PRACTICAL GUIDE

The planting of *Posidonia oceanica*

Technique used in the R&D+i project 'Use of seeds and fragments of *Posidonia oceanica* for the recovery of areas affected by Red Eléctrica de España's activity'

RED ELÉCTRICA DE ESPAÑA

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RED ELÉCTRICA DE ESPAÑA

CSIC

CHAPTER

1

Introduction

REE understands active restoration as the interventions to enable the natural recovery process of an ecosystem following its degradation, damage or destruction.

Clewell et al. (2004)

A restoration project can be either active when it involves techniques such as transplantation or the construction of artificial habitats, or even passive, when it focuses on eliminating the impact that prevents the natural recovery of the ecosystem object of the project (*Shuster,* 2004). The rapid degradation of ecosystems has multiplied the number of restoration projects, although most of them are carried out in terrestrial ecosystems (*Blignaut et al.,* 2013) whereby there is still much uncertainty about the feasibility and convenience of carrying out restoration projects of marine ecosystems (*Bayraktarov et al.,* 2015). Furthermore, marine ecosystems are normally not very visible to citizens in general, which implies that the perception of their degradation and the social pressure to recover them decrease.

The importance of *Posidonia oceanica* meadows in the control of coastal erosion, carbon sequestration or natural water purification, has led these ecosystems to be ranked as those of higher interest for restoration among the coastal systems.

The restoration cost of marine ecosystems is estimated to be 10-400 times higher than that of wetlands and terrestrial ecosystems (*De Groot et al.,* 2013). In economic terms, assessing ecosystem services, we find that the cost-benefit ratio of marine ecosystem restorations, although lower than in terrestrial ecosystems, remains positive (*De Groot et al.,* 2013). Among marine ecosystem restoration projects, the restoration of marine angiosperm seagrass meadows is among the most expensive with an estimated cost of US$ 383,672*Ha^{-1} per hectare planted. In addition, the survival results that have been obtained in seagrass meadow restoration are among the lowest of the marine restoration projects, with an average 38% survival rate of the planted material (*Bayraktarov et al.,* 2015). However, the importance of seagrass meadows in the control of coastal erosion, carbon sequestration or natural water purification, among other things, has led these ecosystems to be ranked as those of greatest interest for restoration among the coastal systems (*Barbier et al.,* 2011; *Greiner et al.,* 2013).

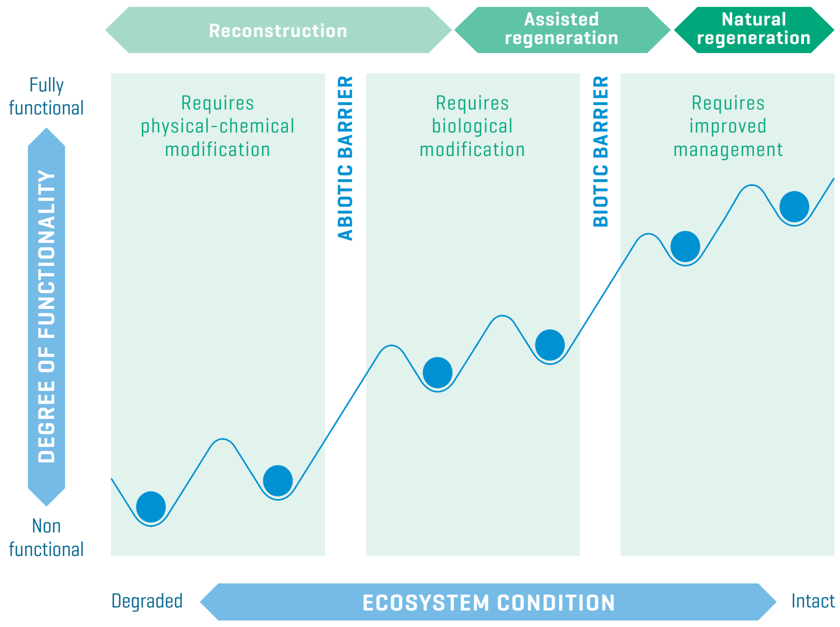
38%

Average survival rate of marine angiosperms replanted in restoration projects

Consequently, the development of seagrass meadows restoration is necessary and has much room for improvement. The most desirable lines of work in the restoration of seagrass meadows are the so-called assisted regeneration options, that is, those aimed at helping the 'repair' of the ecosystem by reinforcing the recovery processes that occur naturally. The replanting of seagrass plant material shed naturally and collected for this purpose is part of this line of work. Without ever forgetting that the most appropriate option for the conservation of ecosystems, and especially those that are under specific legal protection such as *Posidonia oceanica*. Species particularly sensitive to disturbances such as the *Posidonia oceanica* mentioned above, might require

the combination of conservation (reducing impacts) and restoration (*Possingham et al.,* 2015).

Conceptual model of ecosystem degradation and response options from a restoration point of view (Source: International standards for the practice of ecological restoration. Society for Ecological Restoration, 2016)



At present, *Posidonia oceanica* planting techniques are in a development and refinement phase that will allow their application on a larger scale. The bulk of the *Posidonia oceanica* replanting projects have been carried out with a focus on the viability of the planted material, an effect of the planting period on the survival and implementation of small-scale techniques. Transplanting success is quantified by the number of the planted items that survive, although the assessment of the success of a restoration project must consider the recovery of the diversity and functionality of the target ecosystem (*Ruiz-Jaen & Aide,* 2005). The restoration of seagrass meadows, understood as the true recovery of its functions, is an incipient line of work and there are few projects or studies that address it (*Dapson,* 2011). As yet, no *Posidonia oceanica* restoration projects have been conducted using this approach, although the planting of 2 hectares in the Bay of Pollença, Majorca, within the framework of the 'REE Marine Forest' project (2017-2020), incorporates this approach and includes monitoring the recovery of functions during the duration of the project, opening the opportunity to continue well beyond the project's time horizon.

The marine angiosperms have two reproductive mechanisms: vegetative and sexual, which give rise to two types of planting material for restoration, fragments and seedlings respectively, each material has different behaviours and needs. The basic architecture of marine angiosperms is modular or clonal. Clones have leaf-bearing shoots consisting of several leaves and structures responsible for anchorage to the substrate, the roots and the rhizome, the organ from which the shoot emerges/is born. The rhizomes can have vertical or horizontal growth habit, depending on whether the angiosperm marine species have both or only horizontally growing rhizomes (*Hemminga & Duarte,* 2000; *Hartog & Kuo,* 2006).

Prior experiences planting *Posidonia oceanica* with adult plant fragments have stemmed from both vertical or horizontal fragments, whether with one or several shoots, obtaining better survival results with horizontal rhizomes.

Vegetative reproduction consists of the formation of clones, by means of the elongation and ramification of the horizontal rhizomes, the growth of leaf-bearing shoots on them and the fragmentation of the rhizomes as a consequence of the death of the oldest parts to form the clones. This process, also known as 'vegetative fragmentation', represents the main mechanism responsible for contributing to the maintenance of seagrass meadows and their expansion (*Duarte & Sand-Jensen,* 1990). Adult plant fragments are obtained by means of this mechanism: i.e. pieces of rhizome with one or several leaf-bearing shoots and roots, suitable for the planting. Waves can also break up the rhizomes and produce fragments.

Posidonia oceanica has lignified rhizomes of horizontal growth on which the shoots that give rise to vertical rhizomes grow during their development. Previous experiences of planting *Posidonia oceanica* using adult plants have been carried out using both vertical fragments (*Augier et al.,* 1996; *Piazzi et al.,* 1998; *Meinesz et al.,* 1992; *Molenaar & Meinesz* 1992) and horizontal ones (*Piazzi et al.,* 1998) with one or several shoots, with horizontal rhizomes showing better survival rates. More specifically, between one and two years after planting vertical fragments of rhizom, a change was observed in their growth habit going from vertical to horizontal (*Augier et al.,* 1996; *Molenaar & Meinesz,* 1992). In addition, vertical fragments generally showed lower survival rates, less branching and lower root production (*Molenaar et al.,* 1993), the latter being essential for the anchoring of the plant, the incorporation of nutrients and their survival in the medium term (*Balestri et al.,* 2009; *Infantes et al.,* 2011).

Posidonia oceanica horizontal rhizomes show elongation rates (1-6 cm/year) much higher than those of vertical rhizomes (0.1-4 cm/year), although both are among the lowest growth rates of marine angiosperms (*Hemminga & Duarte* 2000). The selection of horizontal fragments for the restoration of *Posidonia oceanica* therefore has advantages over the use of vertical fragments: it facilitates the establishment of the planted material, the consolidation of the clones and the colonisation of adjacent areas.

On the other hand, with regard to seasonal variation in planting works, previous results indicate that planting conducted in autumn with fragments from deep water meadows with two or more shoots show better survival rates than those transplants conducted in summer using fragments from shallow water meadows and having a single shoot (*Genot, Caye, Meinesz, & Orlandini,* 1994; *Meinesz et al.,* 1992; *Molenaar & Meinesz,* 1992). There is the possibility of keeping *Posidonia oceanica* fragments in controlled conditions prior to planting, which enables the plants in worse conditions to be discarded. According to the results of *Meinesz, Caye, Loquès, & Molenaar* (1993), fragments previously kept in the aquarium (for 3 months) show greater survival rates after they are transplanted to the sea (60-100%) than those collected and transplanted directly (25-80%). Furthermore, prior maintenance in an aquarium can represent an adaptation period for fragments whose depth of origin is lower than that of the restoration area, a situation that can compromise the survival of the plantation as a consequence of the lower concentration of chlorophyll in the plants coming from shallower depths (*Genot et al.,* 1994).

Regarding seasonal variation in planting works, previous results indicate that planting carried out in autumn with fragments from deep water meadows with two or more shoots show better survival rates than those transplants carried out in the summer season from shallow water meadows and having a single shoot. There is the possibility of keeping the *Posidonia oceanica* fragments in controlled conditions prior to planting which enables discarding the plants that are in worse conditions.

The survival of fragments rate with several shoots in the cultivation stage is 80-100% during the first 4 months of cultivation (*Balestri et al.,* 2011; *Marín-Guirao et al.,* 2011) and this rate reduces gradually over time: 67-68% at 12 months, 36-56% at 24 months and 42% at 34 months (*Balestri et al.,* 2011; *Meinesz et al.,* 1991). Fragments with a single vertical shoot have lower survival rates in culture (41% after 7 months) although those that survive are capable of generating new roots and eventually turn into rhizomes which will show horizontal growth with ramifications (68% of the surviving fragments will normally branch after two years in culture) (*Meinesz et al.,* 1991).

