest work of *Pinna nobilis* population dynamics research. Each new survey, during relocation of the individuals to perform new measurements, all dead specimens are counted. Owing to the dynamic of successive sampling on the same areas, with all individuals tagged and their positions recorded, and the sessile character of Pinnids, the mortality coefficient (z) can be easily calculated. This coefficient is widely used in ecology and fisheries biology, and can be calculated when there are two counts of individuals of a population (n_1 and n_2) separated by a time lapse. During this time lapse, the loss of individuals will follow the relation:

$$n_2/n_1 = e^{-z(t_2-t_1)} \quad (8)$$

The mortality coefficient can be obtained solving this equation and can be compared among populations or size groups within a population. Solving z for different size groups serves to differentiate sizes with different mortality rates. This is useful to identify the most vulnerable size groups in a population.

Differences in mortality for the different size groups can be checked with a χ^2 -test, supposing random mortality for each group of individuals.

3.4.5.4. Recruitment

Recruitment is one of the least known aspects of *Pinna nobilis* demography, and contrary to mortality, its study is the most time consuming. The methodology recommended herein is based on the successive exploration of delimited areas (circles) where all individuals have been previously located, tagged and measured. By this reason, the study of recruitment implies repeating periodically the most costly work of population dynamics research, the

exploration and tagging of small individuals. Combined with the necessity of measuring again already located specimens for mortality and growth, supposes nearly doubling the effort required to carry out the first field survey. This question should be considered in survey design. Alternatively, subsampling the population of areas explored in the first survey could reduce the effort invested .

But the effort of exploring again the circles in search of new recruits has important benefits. Two counts of high value are obtained, the initial number of individuals in the population (N_0) and the final number of individuals after a time lapse t (N_t). With this data, the instant recruitment rate (r) can be obtained using similar formulation to the calculations of instant mortality rate:

$$N_t = N_0 e^{rt} \quad (9)$$

Using r and m , Malthus parameter, the difference between recruitment and mortality rates ($m - r$) can be obtained. This parameter provides an idea of the evolution in number of individuals of the population. This evolution should be neutral after long periods of monitoring, but variable towards positive and negative values with time.

3.4.5.4.1. Utilisation of seed collectors

The capture of natural seeds (larvae) with collectors can provide a valuable insight on the species reproductive cycles. If combined with circles exploration for natural recruitment estimates, extra information on larval ecology (behaviour, survival rates, etc.) could be obtained . Also, seeds can be grown in protected cages and used for the recovery of endangered populations or to research the viability of repopulation policies. It would be advisable to combine seed collection with the study of some oceanographic parameters as water temperature and density, thermocline formation, dissolved oxygen concentration, etc.

Previous experimentation has shown that seed collection is very variable among the years (De Gaulejac et al, 2003), probably reflecting a subjacent variability of the reproductive effort invested by Pinnids, instead of the consequences of hydrodynamics (i.e. currents) (pers. obs.).

From data available of western Mediterranean climate and *Pinna nobilis* gonad maturation, it is advisable to deploy the collectors around June-July, to recover them on October -November . Each collector is composed by a drag of 50-60 kg, a buoy of 4-5 l to keep the line floating and several plastic sacks such as "onion bags" filled with fishing thread. The onion bags are attached to the line at intervals between 3 to 5 m. Depth of deployment of collectors is usually above 30 m. (Fig 31).



Fig 31. Deployment of collectors in Port -Cros Marine Reserve (France)

If seeds are grown in protected cages, care must be taken in order to choose a suitable place to deploy them . As far as possible, the place should be unpolluted, with good water renewal, but also sheltered and with sediment stability, for young *Pinna nobilis* are vulnerable to sediment burial. Depending on number of individuals, juveniles could be separated in individual compartments inside the cages, or grouped in the same room. Isolation also permits the identification of each specimen and their growth can be studied individually.

The procedure to be followed for the study of population dynamics can be summarized in the following diagram (Fig 32).

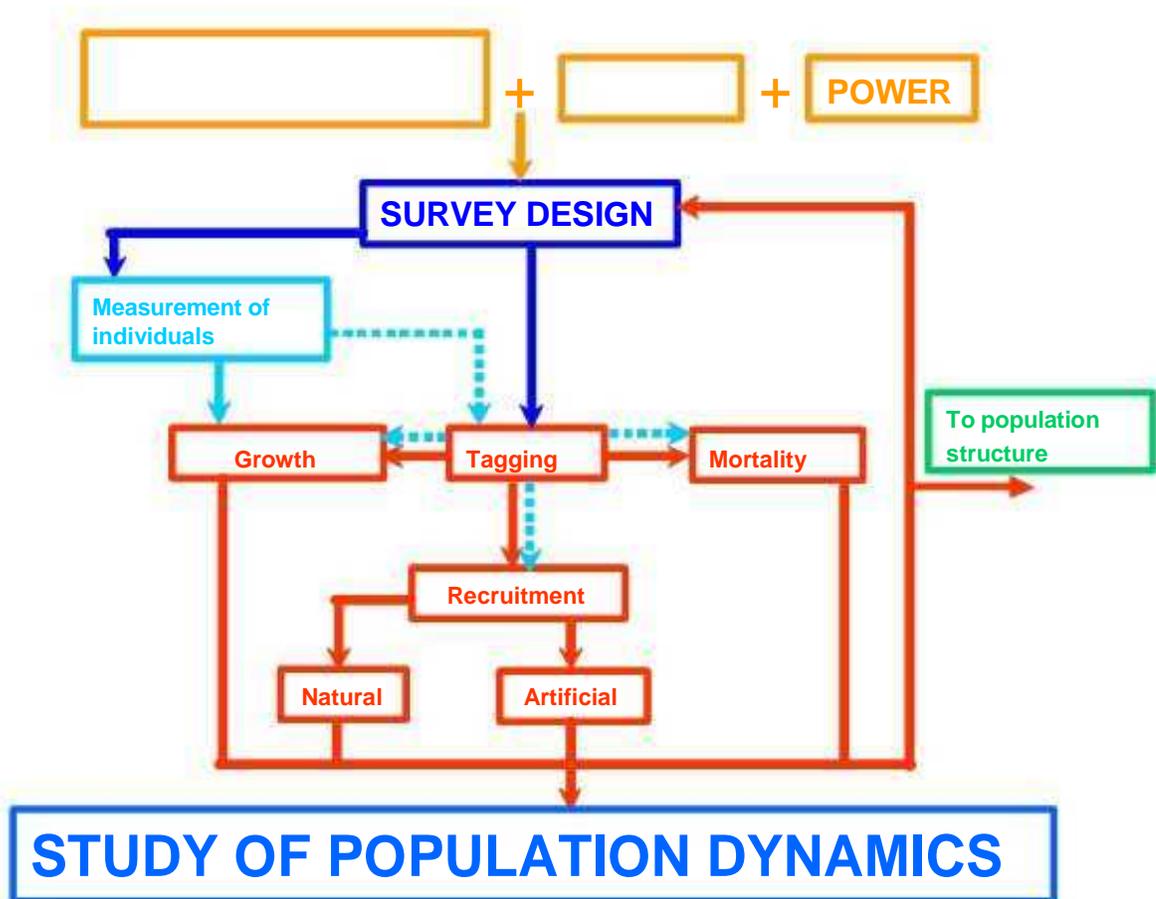


Fig 32. Schematic summary of the steps and methods proposed for the study of *Pinna nobilis* population dynamics.

4. Application of direct techniques for the recovery of endangered populations. Repopulation assays.

The first experiments of *Pinna nobilis* transplantation were made in the Adriatic Sea by Mihailinovic (1955). The intention was growing individuals to commercialise the shell, flesh and byssus. Hignette (1983), transplanted a group of 16 Individuals in Monaco Marine Reserve, and followed their growth during 3 years. Later, De Gaulejac and Vicente (1990) studied the survival of adult and juvenile individuals after transplantation, concluding that specimens larger than 20 cm had serious problems to reattach. The main common feature these studies shared was the limited survival of transplanted individuals, probably owed to the reduced knowledge of *Pinna nobilis* ecology existent at that time. Although no more restocking experiments are found in the bibliography, knowledge on *Pinna nobilis* ecology has been considerably improved, that permits the exploration of new alternatives for individuals' transplantation. The latter is a field of growing interest, for a number of endangered populations could be recovered with this procedure.