The second reproductive mechanism of marine angiosperms is sexual reproduction, by which new individuals are produced, called seedlings, suitable for planting. The seedlings are produced by the germination of a seed from the fruit resulting from a pollinated flower. This mechanism introduces genetic variability in the seagrass meadows and allows the colonisation of new areas through the dispersion of fruits, seeds or seedlings by sea currents. The production of seeds has been confirmed in the 58 species of marine angiosperms identified and among them, we find species whose seeds have a latency period and others, like those of the Posidonia genus, whose germination begins at the moment in which the seed detaches from the fruit (*Orth et al.,* 2000). The fact that *Posidonia oceanica* seeds do not have a latency period has consequences in the restoration projects as it prevents the creation of seed banks or their storage (*Belzunce et al.,* 2008) so that they could not be available for planting when required.

Another difficulty present in the use of seedlings for the restoration of *Posidonia oceanica* is the unpredictable and irregular flowering of *Posidonia oceanica* (*Díaz-Almela et al.,* 2006) and, therefore, of the production of seeds. However, the ease of handling, the reduction of logistical costs in comparison with the planting of adult plants, as well as the high survival rates under certain conditions encourage the use of seedlings in *Posidonia oceanica* replanting projects. Previous planting experiences resulted in 75-80 % survival rates on Posidonia matte after the first year of planting (*Balestri et al.,* 1998; *Domínguez et al.,* 2012) and 44-70 % after 36 months of planting (*Balestri et al.,* 1998; *Terrados et al.,* 2013).

Seeds remain attached to *Posidonia oceanica* seedlings during the first year of life, providing them with the necessary nutrients during the first 6-8 months of their development (*Balestri et al.,* 2009). The ability of the seeds to germinate and survive during the first year depends on the good state of conservation of the seed and the amount of light it receives (*Celdrán & Marín,* 2013). For the purposes of a replanting project, germination under controlled conditions enables the selection of seedlings that are in better conditions for planting.

Since the late eighties, there have been previous experiences of small-scale cultivation of *Posidonia oceanica* mainly with seeds and seedlings. These experiences showed that seed culture could be prolonged for several months with a survival rate above 25 % (*Bedini,* 1997; *Buia & Mazzella,* 1991; *Meinesz et al.,* 1993), and their development is possible under laboratory conditions, with artificial water and closed-circuit pumping system (*Caye & Meinesz,* 1989).

The success of the cultivation of seedlings can be compromised when the seed depletes its reserves, and the seedling needs to incorporate nutrients from the environment in which it is located

(*Balestri et al.,* 2009). Regarding the physical and chemical conditions that favour survival in culture, according to the results of *Caye & Meinesz* (1989), the availability of nutrients favours the branching of the seedlings and the generation of new shoots, although it also seems to reduce the development of the roots and the survival of seedlings in the first year of life (*Statton & Kendrick,* 2014, own data). Survival is also reduced with salinities above 37 PSU (*Fernández-Torquemada & Sánchez-Lizaso,* 2013) and at temperatures of 29 °C (*Hernán et al.,* 2017; *Guerrero-Meseguer et al.,* 2017).

On the other hand, the shoot foliar surface is reduced in one-year-old seedlings subjected for three months to a temperature of 29 °C (*Hernán et al.,* 2017), in the same sense as the results of *Olsen et al.* (2012) for plants of four years of age subjected to water temperatures above 26 °C. It has been demonstrated that *Posidonia oceanica* seeds are photosynthetically active, and the incidence of light on them is essential in the development of the seedling (*Bedini,* 1997; *Celdrán & Marín,* 2013).

Posidonia oceanica seeds are photosynthetically active, and the incidence of light on them is essential in the development of the seedling. The previous culture phase also allows the development of the fundamental root system necessary for the anchoring of the seedlings and helps cover all the nutrient requirements of the seedling following the first year of life.

The culture phase also allows the development of the fundamental root system for the anchoring (and therefore survival) of the seedlings and helps cover the nutrient requirements of the seedling (*Balestri,* 2009) after the first year of life. *Balestri et al.* (2015) proved that the roots developed in seedlings established on average granulometries (sandy) reached greater depths, than those established on rocky substrates which favour the horizontal growth of the roots. On the other hand, *Infantes et al.* (2011) demonstrated that *Posidonia oceanica* seedlings require a root length of approximately 0.35 times the square root of the total surface of the leaf in order to remain anchored and that an erosion of the surrounding sediment greater than 2 or 3 centimetres eventually entails the uprooting of the seedlings. Previous projects involving the planting of seedlings suggests that the Posidonia matte (the interlacing network of rhizomes, roots and sediment formed by *Posidonia oceanica* meadows and that remains when the plants die) is the most favourable substrate for the survival of seedlings (*Balestri et al.,* 1998; *Domínguez et al.,* 2012; *Piazzi et al.,* 1998) although *Posidonia oceanica* seedlings can also become established on rock/solid substrate (*Alagna et al.,* 2013; *Balestri & Lardicci,* 2008). Previous results, therefore, discourage the planting of seedlings on substrates that are not consolidated (*Infantes et al.,* 2011). A recent laboratory study suggests that close contact of developing roots in the seedling with sand when cultured in aquaria is necessary for better root development and possibly improved anchoring and survivorship when planted at sea (*Guerrero-Meseguer et al.,* 2016).

This guide presents a review of the knowledge generated and published regarding the collection, cultivation and transplantation of *Posidonia oceanica*, and also brings together the experience accumulated by researchers from the Research Group of Ecology of Marine Macrophyte from the Mediterranean Institute of Advanced Studies on *Posidonia oceanica* replanting techniques as a result of two fundamental research projects, and a research and development project. The projects from which data has been taken when putting together this guide are the following:

- '*Técnicas de recuperación y expansión de las praderas de Posidonia oceanica mediante reimplante con semillas*' (116/SGTB/2007/1.3). (Techniques for recovery and expansion of *Posidonia oceanica* seagrass meadows through replanting using seeds as a source). Ministry of the Environment (2007/2009)

- '*Restauración de praderas de Posidonia oceanica con plántulas; Influencia de la luz, nutrientes y herbivoría*' (CTM2011-27377). (Restoration of *Posidonia oceanica* seagrass meadows using seedlings; Influence of light, nutrients and herbivory). Ministry of Science and Innovation (2012/2014).

- 'Use of *Posidonia oceanica* seeds and fragments for the recovery of areas affected by REE's activity'. Red Eléctrica de España (2014/2016).

In the first two projects, work was conducted using germinated seedlings with two main goals: assess the effects that the use of different types of anchorages and substrate have on the survival of the seedlings and evaluate the relative importance of bottom-up processes (availability of resources) and top-down (predator pressure) on the survival and development of transplanted seedlings. These projects were carried out on the coast of the Region of Murcia and on the coast of Majorca.

As part of the framework of the third project, in addition to transplanting seedlings, the project also included the replanting of adult *Posidonia oceanica* plant cuttings with the aim of developing an active restoration method for this species. This R&D+i project was leaded and funded by Red Eléctrica de España as a voluntary initiative coinciding with the end of the construction works for the electricity link between Majorca and Ibiza ('Rómulo 2' project). The electricity connection between the islands consists of a double link of 126 km in length, with a submarine section of 118 km along the seabed that reaches depths of up to 800 metres. The *Posidonia*

oceanica seagrass meadows affected by this submarine link are located in those areas where the cable reaches the land on both islands (Ibiza-Talamanca and Majorca-Santa Ponça) and were chosen as the experimental areas on which the transplanting works were conducted.

This guide seeks to disseminate a protocol for the cultivation and transplantation of fragments and seedlings of *Posidonia oceanica* based on the results and knowledge generated through the various projects carried out since 2007, in order to facilitate the implementation of seagrass meadow restoration projects in those cases in which it is recommendable, for example: as a measure to offset or reduce the impact of actions that produce merely mechanical impacts on seagrass meadows (i.e. without actually causing any changes to the environmental conditions).

In no case can a restoration action be considered as an argument to slack off on the protection and conservation measures for seagrass meadows.

To ensure the correct use of this guide, it should be noted that any Posidonia replanting project based on the guidelines set out herein shall necessarily comply with, and fulfil, all the prerequisites and good practices recommended throughout the guide. In this regard, international standards on ecological restoration should be taken into consideration and no replanting projects shall not be conducted in areas where the threat or impact on the seagrass meadow is still present. Moreover, planting works in areas where there was no seagrass meadow previously should not be proposed (*McDonald, Gann, Jonson, & Dixon,* 2016).

CHAPTER

2

Background regarding the cultivation and transplanting of *Posidonia oceanica*

The cultivation of *Posidonia oceanica* from seeds has been conducted on various occasions and under different conditions, although always at a small scale.

Background regarding seedling culture

The majority of cultivation experiences correspond to aquarium tests using fruits either collected from seagrass meadows, floating on the sea surface during the dispersion phase, or found in beach wrack washed ashore.

The collection of fruits among beach wrack has been the main source of the seeds that have been cultivated until now. The efficiency of the collection will depend on several factors. In certain years and under certain conditions (i.e. after strong windy periods during the season of dispersion of the fruits) it is possible to collect a large number of *Posidonia oceanica* fruits from wrack washed ashore on some beaches (*Balestri & Lardicci,* 2008). In the absence of strong winds or storms, fruits can be collected from wrack washed ashore if the beaches are frequented during the period of dispersion that occurs between April and June.

The experiences carried out to date regarding the cultivation during periods of less than 4 months obtain high germination rates and the subsequent survival of seedlings under different culture conditions.

The experiences carried out to date regarding the cultivation during periods of less than 4 months obtain high germination rates and the subsequent survival of seedlings under different culture conditions. However, survival is reduced when the culture periods are prolonged. *Balestri et al.* (2009) demonstrated that the carbon and nutrient reserves contained in the seeds allow the development of the seedling during the first 6 to 8 months after the germination, and that as of that period seedlings need to incorporate carbon and nutrients from the environment to continue their development. Some of the experiences of cultivation of *Posidonia oceanica* seedlings recorded so far are described on the following pages.

Caye & Meinesz (1989) cultivated seeds obtained from dispersed fruits, floating on the surface of the sea, and washed ashore on beaches. In this experience, seeds were cultured for 42 days. Aquariums equipped with a closed-circuit pumping system and artificial seawater, keeping a laboratory environment temperature of 15-20 °C and using natural light (sun through windows). A series of variables were manipulated during the culture period: the light levels received by the seeds (3% and 77% of incident light), the availability of nutrients (with and without added nutrients) and the arrangement of the seeds (half-buried in sand or suspended in the water). The greatest survival rate (90%) and vegetative development of the seedlings took place under conditions with higher light incidence, use of water not enriched with nutrients and seeds suspended in the water column of the aquarium. Similarly,

Bedini (1997) observed the deterioration and subsequent death of seeds grown in semi-darkness (10% of incident light), while seeds exposed to a light regime of 80% of natural incident light developed normally. This work kept the seeds in culture for a year at a constant temperature of 16 °C in aquariums with natural seawater that was partially renewed every month. Two decades later, *Celdrán and Marín* (2013) demonstrated the importance of the photosynthetic activity of the seeds in the seedling development. It was proven that the photosynthetic activity of the seeds is more active than that of the first leaves of a seedling and is responsible for 29% of the growth of the leaves and 42% of the growth of the roots in the first three months of germination.