As endangered population in need of the application of repopulation techniques, we would understand either a population in clear decline or a group of individuals affected by the development of coastal framework. For both, the transplantation of individuals could be a reasonable option both to recover the stock of broodstock and to save the Pinnids. However, in the first case, the restocking should be made together with the implementation of policies to eliminate the factor causing the population decline, or the long term success of the restocking would be committed. This aspect falls out the scope of the present protocol and should be carefully addressed for each situation.

Hydrodynamics and predators are other two factors limiting the success of individuals' transplantation. The procedures to be used for the transplantation are conditioned to keep drag forces (F_d) supported by the individuals under reasonable safety limits. The latter can be calculated to some extent of precision using linear and solitary wave theory and Morison equation. The impact of predators is important when the repopulation is made with juveniles and can be reduced with the utilisation of mesh cages to protect the individuals. In the next paragraphs, the calculations and procedures recommended to carry out repopulation projects are detailed.

According to García -March et al (2007a), the individuals of Moraira can withstand a maximum F_d around 4.5 N, although they inhabit sites where F_d exerted on the shell rarely overcomes 9 N. The key to increase the probability of survival of transplanted individuals would be to emulate these conditions, balancing the trade-off among water hydrodynamics, depth, shell size and orientation. Reducing the stress of translocation and treating the byssus threads as if they were tree roots is also important. An optimum place would have low to moderated hydrodynamics due to water depth or shelter.

For hydrodynamic calculations, data of height (H), period (T_p), and direction of waves in the donor and receptor populations is required. Knowledge of bathymetry and shore exposure is also basic to select the directions of waves directly hitting both sites. Generally, data of swell in the donor and receptor populations is not available, since expensive current-meters should be installed. Alternatively, data of models from nearby oceanographic buoys can be used. This data is usually treated statistically and wave heights are grouped to show

significant wave height (H_s), i.e. the mean of the third part of the highest waves recorded in a time period. To obtain the maximum wave height some extra calculations are required. We propose the utilisation of the model proposed by Denny (1995), method 2, appendix 1, which is described in the following lines.

According to this model, the return period (t_r) (s) is the period one should wait between encountering waves of a given height,

$$t_r = \langle t \rangle / \text{Prob}(H_0 > h) \quad (10)$$

where t is wave period (s), H_0 is the offshore (unbroken) wave height, and h is individual wave height (both in metres).

The probability that the offshore wave height H_0 is larger than a given value h is,

$$\text{Prob}(H_0 > h) = \frac{S_{\text{all}i} S_{\text{all}j} \{ \exp [-2,338(h/H_s)_{i2}] \} (1/t_j) p_{ij}}{S_{\text{all}i} S_{\text{all}j} (1/t_j) p_{ij}} \quad (11)$$

Where p_{ij} is the probability of occurring each significant wave and period in a coast and a determined time period, and is obtained from the distribution of waves measured by the oceanographic buoy.

The denominator of (11) is the reciprocal of the long-term average wave frequency, $1/\langle t \rangle$,

$$1/\langle t \rangle = S_{\text{all}i} S_{\text{all}j} (1/t_j) p_{ij} \quad (12)$$

If we replace the denominator in (11), then

$$\text{Prob}(H_0 > h) = S_{\text{all}i} S_{\text{all}j} \{ \exp [-2,338(h/h_s)_{i2}] \} (1/t_j) p_{ij} / 1/\langle t \rangle \quad (13)$$

And therefore, simplifying equation (10), t_r can be calculated as,

$$t_r = S_{\text{all}i} S_{\text{all}j} \{ \exp [-2,338(h/h_s)_{i2}] \} (1/t_j) p_{ij} \quad (14)$$

Values of H_0 are calculated using an iterative method, assigning a value to h in which the t_r in seconds coincides with the considered time lapse (e.g. 3.1536×10^7 s for a year or 86400 s for a day).

To calculate wave shoaling, the following equation is used,

$$H_i = H_0 \left\{ \frac{\sinh(2k_i d_i) [2k_0 d_0 + \sinh(2k_0 d_0)] / \sinh(2k_0 d_0) [2k_i d_i + \sinh(2k_i d_i)]}{\tanh(k_i d_i)} \right\} \frac{\tanh(k_0 d_0)}{\tanh(k_i d_i)} \quad (15)$$

Where H_0 is maximum wave height offshore at a depth d_0 , H_i is the maximum wave height inshore at a depth d_i , \sinh , \cosh and \tanh are hyperbolic sinus, cosine and tangent, k_0 and k_i are offshore and inshore wave numbers ($k = 2\pi/l_w$) and l_w is wave length, calculated with the equation,

$$l_w = (g t^2 / 2\pi) (\tanh[4 \pi^2 d / t^2 g])^{1/2} \quad (16)$$

Water speeds are calculated using linear wave theory for unbroken waves when water depth is $>3/4$ wave height and solitary wave theory for breaking waves, with the equations,

$$u_1 = (\pi h_0 / t) [1 / \sinh(kd)] \quad (17)$$

$$u_2 \sim 0.3 [g(h+d)]^2 \quad (18)$$

where h is wave height, t is wave period, k , the wave number, d is water depth and g is acceleration due to gravity (9.81 m/s^2). When the individuals inhabit meadows of *Posidonia*, the roots and leaves of the seagrass reduce water speed at bottom level. Some authors (García -March et al, 2007a) have applied a factor of $1/2$ to correct for this water speed reduction.

Finally, drag force (F_d) is calculated solving Morison equation,

$$C_d = 2F_d / d v^2 A_s \quad (19)$$

where C_d is drag coefficient, d is water density (1025 kg/m^3 for seawater) v is water speed and A_s is the surface of the object projected in a plane.

Drag coefficients for the lateral and dorso -ventral positions of the shell of *Pinna nobilis* where studied by García -March et al (2007a), and for different water speeds can be approximated by the following equations ,

$$C_{d \text{ lateral}} = \exp^{-0.2482 + (0.1703/v)} r^2 = 0.94 \quad (20)$$

and

$$C_{d \text{ dorso-ventral}} = \exp^{-0.3024 + (0.0685/v)} r^2 = 0.95 \quad (21)$$

A rough approximation of shell surface area (SSA) of individuals can be obtained using the equations relating H_t and SSA calculated for the individuals of Moraira population, for lateral and dorso-ventral shell orientation García March et al (2007a),

$$S_{\text{lateral}} = 0.2567 * H_t^{1.9180}, R^2 = 0.99. \quad (22)$$

$$S_{\text{dorso-ventral}} = 0.0734 * H_t^{1.8954}, R^2 = 0.98 \quad (23)$$

All these equations can be used to calculate the optimal site for the transplantation, position, water depth, burial depth and orientation of each specimen, depending of its size . In any case, exposed locations, sites with important sediment movements or enhanced particle deposition should be avoided a priori for the transplantation. On the other hand, places where some Pinnids already live are good candidates to be receptors for the transplanted individuals. The new specimens will help to improve the success of reproduction and the probability of natural recovery of the population.

The process of transplantation itself should be fast, and exposition of individuals to air should be avoided as far as possible. For adult individuals, byssus threads should be conserved and, in fact, it is recommended to transplant the individuals with 20-30 cm of sediment around the buried anterior part, including shoots and roots of *Posidonia* if the donor population inhabits a meadow of the phanerogam. A hole must be made in the place of transplantation where both individual and sediment can be deposited. Once transplanted in the appropriate site, orientation and burial depth, the sediment should be secured with a plastic mesh fixed with thin stainless steel pegs (~ 20 cm long) driven in the seafloor. If young juveniles from collectors are used for repopulation, the most important is to protect individuals with mesh cages until they reach an appropriate Ht to diminish the probability of being preyed by octopuses and sea breams. It is advisable to keep the cages at least until they reach 30 -35 cm of maximum length. The hydrodynamic calculations should ensure that the transplanted specimens can enlarge the shell under safety limits of F_d and that orientation is appropriate, for individuals do not change their position once they are implanted.