Worse survival results were obtained by *Buia & Mazzella* (1991), with a survival rate of the seedlings of 28% after a cultivation period of 8 months. The seeds were collected from material found among beach wrack and cultivated in aquariums equipped with an open-circuit pumped seawater system, using natural lighting and laboratory environment temperature (this work does not detail the ranges of temperature and light reached).

Balestri et al. (1998) cultivated seeds obtained by collecting fruits in beach wrack and cultivated in aquariums (20 L) in the laboratory for two months, using natural and aerated seawater which was renewed weekly. A constant temperature of 16 °C was maintained with a 16-hour photoperiod of white fluorescent lamps (30 μmol m^{-2} s^{-1}). The seeds were suspended in the water column and 88% germinated normally. At the end of the culture period, the seedlings had 3 leaves, 1 primary root and 2 adventitious roots, the maximum length of the leaves was 2.4 cm, the width of the leaves, 3 mm, and the length of the primary and secondary roots, 3 and 3.5 mm, respectively.

The photosynthetic activity of the seeds is more active than that of the first leaves of a seedling first leaves and is responsible for 29% of the growth of the leaves and 42% of the growth of the roots in the first three months of germination.

Belzunce et al. (2008) cultivated the seeds in covered, semi-transparent plastic containers in which a filter paper base was set, accordion-style folded and saturated with sterilised natural sea water. The seeds were placed on the folds. The containers were kept for 4 weeks in a germination chamber at 17 °C with a 24-hour photoperiod and illumination of 30 μmol m^{-2} s^{-1}.

Terrados et al. (2013) collected seeds obtained by collecting fruits washed ashore in the beach and cultivated them for 2-3 months. Aquariums (80 L) were used in a closed-circuit pumped seawater system, using natural seawater, illuminated with 500 W halogen lamps (irradiance> 100 μmol m^{-2} s^{-1}) and with a 12:12 hr photoperiod (light : darkness cycle) at a temperature of 21 °C. The seeds were placed on a bed of fibreglass wool.

Guerrero-Meseguer et al. (2016) cultivated seeds (36 PSU, 21 °C, 100 µmol m^{-2} s^{-1}, 10 h : 14 h dark : light photoperiod) in 10 L aquaria either in sand or in a hard substrate (glass) during four months. After that phase, seedlings were planted in sand in aquaria under the same environmental conditions of previous culturing and monitored for one additional month. Three levels of seed burial were also assayed (none, 0.5 cm –half- and 1.5 cm –complete-). Root development was promoted when seedlings developed in sand compared to glass, and after planting in sand those that were cultured in sand in the previous phase showed an increased development of the root system. Leaf development of seedlings growing in glass was, however, greater than that of seedlings growing in sand. Seedling development was similar among the different seed burial levels.

In addition to the influence of the cultivation time and the incidence of light on the seeds, it has also been observed that a salinity higher than 37 PSU (Practical Salinity Unit) reduces the germination process of the seeds and the development of the seedlings (*Fernández-Torquemada & Sánchez-Lizaso*, 2013) and that increasing water temperatures above 26 °C tends to reduce the foliar growth of four-year-old plants (*Olsen, Sánchez-Camacho, Marbà, & Duarte*, 2012), reduces their leaf development and increases their mortality (*Hernán et al.*, 2017; *Guerrero-Meseguer et al.*, 2017).

A salinity higher than 37 PSU (Practical Salinity Unit) reduces the germination process of the seeds and the development of the seedlings.

Background regarding the cultivation of adult plant fragments

The maintenance or cultivation of *Posidonia oceanica* fragments in aquarium conditions has always been a difficult task that has obtained poor results in most cases.

Meinesz et al. (1991) cultivated vertical shoots in an aquarium for two years. The aquariums had a volume of 750 L and a depth of 0.9 m, and were filled with natural seawater, 1/3 of which was renewed every 4 months. The water was sand-filtered and was also aerated with an air compressor. The temperature oscillated between 18 °C and 22 °C and natural light coming through a transparent cover was used. The rhizomes were planted by inserting them individually in planting pots with sand (3 rhizomes per pot). The length of the rhizomes was 3-4 cm. Mortality was 50 % at 4 months, 59 % at 7 months and 64 % after 24 months. The rhizomes initially maintained their vertical growth habit but gradually changed it to horizontal resulting in elongations of 6-8 cm after two years. All fragments formed new roots 13-16 months after transplantation. The percentage of branched fragments after two years was 68 % and the number of new branches ranged between 1 and 4.

Meinesz, Caye, et al. (1993) indicate that they were able to maintain fragments in the laboratory for 28 months, although they do not describe the aquariums, or the environmental conditions used.

Marín-Guirao et al. (2011) cultivated rhizome fragments with 40-60 shoots collected in seagrass meadows by scuba divers and kept in 1500 L aquariums for two months with a 100 % survival rate. Environmental conditions: closed-circuit recirculation system filled with natural seawater; salinity, 37 PSU; temperature, 21 °C; lighting, 400W halogen lamps (irradiance, 300 µmol m^{-2} s^{-1}); photoperiod, 12h light : 12h darkness. This study demonstrated the negative effects of salinities higher than 38 PSU on the photosynthetic rate of the adult plant fragments of *Posidonia oceanica*.

Balestri et al. (2011) used fragments of *Posidonia oceanica* rhizome collected from among beach-wrack immediately after being washed up on the beach (<7 hrs) by winter storms. The fragments were cultivated in 5000 L tanks in the open air and with full sunlight and filled with seawater that was continuously pumped from the sea using an open-circuit system. The depth of the water was 1.5 m; salinity, 37.5-37.8 PSU and the temperature ranged between 12 °C and 29 °C. The fragments were arranged on plastic grids and suspended 0.5 m below the surface of the water in the tanks. The study considered the cultivation of vertical fragments, with 2 shoots and 10-12 cm of rhizome length, and also horizontal fragments, with 4-5 shoots and 15-17 cm of rhizome length. Additionally, fragments of rhizome with 6-9 shoots were used to produce new fragments, with 1-4 shoots, by using cuttings of the latter.

The overall survival rate of the fragments was 80-97 % at 4 months after their collection, 67-68 % at 12 months, 44-50 % at 22 months and 42 % at 34 months. The first new root was formed 4 months after the collection of the fragments. The fragments produced new roots in spring and summer, and new shoots only in spring. The growth in length of the rhizomes was 0.9 cm $year^{-1}$. The percentage of fragments that had branched was 10-24 % at 12 months, 37-55 % at 22 months and 60 % at 34 months. The number of new shoots produced was 1-2 per fragment, but it was not enough to offset the loss of shoots in the fragments. There was also no relationship to be found between the type of fragment (vertical/horizontal) and initial size of the fragment and its capacity to form new shoots.

Background regarding the transplantation of seedlings

Prior studies on the planting of seedlings indicate that dead *Posidonia oceanica* matte maximises the survival of seedlings and that mobile substrates such as gravel and sand are unsuitable for transplantation. In addition, the vegetative development of the transplanted seedlings on a suitable substrate is comparable to the development reached by the seedlings settled by natural processes. On the other hand, the sufficient availability of light for the seedlings is an essential factor in the success of the plantations, as happens in the cultivation phase.

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Meinesz et al. (1993) experimented with twenty seedlings obtained from aquarium seed culture that were planted on dead Posidonia matte after a 14-month cultivation period in an aquarium. The planting technique is not described, though it is known that the seedlings were planted at a depth of 11 m and had survival rates of 50% after one year, 45% after two years, and 20% after three years.

Balestri et al. (1998) used seedlings obtained through seed culture in an aquarium; fruits were collected among beach wrack. Seedlings, 2 months old, individually wrapped in gauze were fixed to a plastic grid of 25 x 25 cm and with 1 cm square meshing (5 seedlings per grid). Subsequently, the grids are fixed to the substrate (gravel or matte) with four 20 cm metal bars placed in the corners. The anchoring was successful because none of the grids were lost during the three years the experiment lasted. The depth was 10 m. The survival of seedlings on matte was >80% after one year of planting and 70% after three years. The survival rate on gravel was substantially lower (<25% after one year, 0% at three years). The surviving seedlings grew until reaching a rhizome length of 4.5 cm by the end of the experiment; number of leaves, 4.4; maximum length of the leaves, 16 cm, and width, 7 mm. 14% of the seedlings had branched after 3 years. As a contrasting element, the survival of seedlings established naturally in the area was 66% after three years. The rest of the vegetative characteristics were also similar to those of the transplanted seedlings.

Terrados et al. (2013) transplanted 2-3-month-old seedlings germinated in aquarium from fruits washed ashore or collected among beach wrack. A first experiment evaluated the influence of the planting substrate used (dead matte – no leaf canopy-, and matte within

a seagrass meadow ´leaf canopy present-) and level at which planting is made (below/above the surface of the sediment) on the survival and development of the seedlings three years after the transplant. The seedlings were placed individually in plastic grid pots that were anchored to the substrate with two metal bars. A second experiment compared the survival rate after two years of transplanting the seedlings with the described pots and transplanted seedlings without any type of anchoring (seed fully buried). All transplants were conducted in the month of July.

One year after the transplantation works, the survival of the seedlings in dead matte was 75% and 22% in matte found within the live seagrass meadow. Two years later, most of the transplanted seedlings within the seagrass meadow had died. The survival of the seedlings in dead matte was 44%. The level at which planting was performed did not affect the survival of the seedlings and as of the second year of life began to branch out, but only those that were transplanted below the surface of the sediment, 50% of the seedlings, produced between 1 and 4 shoots three years after the transplantation.

The survival of the seedlings two years after transplantation was 37% in the seedlings with anchoring and 26% in the seedlings without anchoring, although this difference was not statistically significant. Some seedlings branched as of the first year of age giving way to 51% of the seedlings producing between 1 and 3 new shoots after two years.

Background regarding the transplantation of fragments

The transplantation of adult *Posidonia oceanica* plant fragments has been, for some time now, the object of various studies and projects in which different techniques and conditions have been tested. Most of these studies obtained the fragments for transplantation directly from live seagrass meadows, this technique is specifically and emphatically discouraged in any case and is currently prohibited by the Berne Convention. *Posidonia oceanica* is catalogued in the list of wild species under the special protection regime of the Spanish Ministry of the Environment, RD 139/2011 of 4 February. Valuable information has been obtained from these studies/projects that can be applied in future plantations, such as collecting the fragments at greater or equal depths to the transplantation area or choosing horizontal fragments.