5. Biometry of empty shells

The utilisation of shell biometry in Pinnids has been traditionally focused to study population growth rates. Commonly, the posterior adductor muscle scars (PAMS) have been used to estimate these parameters, as well as the age of individuals. Each PAMS is a curved print or ring, formed around the posterior adductor muscle in the dorsal nacre lobe (Fig 33). Moreteau and Vicente (1980) demonstrated that Ht and PAMS positions were highly correlated and later Richardson et al (1999) correlated the seasonal temperature changes of occidental Mediterranean with regular periods in the proportions of stable oxygen isotopes recorded in the external calcitic shell -layer of *P. nobilis*. The minimum temperatures observed from the d18O: d16O proportions of the shell usually occurred around one internal PAMS. The authors concluded that each PAMS was deposited annually and that one or sometimes two PAMS were undetected in adult individuals. Having the relationships of PAMS with total size and PAMS with age, growth curves have been calculated using length -at-age data from thereafter (Richardson et al 1999, 2004; Kennedy et al 2001). However, recent works by García -March and Márquez-Aliaga (2006b) have demonstrated that the number of obscured marks is much larger and variable among individuals. Some specimens show 6 obscured PAMS whereas others only two. Furthermore, the annual periodicity of PAMS has been called in question. *Pinna nobilis* shows a clear inner register through the dorsal nacre lobe, coinciding with external straight lines. Internally, nacre tongues directed in posterior direction are observed periodically. Each nacre tongue is also accompanied by two calcite stripes starting on the tongue and from it, which also are directed in posterior direction. These calcite stripes are distinguishable by their different coloration (dark and pale) (Fig 34). Data indicates that inner register is indeed formed annually, but it is layered before (in time) than PAMS, and do not coincide in position with them. Therefore, PAMS would not be formed annually.

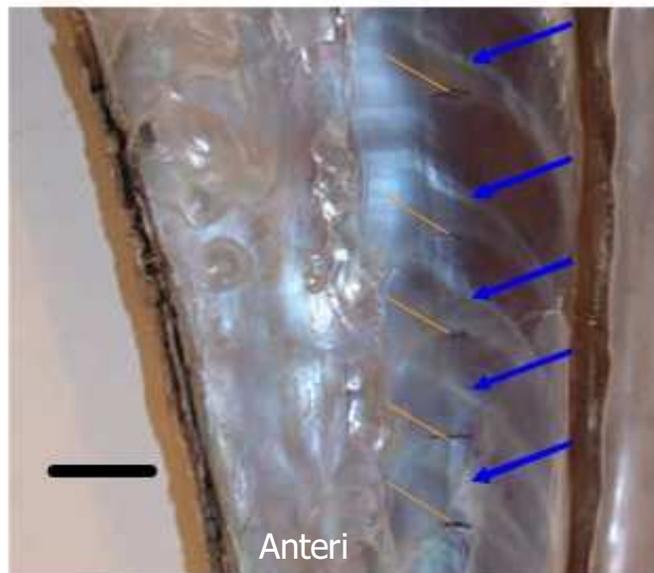


Fig 33. Positions of rings (orange arrows) and straight lines (blue arrows) of the posterior adductor muscle scar register in the dorsal nacre lobe of *Pinna nobilis*. The dorsum of the shell is at the left and anterior at the bottom. Scale bar 20 mm.

Although inner register is externally observable as straight lines, unfortunately these are confused with external rings in old individuals. Therefore, to study individuals' age from empty shells, inner register is the only print to be measured accurately.

To “read” the inner register it is necessary to cut the shells through the middle of the dorsal nacre lobe with a power saw, and polish the surfaces of the cuts to 1200 μ with Carborundum. It is advisable to include the shells in polyester resin (e.g. STRATIL AL-100) to protect the shell surfaces from excessive tensions during the process of cutting. Polished surfaces are studied with binocular lens and age estimated counting the register. Total sizes can be correlated with the positions of the inner register using linear regression between Ht and distance of inner register to the umbo, as made by Moreteau and Vicente (1980) between Ht and PAMS. This provides a number of sizes separated by constant time intervals (years) for each specimen, which can be fitted to VB growth equations using either tag-recapture or size-at-age methods if the first year of live is clearly identifiable.

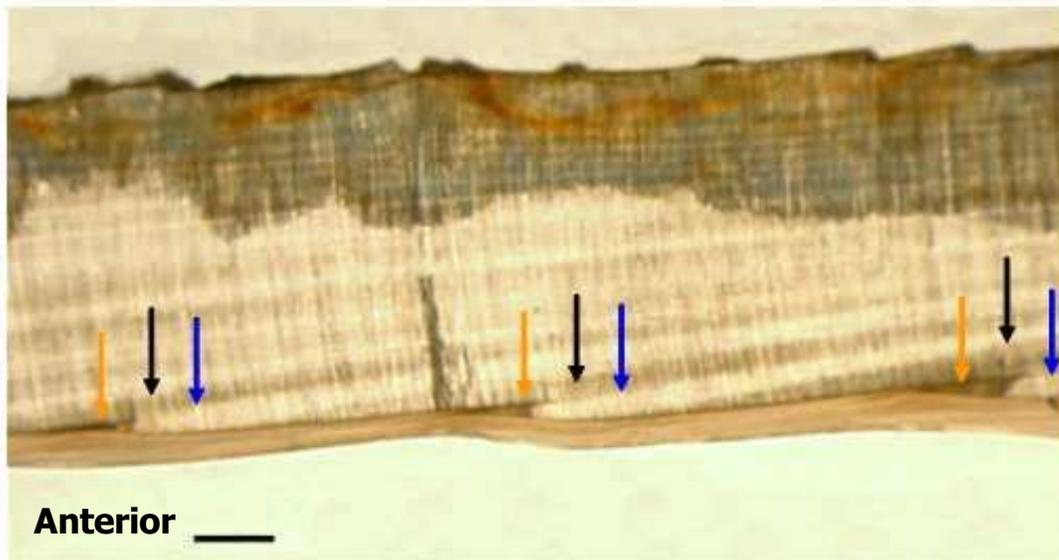


Fig 34. Thin sheet showing the inner register of the dorsal nacre lobe of *Pinna nobilis*. Orange arrows, nacre tongues. Black and blue arrows, calcite stripes. Scale bar 1 mm.

Some remarks must be addressed about the utilisation of empty shells to estimate population growth rates. *Pinna nobilis* is a polymorphic species and the causes of this morphological plasticity are still focus of intense research. Abrasion of the umbo shortens the shell, and individuals counteract this shell destruction by i) the migration of soft tissues in posterior direction and ii) the active reconstruction of new umbonal cavities to keep the triangular anterior shape. This produces an actual shell “reshaping” that is probably influencing shell morphology (García -March and Márquez-Aliaga, 2006a). Consequently, for the same population and depth range, individuals with the same Ht but different shape usually do not have the same age, and biases can be introduced in the calculations. The recommendation is to be aware that the techniques to use empty shells to estimate growth rates are not yet completely depurated, and results could be biased.

To reach a reasonable sample size, it is advisable to gather all empty shells of *Pinna nobilis* individuals found during field surveys, taking for granted that the appropriate permissions are solicited to the pertinent governmental authority. If necessary, some surveys should be designed to collect these shells. Explorative sampling is the best way to search for dead individuals. If these searches are made after strong storms, the probability of finding dead specimens is much increased. The waves detach dead specimens and lay them in channels or near steps, where they are easily located, but also unbury specimens that died in previous years and were already covered by sediments. Under these circumstances, it is not rare to collect more than 15 empty shells in only one SCUBA dive (pers. obs.). During collection of dead individuals, researchers must make sure that the Pinnids found are actually dead, for some times detached specimens are still alive!