Both the individual fragment anchoring as well as the use of meshed grids/pots have shown good results if they are secured to the seabed adequately. Most plantations have been conducted between June and November with no clear effect on the survival of the transplanted material, although the bibliography recommends that the planting of fragments be carried out in autumn and spring.

Meinesz et al. (1992) used vertical *Posidonia oceanica* rhizomes collected directly from the seagrass meadow and planted them in June at a depth of 5.3 m on matte. The rhizomes were fixed horizontally in groups of 18 to plastic grids (50x35 cm) with 1 cm square meshing which in turn were secured to the seabed with 6 metal pegs. The impact of the length of the rhizome (7, 12 and 17 cm) and the month the transplantation works were conducted (March, June, September, December) on the survival and vegetative development of the fragments was assessed.

Both the individual fragment anchoring as well as the use of meshed grids/pots have shown good results if they are is secured to the seabed adequately. Most plantations have been conducted between June and November with no clear effect on the survival of the transplanted material, although the bibliography recommends that the planting of fragments be carried out in autumn and spring.

The system used to secure the plastic grids worked well because only 2 were lost. The mortality at 12 months ranged between 3% and 47% without differences being noted regarding the initial size of the rhizome. The mortality of the fragments transplanted in March, June and December had mortality rates of 33%, 23% and 29%, while those transplanted in September was just 5%.

The fragments formed roots only in summer. After one year, between 3% and 69% of the fragments had roots while between 3% and 28% had branched. Those transplanted in September showed the lowest branching (6-28%) and those in June the highest (19-25%). As a result of this study, it is recommended that transplantations of vertical rhizomes (10-15 cm in length) be carried out in autumn (at temperatures below 20 °C) for fragment mortality was lowest.

At present, the direct extraction of fragments from live seagrass meadows to be used later for transplantation is not permitted. Any damage to the meadow is currently prohibited. *Posidonia oceanica* is protected by the Berne Convention and catalogued in the list of wild species under the special protection regime of the Spanish Ministry of the Environment, RD 139/2011 of 4 February.

Molenaar & Meinesz (1992) transplanted vertical rhizomes of *Posidonia oceanica*, collected directly from seagrass meadows found at depths of 3 m and 30 m, and planted them at depths of 3 m, 14 m, 20 m, 29 m and 36 m. The transplants were carried out in August. The fragments were fixed horizontally in groups of 18 to plastic grids (60 x 17 cm) with 1 cm square meshing which in turn were secured with metal pegs to the bottom of *Posidonia oceanica* matte.

Three of the 10 plastic grids were lost, the fixing of the grids failed. Eleven months after planting, the fragments originally collected at depths of 30 m had survival rates of between 93% and 100% when transplanted at depths between 3 m and 20 m, and 72% when transplanted at 36 m. After the same period (11 months) fragments originally collected from shallow seagrass meadows at a depth of 3 m had survival rates between 5% and 41% when transplanted at depths of 14 m and 29 m, and only 3% when transplanted at 36 m.

All fragments originally collected at a depth of 30 m showed a change in their growth habit (going from vertical to horizontal) whereas this did not occur with most of those fragments originally collected at a depth of 3 m (only those transplanted at a depth of 14 m branched off, 44%). The fragments collected at a depth of 3 m did not branch and only one batch (those transplanted at 14 m) produced roots. The fragments originally collected at a depth of 30 m branched more (22-42%) at depths of between 3 and 20 m compared to 36 m (11%). Something similar happened with the formation of roots.

Molenaar et al. (1993) compared the survival and development of three types of *Posidonia oceanica* fragments: vertical rhizomes with 1 apex (leaf-bearing shoot), vertical rhizomes with two apexes (2 leaf-bearing shoots), horizontal rhizomes with 3 apexes (1 terminal and 2 lateral -i.e. with 3 leaf-bearing shoots). The fragments were collected and transplanted in August, at a depth of 14 m, on top of matte and using meshed plastic grids (60 x 17 cm) with 1 cm square meshing, each plastic grid had 18 vertical or 12 horizontal fragments attached.

At present, the direct extraction of fragments from live seagrass meadows to be used later for transplantation is not permitted. Any damage to the meadow is currently prohibited.

Eleven months after the transplantation works the survival of the horizontal fragments was 100%, 49% had formed new shoots (branching), and 97% had formed new roots. In the case of vertical rhizome fragments with two shoots, the percentages were 94%, 8% and 62%, respectively. The survival rate of vertical rhizome fragments with only one apex was 31%, with 9% fragments showing branching and only 14% produced new roots.

Meinesz, Caye, et al. (1993) planted fragments of *Posidonia oceanica* rhizome collected directly from seagrass meadows of different types and origins throughout the Mediterranean. The plantation works were carried out between September and October in different years. They were planted in the same area of dead matte within a protected marine reserve, each fragment individually planted with the help of an anchoring system consisting of a type of metal hook and monitored for two years. Fragments previously cultivated in an aquarium between 4 and 28 months, showed a greater survival rate after their transplantation to the sea (60-100 %) than those collected and directly transplanted (25-80 %). Higher mortality was observed in those fragments that came from depths lower than that of the transplant depth (11 m).

Molenaar & Meinesz (1995) evaluated differences in survival, growth and branching of *Posidonia oceanica* rhizome fragments of different types (vertical, horizontal) depending on the distance between the transplanted fragments (between 5 cm and 20 cm), the spatial arrangement of the fragments (circles of increasing size, parallel lines with the apices facing each other or facing the same direction) and the type of substrate (dead matte, sand without vegetation, sand with *Cymodocea nodosa* (little Neptune grass). The plantation works were carried out in April, July and October without evaluating if the month of planting had any kind of effect.

The transplantation of adult *Posidonia oceanica* plant fragments has been, for some time now the target of various studies and projects in which different techniques and conditions have been tested. Valuable information has been obtained from these studies/projects that can be applied in future plantations, such as collecting the fragments at greater or equal depths to the transplantation area or choosing horizontal fragments.

The results revealed that the survival of the fragments was greater when they were planted at close distances (5-10 cm), in circles or in parallel rows. Transplants performed on sand without vegetation did not survive due to bioturbation (*Pestarella tyrrhena* – mud shrimp).

Augier et al. (1996) describe the results of *Posidonia oceanica* transplants performed using rectangular cement frames that hold the fragments (30-35 vertical fragments obtained from the natural fragmentation of larger pieces) between two layers of wire mesh. During the first two years, the apexes branch out and from then on horizontal rhizomes appear that after ten years have formed small patches of *Posidonia oceanica*.

Piazzi et al. (1998) describe an experiment in which the influence of the site (three locations), depth of origin of the fragment (5, 10 and 20 m) and type of fragment (vertical, horizontal) on the survival and development of fragments collected was evaluated and planted in one of these locations. The length of the rhizome was 8-9 cm in both types of fragments while the number of shoots was 1 in the vertical rhizomes and 2 in the horizontal ones. The rhizomes were fixed in groups of 17 to a plastic grid (unit of transplant) that was anchored to the seabed. The transplant site had a *Posidonia oceanica* meadow in the past. Therefore, dead matte was the substrate in the plantation area. The transplants were carried out at a depth of 10 m between June and September.

The loss of planting units after 3 years was 5 %. No differences were detected in the survival and development rates of the fragments according to the location and depth of origin, but differences were seen depending on the type of fragment. Horizontal fragments had a longer survival rate (76 % versus 59 %), rhizome length (9-30 cm versus 6-20 cm), elongation of the rhizome (70 % versus 22 %), time for the formation of new leaf shoots (5-6 months vs. >12 months), number of branches (9.6 vs. 1.3) at the end of the experiment. 5 % of the fragments flowered at the end of the experiment, but only the vertical fragments produced fruit.

Boudouresque (2001) refers to the work of *Charbonnel, Molenaar, & Gravez* (1995) in which a survival rate of 84 % is achieved for *Posidonia oceanica* fragments four years after they were transplanted in the Bay of Prado (Marseille) and also the number of shoots per fragment duplicated. No data of the technique used is given. It is also commented that spring is the best time for the transplant of fragments of horizontal rhizome giving as reference the doctoral thesis of *Molenaar* (1992).

Balestri et al. (2011) used accumulations of fragments caused by storms (recommended technique) that after being cultivated in open-air tanks for two years are transplanted to the sea during November. The substrate into which they were transplanted is a human-made reef, at a depth of 1.5 m, formed by rubble of calcareous rocks. The survival rate of these fragments, with an average of 3.3 shoots per fragment, was 50 % after one year and 40 % of these survivors produced at least one new leaf-bearing shoot.

CHAPTER

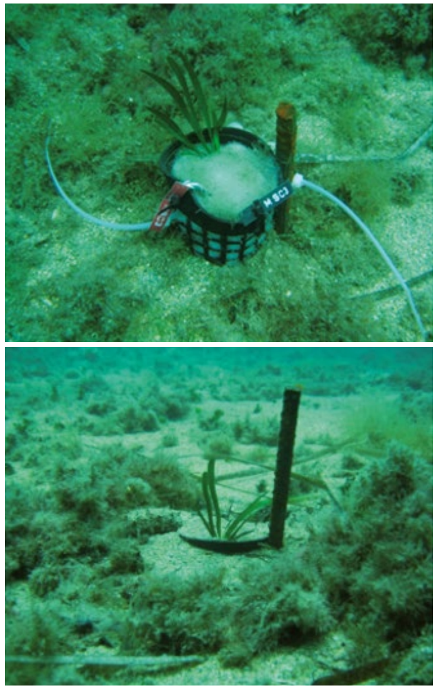
3

Proposed planting technique

Among the variables assessed in the replantation techniques of *Posidonia oceanica*, we can find the types of substrate, the use of human-made anchoring systems or the availability of nutrients in the first implantation years.

The experiences of planting in the project of 'Techniques for the recovery and expansion of *Posidonia oceanica* meadows by replanting through the use of seeds' tested the survival and development of *Posidonia oceanica* seedlings in two types of substrate: on dead matte and in live seagrass meadows. In both substrates the seedlings were secured with the help of a human-made anchoring system consisting of a plastic pot with fibreglass wool as a substrate, fixed to the seabed by means of iron bars. Two different planting

Dead matte (absence of foliar cover) is the most suitable substrate for the replanting of *Posidonia oceanica* seedlings, and the use of added anchoring systems was ruled out.



Close-up images of how seedlings are planted on matte: anchored on top of the substrate allowing the incidence of light on the seed (up), anchoring and buried within the substrate preventing the incidence of light on the seed (down).

levels were used in both the matte substrate and in the seagrass meadow: one directly on the substrate allowing the incidence of light on the seed and another one encased within the substrate.

On the other hand, the differences in the survival rates of the seedlings were tested when a human-made anchoring system was used to secure them to the seabed and when they were planted without the need for added anchoring structures.