6. Methods to study anthropogenic impacts on *Pinna nobilis* populations

There is a generalised consensus of the negative impact of human activities on *Pinna nobilis* populations. It is admitted that pollution can affect the larvae (as for many other invertebrates) (Vicente, 1990; Vicente and Moreteau, 1991) and the development of coastal framework has been responsible of the destruction of vast extensions of seabed covered by *Posidonia oceanica* meadows, the preferential habitat of *P. nobilis*. The collection of individuals by amateur divers has been another cause of the species decline. It is common to find empty shells as decoration in coastal tourist restaurants, or sporadically commercialised in souvenir shops. Commercial fishing has been another major cause of population decline. Even today, nets of local fishermen are entangled on large individuals (pers. obs.). But the effects the trawlers have had on deep populations are probably incalculable. Nearly two decades ago, guards of Columbretes Islands Marine Reserve had to help a trawler arrived to "Illa Grossa", the main island of the Reserve, because the fishermen could not haul in the nets, full of large *Pinna nobilis* (Santiago Sales, Guard of the Reserve, pers. com. 2004). Probably an entire population had been killed that day. Boat anchoring breaks the posterior extensions of the shell, giving rise to shell malformations. Pronounced breakages of the shell led to increased mortality rates, despite the elevated capacity of shell reconstruction shown by Pinnids.

There are many proves of the harmful effects of human activities on *Pinna nobilis*, but, however, there are little, if any, experiments to evaluate quantitatively these effects. This is mainly owed to the technical and theoretical complications of this type of studies, but also to the fact that until recently the research on this species has been strongly neglected.

The development of studies to asses human impacts on one population is constrained by the previous knowledge about the structure of that population. If the latter is unknown, the effects of an impact are difficult to evaluate, and it is necessary to compare with other similar populations in similar environments. Commonly, it is difficult to predict the occurrence of an impact on a population and, therefore, only through the accurate knowledge of the species ecology will it be possible to approximate its degree of deterioration. Consequently, the best policy to study human impacts on *Pinna nobilis* populations is the improvement of the knowledge of undisturbed populations in different environments.

The aggressions suffered by the populations of *Pinna nobilis* are varied, but they can be grouped into chemical and physical. The chemical impacts to be considered herein are those owed to marine sewage outfalls, but for other similar focalised impacts, the methodology proposed is identical. Considered physical impacts for which specific sampling methodologies are described herein are the effects of fishing gears, boat anchoring and collection by SCUBA divers.

6.1. Chemical impacts, study of the effect of marine sewage outfalls

Chemical pollution from sewage outfalls is usually focalised and the intensity of the impact on a population of Pinnids is expected to follow a gradient towards the sewage outfall. The best sampling methodology is the uniform layout of sampling stations around the source of pollution and the comparison between the population structure of the Pinnids found in each

station. If there are important natural gradients (e.g., water depth or substrate type, etc.) it is advisable to use a covariate in the ANOVA, to minimize the variability introduced by them. Differences between density of individuals and size structure can be detected in the tests. Also, some chemicals can produce shell malformations, and the degree of occurrence of these malformations can be a good estimator of the impact. Random sampling of some individuals at each station, to carry out anatomical and chemical studies at the laboratory, may be helpful to identify the effects the pollutants produce on the individuals. Additionally, substrate and water samples may be gathered to compare the anatomical and chemical studies of the tissues, with the levels of pollutants in substrate and water.

The effects on *Pinna nobilis* larvae can be researched using seed collectors as those described in section 3.4.5.4.1., situated at different distances from the outfall.

6.2. Physical impacts

6.2.1. Fishing gears

Trawlers can produce important damage on *Pinna nobilis* populations. In the European Union, the utilisation of this type of net is forbidden in depths less than 50 m, which initially would reduce the impact of this type of fishing gear. However, some boats do not respect the legislation and artificial reefs have been installed in some sites to avoid their action. In El Campello (Spain, Western Mediterranean), the recovery of a *Pinna nobilis* population was used to study the evolution of the benthic community after the installation of an artificial anti-trawling reef (Barberá et al, 1996).