The seedlings transplanted directly on the matte substrate without anchoring branched better and developed more and longer leaves.

Close-up image of anchoring system of seedlings on matte substrate: without anchoring (up), with human-made anchoring (down).

Two years after the transplanting works, no differences were found in survival rates between the seedlings secured by human-made anchoring methods and those that used natural anchoring.

As a result of these experiences, it was determined that the dead matte (absence of foliar cover) is the most suitable substrate for the replanting of *Posidonia oceanica* seedlings and the use of added anchoring systems was ruled out, as these did not provide significant improvements in the survival rate of the planted items. The plantation on sand substrate had already been tested in own pilot experiments (*Infantes et al.*, 2011) which resulted in the loss of all the material planted at depths greater than 20 metres.

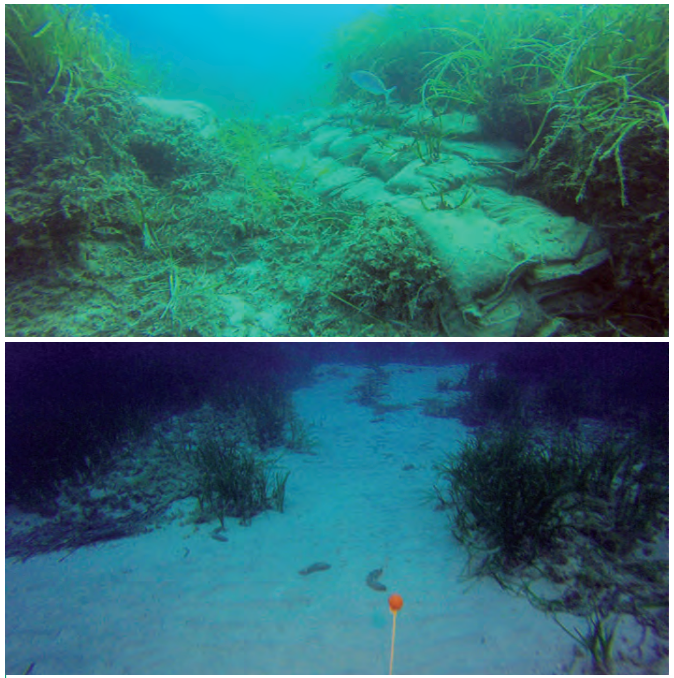
Subsequently, the project 'Restoration of *Posidonia oceanica* seagrass meadows using seedlings; Influence of light, nutrients and herbivory' evaluated whether increasing the availability of nutrients in the first years following the planting of a seedling would benefit survival or development rates. On the other hand, the impacts of herbivory on the development of transplanted seedlings was tested.

Close-up image of seedlings with a natural concentration of environmental nutrients (up) and seedlings with enriched concentration of nutrients (down).

Seedlings planted in an environment with added nutrients developed a greater number of leaves which were both wider and longer. Nevertheless, the added nutrients appeared to reduce survival rates.

The technique used in the R&D+i project 'Use of seeds and fragments of *Posidonia oceanica* for the recovery of areas affected by REE's activity' and described in detail in this guide was tested on two types of substrate near a seagrass meadow of *Posidonia oceanica:* sand and consolidated sand with gravel sacks. Sand is the natural substrate that was disturbed after cable laying works of electrical interconnection between Mallorca and Ibiza.. As already mentioned in the background regarding transplantation works, unconsolidated/mobile sediment substrates are not suitable for the survival of seedlings. On the other hand, there was a need to recover the profile of the seabed in those sections where the trench of the cable generated a slope in the seagrass meadow matte to avoid the collapse and the loss of greater surface of seagrass meadow; this led to the installation of burlap sacks filled with gravel to stabilise the profile. These bags of biodegradable material, filled with gravel, constitute the second substrate on which the plantations were carried out.

The steps necessary to implement the planting technique developed for the R&D+i project 'Use of seeds and fragments of *Posidonia oceanica* for the recovery of areas affected by REE's activity' are described in detail on the following pages.



Close-up image of sandy substrate and consolidated substrate with burlap sacks filled with gravel to provide anchoring support to the plants.

72%

Survival rate of the adult plant fragments one year after the transplant

20%

Of fragments generated new leaf-bearing shoots one year after plantation

80%

Survival rate of transplanted fragments on a consolidated substrate using gravel sacks (60% obtained on sand sacks)

CHAPTER

4

Phases prior to the planting works

The selection and characterisation of the area, the permitting process, collecting the material for planting, and the preparation and transport of the material for planting are some of the operations to be fulfilled before the replantation.

Selection and characterisation of the area

Firstly, we must ask ourselves what objectives we are seeking with these replanting projects, which will help us to narrow down the scope of the project.

The area to be replanted must be selected, delineated, characterised and documented. Selecting zones that already have some form of legal environmental protection can speed up the necessary documentary processing for the transplanting works. All historical data regarding the area to be restored and the causes that led to the loss of the seagrass meadow concerned (which is the object of the replanting works) must be known. It does not make sense to start a restoration project of this type if the causes of the impact are still present or will be present again in the future; nor does it make sense if the areas affected have not recovered after the initial impact. The actual biotic community in the area concerned serves as an indicator of water quality and the availability of light. The presence or absence of seagrass meadows or patches of *Posidonia oceanica* in good condition in the vicinity is proof of the adequacy of the environment for the development of Posidonia. It is recommended that these observations be supplemented with direct measurements regarding the availability of light and characterisation of the sediments (organic matter content and granulometry) in the area to be replanted.

When performing the characterisation of the area that is the focus of the project prior to the start of the replanting project, the following should be considered;

1. Environmental conditions

Type	Reference values	Observations
Interstitial water quality	NO_3+NO_2+NH_4 - 300-645 μM PO_4 – 9 -43.4 μM H_2S <10 μM	Annual average, measurements taken in the first 5 cm of sediment (*Alcoverro, Duarte, & Romero*, 1995; *Alcoverro & Romero*, 1997); (*Calleja, Marbà, & Duarte*, 2007)
Light availability	>338 $\mu E\ m^{-2}\ s^{-1}$	Measured in summer at the depth of the transplanting site. (*Gacia et al.*, 2012; *Serrano, Mateo, & Renom*, 2011)
Type of substrate	Posidonia matte	
Characterisation of sediments: Organic matter	< 5%	(*Cancemi, De Falco, & Pergent*, 2003; *Invers, Pérez, & Romero*, 1995)
Characterisation of sediments: Granulometry	Sand and silt. No mud	
Bathymetry	0-40 metres	According to the spatial distribution of the seagrass meadows in the area to be replanted
Surrounding biotic community	Native community of photophilic algae	No proliferation of invasive species. It is recommended that the presence of *Caulerpa prolifera* and *Caulerpa racemosa* (both species of green algae) be limited (*Holmer, Marbà, Lamote, & Duarte*, 2016)
Hydrodynamics	Hydrodynamic situation re-established if it had been modified	

2. Other conditioning factors

- Replanting shall only be considered in areas where Posidonia has existed previously and preferably where it is still established naturally, even if it has suffered some type of impact.

- Replanting shall only be considered in the absence of impacts. When the causes that generated the impact on the seagrass meadow have ceased and under the expectation that they will not reoccur.

- The management of potential future impacts on the replanting area shall be identified and planned.

- It is necessary to implement a programme for monitoring the survival of the planted items. Survival rates should be assessed at least once a year during the years following the planting works, and the ideal scenario is that monitoring will continue for several years after the planting works. The planting of Posidonia is not a valid objective in itself; the goal is to help its recovery. Therefore, it is necessary to assess the success of the planting project.

The presence or absence of seagrass meadows or patches of *Posidonia oceanica* in good condition in the vicinity is proof of the adequacy of the environment for the development of Posidonia.

- Monitoring the recovery of the biotic community associated with the transplanted seagrass in the years following the planting works is desirable but not mandatory.

Permitting process

Prior to the start-up of a *Posidonia oceanica* replanting project, the appropriate permits from the competent administrations need to be processed, as required. If the replantation is going to take place in Spain in this respect it is necessary to consider the following:

- The Environmental Administration, normally belonging to the autonomous community concerned, that is responsible for the protection of the *Posidonia oceanica* seagrass meadows.

- Coastal Authorities, as the entity responsible for authorising projects that involve occupation of the seabed. A replanting project may require this permit both for replanting work itself, if the planting uses a human-made anchoring system, and for the protection of the replanting area with buoys or equivalent.

- The administrations or entities affected regarding the area selected for replanting, namely: SCI (Site of Community Importance), port area, military zone, area affected by airport activities, marine reserve, etc.

Collecting material for planting

As already mentioned, among the species whose habitat is protected by law, *Posidonia oceanica* is also a protected species and the collection of plant specimens, either adult plants or seeds, is prohibited by the Berne Convention, it is also a species covered by a Special Protection Regime according to national legislation (RD 139/2011 of 4 February). Therefore, according to Law 42/2007, of 13 December, on Natural Heritage and Biodiversity, it is expressly prohibited to 'collect, cut, mutilate, uproot or intentionally destroy' *Posidonia oceanica* as it is a species included in the List of Wild Species covered by a Special Protection Regime. Therefore, collection works prior to a replanting project must be justified before the appropriate competent environmental authority, as required, and the collection works must be formally authorised. The collection of drift fragments generated through natural processes, such as storms, is recommended as a method of obtaining the planting material.

The collection of fragments:

Where: Recommended areas for surveying are clearings in simple meadows with substantial patch formation or 'return river' meadows.

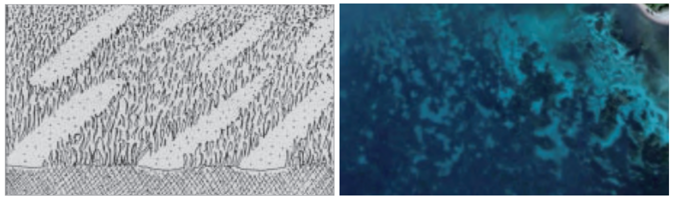
How: Divers collect from accumulations of drift fragments found in clearings of patch meadows.

Fragment type: horizontal growth habit with at least two vertical shoots

When: In autumn/winter after a storm.

Collection of *Posidonia oceanica* fragments:

Posidonia oceanica seagrass meadows can generate diverse underwater seascapes, ranging from simple meadows with smooth bathymetries to hilly meadows, micro-atolls/reefs, or tiger meadows among others (*Boudouresque et al.,* 2006). All of them interspersed with areas without live Posidonia - areas of matte - normally covered by a layer of sand and which may or may not be colonised by faster growing macrophytes. The frequency and size of these areas vary according to the type of meadow and are the areas of natural accumulation of fragments naturally uprooted from the meadow by the effect of storms. Gaps in mature hilly meadows, simple meadows with substantial patch formation or 'return river' meadows are suitable sites for the collection of material.



Close-up image of patch and 'return river' seagrass meadow morphologies (Source: Boudouresque et al., 2006).

The collection of material is carried out manually by scuba divers. The collection of fragments of horizontal growth habit with at least two vertical shoots should be prioritised, which will maximise survival during the cultivation and planting works of the material (*Molenaar et al.,* 1993, *Piazzi et al.,* 1998).

It is recommended that the collection process be undertaken in winter when the incorporation of nutrients through the leaves is at a maximum level (*Alcoverro, Manzanera, & Romero,* 2000) although it is also possible to carry out collection works during the autumn and spring seasons depending on the wave and weather conditions of the year.

Image showing the collection from a natural accumulation of fragments (left). Close-up image of the standard type of fragment used in the plantation (right).

Collection of *Posidonia oceanica* seeds:

The *Posidonia oceanica* seed collection season is restricted to the last months of spring and is irregular, as flowering does not occur every year (*Balestri*, 2004; *Díaz-Almela et al.*, 2006). In warmer and/or shallower areas, fruits will ripen sooner (*Buia & Mazzella*, 1991), so the fruit collecting season will change according to the Mediterranean area where the replanting project will take place. The collection period could take place between mid-April to the end of June.

The collection of seeds:

Where and how: Sifting through beach-wrack and washed ashore on beaches which due to their orientation have been favoured by winds blowing inland. Equipped with a container where the wet seeds can be stored.

When: At the end of spring and beginning of summer, depending on the area of the Mediterranean, the collection period can vary between the end of April and the middle of June.

Images of seeds among beach-wrack and the seed collection process.

Image of damaged seeds not suitable for culture.

The collection is carried out by sifting through beach-wrack where there is a natural accumulation of mature fruits that, after having detached from the plant, drift to the coast due to wind currents. These fruits will be viable for less than 24 hours once washed ashore on the sand, so they are considered fruits that can no longer sustain existant meadows or form new ones. As the fruits float and are scattered by the wind, the beaches where the collection efforts must be concentrated must be chosen according to the wind conditions during the 12-24 hours prior to collection.

General conditions for the cultivation of fragments and seeds

Both for adult plant fragments and for seeds, it is necessary to have a continuously flow of seawater, which helps to maintain natural salinity (around 37 PSU), this reduces the proliferation of algae and infections in the planting material. In the case of seedling cultivation, if the continuous flow of seawater is not available, the culture facility can be run in a closed-circuit pumped seawater system, in which case it will be necessary to correct the salinity level of the water on a weekly basis and renew entirely the aquarium water every month. If the water flow is sufficient, it will not be necessary to have a water aeration system. Filtering the water and applying ultraviolet radiation before introducing the water will help maintain water quality and reduce the proliferation of algae and infections. In any case, it is recommended to maintain the water temperature between 20º C and 22º C and always below 24º C.

As part of the maintenance tasks during the cultivation phase, the fragments and/or seedlings will be inspected every 2-3 days to remove fragments and seedlings from the aquariums that are deteriorated or dead and thus avoid the spread of infections to healthy material. The growth of epiphytic algae will also be controlled, which can negatively affect the development of the fragments and, especially, of the *Posidonia oceanica* seedlings. The epiphytes will be removed from the leaves manually to avoid damaging the leaves. In addition, invertebrates (e.g., gastropods, isopods, amphipods) that could have been introduced into the cultivating system and are potential predators of *Posidonia oceanica* will be eliminated manually whenever possible. The control of predators in the cultivation of seedlings is simple, as it is not frequent to find fauna that is alive in the fruits washed ashore or collected after marine storms. However, fragments will generally be accompanied by a countless number of invertebrates lodged between the leaves and rhizomes. Given that seedlings and seeds are normally preferred by herbivores rather than the adult plant, it is recommended that the cultivation of seedlings and fragments does not occur in the same controlled environment.

The cultivation phase of fragments in projects in which large quantities of plants are handled can be eliminated, thus lowering the costs of the project. However, it is a recommendable stage – as it allows fragments that become sick or weak to be eliminated/discarded.

The seedling culture phase is necessary to allow time for germination and root development.

Type of material	Water flow	Temperature	Salinity	Light	Density
Fragments	Open	20-22 °C	37 PSU	300 μmol $m^{-2} \cdot s^{-1}$	350 leaf shoots·m^{-2}
Seedlings	Open Closed	20-22 °C	37 PSU	64-89 μmol $m^{-2} \cdot s^{-1}$	160 seedlings·m^{-2}

Cultivation of adult plant fragments

Although not essential, the cultivation of fragments prior to planting is a recommendable stage, as it acts as a screening process for plants in poor condition. Irradiance conditions around 300 µmol m^{-2} s^{-1} will be sought at the level of the fragments to ensure the light saturation point of photosynthesis (*Alcoverro, Manzanera, & Romero,* 1998; *Gacia et al.,* 2005) with a photoperiod (light/darkness cycle) according to the time of year of the cultivation. Alternatively, fragments could be grown outdoors, using sunlight as a source of illumination (*Balestri et al.,* 2011), but the increase in temperatures in the summer months should be monitored.

The presence of a substrate was not necessary for the cultivation of the adult plant fragments since they can be cultivated using plastic grids or trays suspended below the surface of the water. The average density during the cultivation stage that was used was about 350 shoots per square metre, equivalent to the shoot density of a low-density seagrass meadow (*Giraud,* 1977), and that is equivalent to approximately 100 fragments per square metre (3-4 shoots per fragment).

The minimum time for the cultivation of fragments prior to the planting of seedlings ranges between 6 and 8 weeks from germination to allow sufficient time for the development of roots.

The control of the fauna that may have been introduced into the cultivation system with the fragments can cause difficulties, especially if the cultivation period is prolonged over time. In case of proliferation of gastropods, manual elimination is possible. If on the contrary there is an increase in the abundance of crustaceans or polychaetes, it is possible to try to introduce some type of natural predator into the environment at a density appropriate to the volume of the cultivation environment being used, although we cannot assure its effectiveness based on our experience in the cultivation process.

Cultivation of seedlings

In the case of seeds, the cultivation phase is essential to allow the germination and the development of roots of the seedling that will favour the success of the planting works. The minimum time of growth and development prior to planting seedlings is estimated at between 6 and 8 weeks from the time of germination in order to allow time for the development of the root system. The growth and development of seedlings can be done in aquariums of different sizes, the seeds should be separated from each other by approximately 7-10 cm (maximum density approximately 40 seedlings * 0.25 m^2), with light availability greater than 64-89 µmoles of photons *m^{-2}*s^{-1} to ensure the light saturation point of photosynthesis (*Hernán et al.,* 2016, 2017). Alternatively, the seedlings could also be cultivated in open waters in trays,

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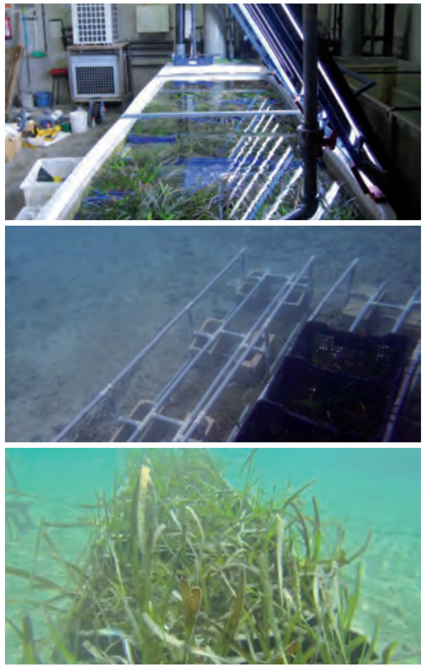
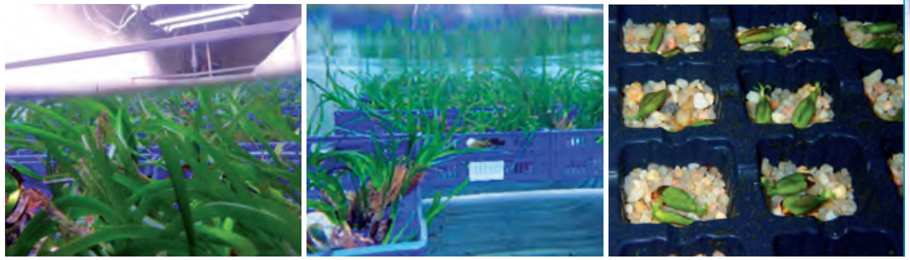


Examples of aquarium facilities for the cultivation of fragments: continuous seawater flow and in trays/baskets in open waters.

but it is probably necessary to use shading mesh and the increase in temperatures in the summer months should be monitored.

A gravel or sand substrate in contact with the seeds during germination will favour the development of vertical roots that will increase the resistance of the plantation sites to hydrodynamics (*Balestri et al.,* 2015; *Guerrero-Meseguer et al.,* 2016), provided that the plantation is executed on consolidated substrates (*Infantes et al.,* 2011). The seeds are photosynthetically active, so the incidence of light on the seeds is essential for the germination and development of the seedlings, the seeds must not be buried (*Celdrán & Marín,* 2013) during the cultivation phase.



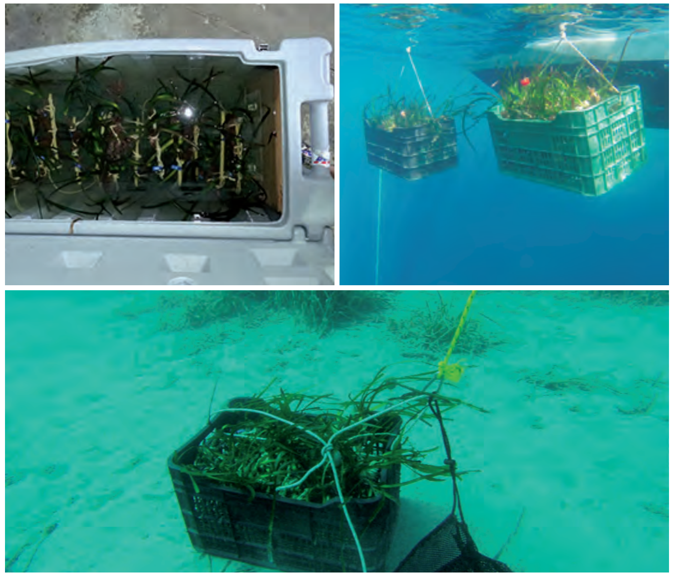
Bottom right: close-up image of seed culture in gravel. The two images at the left: close-up image of a facility for the cultivation of fragments.