In sites where trawling is still carried out at forbidden depths, even over *Posidonia oceanica* meadows, the populations of *Pinna nobilis* can be strongly reduced. One way to study the impacts of trawlers in these zones is the utilisation of artificial models of *Pinna nobilis* deployed regularly in the fished zone. Controls may be established in similar environments, and the destruction of the models, compared to those in undisturbed sites, may be a good quantitative estimation of trawling impact.

Entanglement of trammel nets with *Pinna nobilis* specimens is also a common cause of mortality of large individuals. The best way to estimate its impact is asking for the collaboration of fishermen, who could report the number of occasional captures. The release of captured specimens may suppose, at least, the survival of some of them.

6.2.2. Boat anchoring

In sites where boat anchoring is a common practice, it is difficult to find adult individuals, probably owed to the mortality produced by the anchors. It is important to make an inventory of the remaining stock of *Pinna nobilis*, tagging and positioning all specimens, and monitoring them. Additionally, the deployment of models of Pinnids, as suggested to study trawling effects may be helpful to evaluate the impact of anchoring. The models must be surveyed periodically during the high season and the rate of hits on models calculated.

6.2.3. Collection by divers

It is difficult to assess the impact of collection by amateur divers. In open waters, the possibilities are limited owing to the vast extension that should be surveyed and to the insufficient knowledge on *Pinna nobilis* ecology. Differentiating between collection and other impacts requires quantitative knowledge of the latter, which is usually lacking. One option to minimize variability is to carry out the studies in populations located in bays close to the shore and near recreational bathing sites.

Pinnids can be tagged and positioned. Some of them are signalled underwater with a visible mark or buoy and the rest kept as control. Signalled specimens are more accessible to amateur divers and the rate of mortality between control and signalled populations compared. Mortality is expected to be larger in signalled individuals, for personal experience indicates that signalling by scientist does not ensure a reduction in the collection of Pinnids, but specimens are more easily located.

7. Conservation and measures of protection

Education at the level of diving clubs and amateur divers is fundamental. Informative measures oriented to improve public and tourist awareness is also basic to transmit the importance of the conservation of this species and biodiversity in general. The distribution of leaflets in tourist zones during the high season may be an affective measure to fulfil this objective. The location, identification and tagging of *Pinna nobilis* individuals in populations close to the shore, and the deployment of underwater educational panels has demonstrated to be an effective measure for Pinnid protection in Port -Cros Islands Marine Reserve. The “Sentier Sous -Marin” (Port-Cros, France) , created in 1979, is visited by 2000 amateur divers each year, and its utility has been more than justified with time (<http://www.portcrosparcnational.fr/visite/portcros/animation/>).

Under a scientific approach, the development of doctoral programmes and meetings will help to improve the knowledge on the ecology of this species and to share information between scientists involved in this field of research. In this regard, the revival and implementation of initiatives like the R.E.M.O.E.P.P. (Mediterranean Network for Observation, Study and Protection of *Pinna*), created in 1991, would help to join efforts and exchange knowledge between specialist, fulfilling the objectives of a global understanding of this species ecology and population dynamics, unifying research methodologies and establishing the priority knowledge-gaps of *Pinna nobilis* ecology to be researched.

Under a practical point of view, trawling should be avoided in shallow waters, but also exploration of deep *Pinna* populations should be carried out, for the knowledge about these populations is scarce and mapping them may be a positive measure to select zones to reduce fishing pressure. Deployment of trammel nets should be avoided in zones with high densities of Pinnids to diminish the probability of accidental entanglement of large individuals.

The impacts of boat anchoring may be strongly reduced with the installation of mooring buoys. Modern anchorage systems also reduce the impacts on the seabed of dead weights. Environmental-friendly anchorages have been successfully used in different MPAs of the Mediterranean (Port Cros Marine Reserve, France, and Sierra Helada Natura 2000 site, Spain, etc.) and its utilisation reduces successfully the impacts of boat anchoring on the seabed.

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