Preparation of material for planting

The adult plant fragments need to have their anchorages to the substrate strengthened before they are planted. In this regard, each fragment will be previously attached to an iron stirrup (*Verduin & Sinclair,* 2013) whose central part is covered with beeswax so that the corrosion of the metal does not alter the environment of the roots of the fragments. The dimensions of the stirrups will be 20 centimetres in the central part and 25 centimetres in the legs. It is recommended that the system for tying and attaching the fragments be carried out using some type of flexible material (e.g. biodegradable rope) rather than a rigid system (e.g. adjustable plastic clamps) to prevent breakage of the plant material at the friction points. The materials of the tying system must be resistant to immersion in salt water for several years. Note: Seedlings do not need preparation prior to planting.

Top: close-up image of anchoring system of fragments during the testing phase of the planting technique. Centre: fragment planted in Posidonia matte.

Botton: image of a fragment plantation site in a linear arrangement on consolidated substrate using burlap sacks filled with gravel.



Close up of fragment transportation on land and underwater.

Close-up images of seedling transportation.

Transport of planting material

The transportation of seedlings to the plantation area does not entail difficulty. It will always be done with the plants submerged in seawater, either in a rigid container or in reclosable zipper bags, the latter have the advantage that they can be used directly to transport the planting material when the divers carry out the planting works on the seabed.

Transporting *Posidonia oceanica* fragments to the planting area implies greater difficulty. The adult plant fragments, like the seedlings, must be transported submerged in seawater, but fragments are more voluminous, delicate and are tied to rigid iron stirrups. For this reason, it is convenient to have a transport system in which the fragments can be adequately organised and remain relatively immobilised until the planting area is reached.

CHAPTER

5

Phases during the planting process

Posidonia planting involves labelling the seedlings or the fragments, controlling the siting and layout, monitoring the survival and the development as well as taking measures to reduce bioturbation.

Labelling

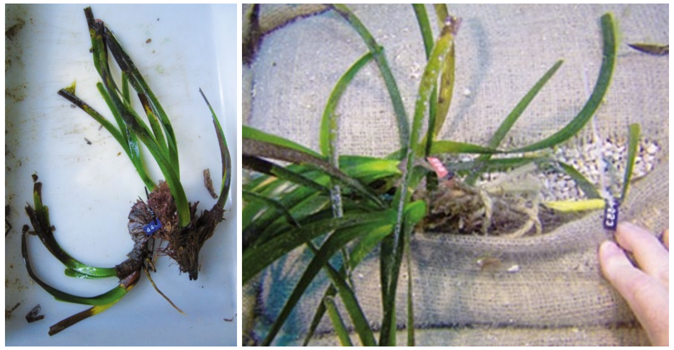
To describe the detailed evolution of the plants, it will be necessary to characterise the seedlings or fragments, or a representative portion of material that will be defined according to the dimensions of the project, before planting works take place and keep an identification record of the individual plants for subsequent monitoring. It is recommended that the identification of the seedlings be conducted by location without adding any physical labelling to the seedling, which would most likely cause damage to the seedling (tissue breakage). The location of each seedling will be identified with a coordinate system: e.g. the seedling in first position 'A1' will be located in 'row' A and 'column' 1, the seedling 'A2' (located to the right of the previous one) will be in the second position of the 'row' A (see diagram below). This method will remove the need for adding physical labelling to the fragile structure of the seedlings. For this method to work, the way to mark each group of transplanted seedlings should indicate, not only the location and number of the group, as already mentioned, but also the way in which the monitoring process should be carried out.

It is recommended that the identification of the seedlings be conducted by location without adding any physical labelling to the seedling, which would most likely cause damage to the seedling (tissue breakage).

A1	A2	A3	A4	A5
B1	B2	B3	B4	B5
C1	C2	C3	C4	C5
D1	D2	D3	D4	D5
E1	E2	E3	E4	E5

Table for the identification, by seedling position, for monitoring works after the planting process. A clearly identifiable stake or marker, represented by a red dot, will be placed to indicate the correct side where to begin the monitoring of the group of seedlings.

In the case of fragments, labelling with adjustable plastic clamps and labels with relief printing (ink printing is not to be used) is recommended. The clamps will be tied to the stirrups and identification can be reinforced with a secondary labelling (always respecting the same numbering) on the rhizome of the fragment. The reason for double labelling is to ensure that we do not lose the identification of fragments to be monitored. In case of placing a second label on the fragment, it will be placed on the rhizome segment between a group of apical shoots and the first vertical leaf-bearing shoot of the fragment and the clamp will fit loosely around the rhizome. Maintaining this position will enable us to know if new shoots have been generated from the apical group in successive follow-up monitoring processes. Light coloured labels (e.g. yellow) are easier to detect and read underwater. It is recommended to avoid greenish, bluish or brown colours for labelling.

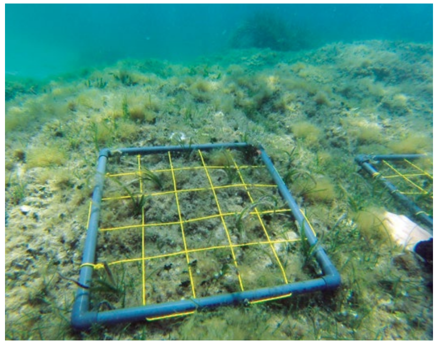


Close-up image of labelling on a fragment and double labelling on a planted fragment.

Siting and layout

The planting of seedlings and fragments should be carried out on dead *Posidonia oceanica* matte. In the case of fragments, the distance between them must ensure that the stirrups are not obstructed and allow an individualised monitoring of each fragment. In the plantation described, the distance between fragments was 20 centimetres. Previous studies indicate an increase in survival at distances of 5 centimetres between fragments (*Molenaar & Meinesz,* 1995) although plantings at distances of 20 centimetres between fragments also offer good survival rates. Regarding seedlings, it is recommended that a distance of between 5-10 centimetres be kept between seedlings.

Close-up image of planting plot of seedlings on matte substrate.



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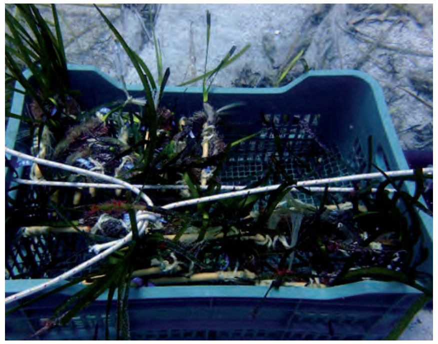
The seedlings are transplanted manually. This process is best helped by using one's fingers so that the roots of the seedling get lodged in the substrate. Once placed, gently press on the substrate around the seedlings to increase root contact and stimulate growth.

The planting of the fragments attached to the stirrups will be done by pressing down on the stirrup. Depending on the difficulty of penetration of the substrate, tools can be used that increase the force exerted (hammer), always using it exclusively on the stirrup. In no case shall the fragment receive any direct pressure. It should also be ensured that the roots of the fragment remain in contact with the surface of the substrate.

The layout of the planted material (seedlings or fragments) can have a linear or grid arrangement. To facilitate the monitoring of the planted material it is recommended that each group of plants is marked with stakes or buoys, the number of plants in each group should also be indicated. Each group of plants must be identified by a numbered label or other agreed code. To monitor the survival and development of the planted material, it will be necessary to individually identify the total number of seedlings or fragments or a representative portion of the total (e.g. 20%) in the case of larger-scale replanting works.

To monitor the survival and the development of the planted material, it will be necessary to individually identify the total number of seedlings or fragments or a representative portion of the total.

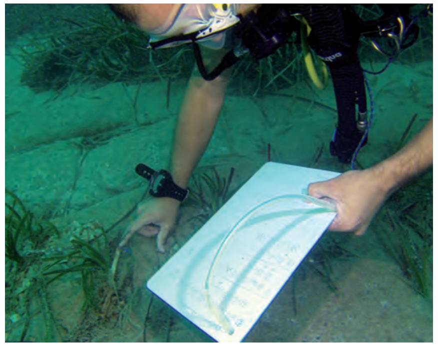
A methodical organisation of the material to be planted is recommended before starting the dive in order to reduce immersion times and avoid errors under water. The fragments or seedlings must be previously grouped in planting sets. An adequate transport system will be of great help (see the section on transport of planting material). One possibility is reclosable zipper bags for the seedlings, with one bag for each set of planting material. Also, plastic crates of 40-50 cm deep with a plastic grid with 1 cm square meshing adapted to half the height



Close-up image of a plastic crate for the orderly transport of fragments during dives.

of the crate, where the legs of the stirrups are inserted and allow the fragments to be placed and transported in an orderly fashion. During the dives for the planting works or for monitoring, a clipboard with the diagram/layout of the plantation should be taken and, as appropriate, with the number of plants that should be found in each group. This will be of great help and will speed up the work under the water.

Close-up image of a clipboard for the orderly monitoring of the plantation.



Efforts should be made to ensure that the planting groups are equivalent, by mixing fragments of different sizes, different collection lots (by donor seagrass meadows or date of collection) if there are any, and to avoid grouping together the larger fragments or those from the same origin.

Close-up image of a planting plot with the arrangement of fragments in sand (top) and on consolidated substrate using gravel sacks (botton).

Characterisation and monitoring

The descriptors that can be recorded before the planting works take place in order to conduct a detailed monitoring of the fragments over time are: the number of shoots per fragment, the number of leaves per shoot, the length of the longest leaf of each shoot, the width of the second youngest leaf of each shoot, the number and type of bite marks due to herbivores per shoot, the length of the rhizome between the apical group and the last vertical shoot. If the fragment has more than one branch, the measurements are taken for each branch. The minimum measurements to be registered prior to planting are the following: number of leaf-bearing shoots of the fragment (including the apical group and the vertical shoots), number of leaves per shoot and length of the rhizome between the apical group and the last vertical shoot.

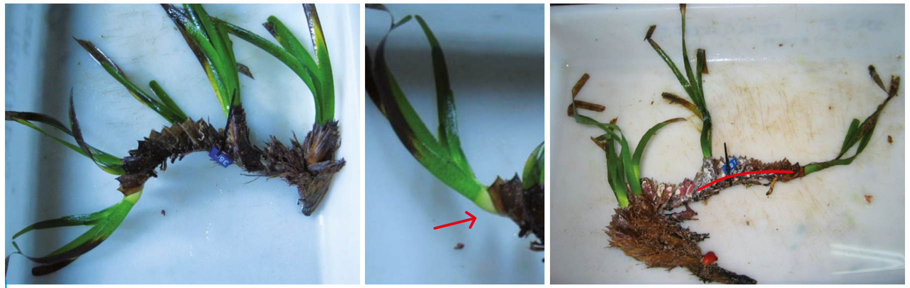


Photo of fragment for planting with apical group and 4 vertical shoots (left).

Close-up image of apical group. The base of the leaf (sheath) is indicated from where the length of the longest leaf begins to be measured (centre).

Close-up image of the measurement of the rhizome length between the apical group and the first vertical shoot (right).

The seedlings descriptors that can be recorded before the planting works begin are: length and width of the seed, number and length of all the roots, number and length of all the leaves, width of the leaves. The minimum measurements to be registered prior to planting are the following: length and width of the seed, number of leaves of the seedlings and number of roots.

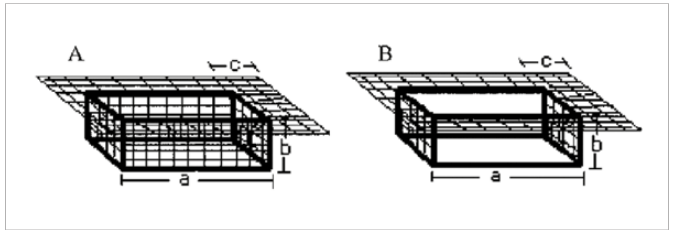
These descriptors will provide information regarding whether the planted material (seedling or fragment) is developing new leaves or shoots, and to see if the rhizome is growing. The measurement of herbivore bite marks is not necessary, but it is recommended, especially in plantations that may be compromised by herbivory.

In the monitoring process following the planting works, the same measures can be maintained, except for measurements on rhizome and roots. In the case of the seedlings if they have branched out, the measurements for each branch will be taken.

Descriptors allow us to know if the planted material (seedling or fragment) is developing new leaves or shoots besides checking if the rhizome is growing.

Measures to reduce bioturbation

In plantations of seedlings that are easily uprooted, it is recommended to take measures to avoid the effects of bioturbation. For example, by installing cages or grids with meshed openings of less than 2 cm square, around the groups of transplanted seedlings will discourage the access of crawling benthic organisms (e.g. sea cucumbers, starfish...). According to *Alves, Chícharo, Serrao, & Abreu* (2003) if an additional meshed grid is installed horizontally or with a negative inclination over the meshed cage (see diagram below), access by macroinvertebrates will be made even more difficult.



Detailed image of macroinvertebrate exclusion system (A) and of enclosure designed for controlling a possible cage effect (B). Source: Alves et al., 2003.

Close-up image showing bioturbation: sea star dragging plantation seedlings.

CHAPTER

6

Financial cost of the planting technique

The financial cost set out herein has been established based on the experience related to the contracting of the R&D+i project 'Use of seeds and fragments of *Posidonia oceanica* for the recovery of areas affected by REE's activity' and the Red Eléctrica Marine Forest restoration project (more information http://www.ree.es/en/).

Currently, the technique outlined in the guide is in its infancy or introduction stage, so it is expected that if it advances into its mature stage the unit costs detailed below will decrease considerably.

The following table breaks down the estimated price per item planted, which varies according to the total area planted; bearing in mind that the distribution of planted areas is uniform over the entire surface and the density of planting is constant (i.e. 1600 seedlings or fragments per ¼ hectare). The cost includes materials, human and technical resources and associated logistics, and is broken down by fragment and seed.

Activity	Item cost (€) / 2,500 m²	Item cost (€) / 10,000 m²
Fragments planted	€ 26.68	€ 12.40
Seed units planted	€ 22.16	€ 8.17

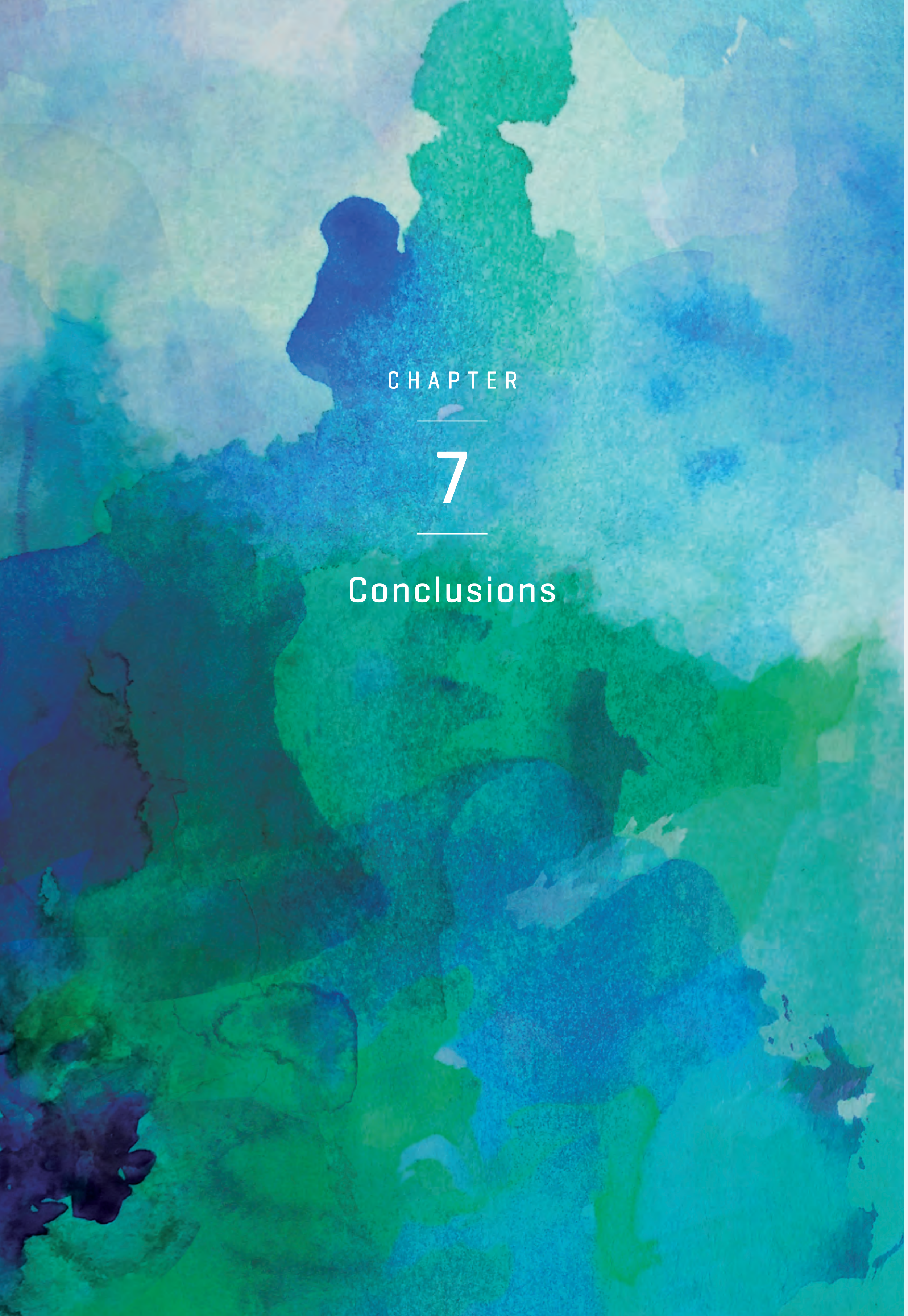
The greater the scale of the planted area can represent a cost reduction:

46% IN FRAGMENTS

36% IN SEEDS

The costs are estimated for a fieldwork campaign in which the surface area indicated in the table is planted. The greater the scale of the planted area can represent a cost reduction of 46% in fragments and 36% in seeds.

Taking the Red Eléctrica Marine Forest as a reference (two hectares of area planted), in the case of planting 2,500 m² / campaign, the total cost would be 341,504 euros for fragments and 283,648 euros for seeds. By contrast, in the case of planting 10,000 m² / campaign, the total cost would be 158,720 euros for fragments and 104,576 euros for seeds.



CHAPTER 7 Conclusions

The technique described in this guide gathers much of the knowledge generated so far on *Posidonia oceanica* transplantation and applies it in a technically and financially feasible methodology.

The economic value of the ecosystem services that one hectare of seagrass meadow of *Posidonia oceanica* represents and that could be valued from a monetary point of view, has been estimated at somewhere between €284-514/ha/year, which implies that the economic value of goods and benefits provided by *Posidonia oceanica* seagrass meadows ranges between 25.3 million and 45.9 million €/year (*Campagne et al.,* 2015). Added to this value is the valuation of non-market ecosystem services which, because they are not translated directly or indirectly into consumer goods and are not known to the public, are difficult to translate into monetary units (*Börger & Piwowarczyk,* 2016). According to the calculation of cost per item planted, based on the results of the project, stands at 8.17 - 22.16 euros/seedling and 12.40 - 26.68 euros/fragment. The cost of replanting one hectare of *Posidonia oceanica* will depend on the planting design and the desired density of items.

Posidonia is a very slow-growing plant, and the outcome of the restoration measures will not necessarily give way to a meadow of dimensions equivalent to the seagrass meadow affected by the work for several centuries.

This technique can be part of measures that restoration actions that generate merely mechanical impacts (i.e. low modification of the environmental conditions, minor change of substrate features), and can be applied using two possible approaches. On the one hand, the substrate can be consolidated to facilitate the natural recolonisation of *Posidonia oceanica,* which has been proven to be possible once the environmental conditions are restored (*Bryars & Neverauskas,* 2004; *Di Carlo, Badalamenti, Jensen, Koch, & Riggio,* 2005). On the other hand, active replanting measures can be taken to accelerate the recovery of the affected seagrass meadow. In any case, we must not forget that it is a very slow-growing plant (*Marbà et al.,* 1996) and that the outcome of the restoration measures will not give way to a meadow of dimensions equivalent to the seagrass meadow affected by the work for several centuries (*Kendrick et al.,* 2005).

It is essential to emphasise that the success of the results of replanting works using the described technique has been assessed in terms of the short-term survival rate. This is a frequent feature in restoration projects of marine ecosystems, however, restoration projects must, by definition, include a method by which to measure the recovery of functions of the restored ecosystem, in terms of diversity, structure and processes (*Ruiz-Jaen & Aide,* 2005). If the measure of the success of the replanting projects is based for the moment on the survival of the planted items, it is because the restoration of coastal ecosystems is still in an initial phase that needs to evolve towards monitoring and assessment measures based on the recovery of the biotic community associated with the seagrass meadow and the ecosystem processes and services involved.

To view the video please scan this QR code

The purpose of this guide is to collate and analyse the main studies carried out on the collection, cultivation and transplantation of *Posidonia oceanica* written to date. The guide incorporates the experience obtained after carrying out the R&D+i project 'Use of seeds and fragments of *Posidonia oceanica* for the recovery of areas affected by Red Eléctrica de España's activity', and it is expected that in the coming years it will be expanded on to include the experience obtained through the 'Red Eléctrica Marine Forest' project.

This practical guide for the planting of *Posidonia oceanica* primarily aims to disseminate the techniques described and encourage their implementation in different degraded areas present in the Mediterranean. It is the desire of the entire team that worked on this project that this planting guide be reproduced and enriched through the addition of other experiences so as to identify aspects that can be improved and to optimise efficiency in the planting of *Posidonia oceanica* thus contributing to the conservation of a unique habitat of high ecological value.

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