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GROWTH PERFORMANCE AND PHYSIOLOGICAL TRAITS OF *POSIDONIA OCEANICA* EXPOSED TO A HYPERSALINE ENVIRONMENT

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1. Introduction

The endemic seagrass *Posidonia oceanica*, widely distributed along the coasts of the Mediterranean Sea, is extremely sensitive to both low and high noise levels often associated with human and natural factors, which are able to determine changes in various environmental parameters, such as temperature, salinity, light and pH (Procaccini *et al.*, 2003; Boudouresque *et al.*, 2006; Ralph *et al.*, 2006; Marbà, 2010). Environmental changes, although moderate, are important, because they determine the structure, function and distribution of seagrass communities (Ruiz *et al.*, 2009).

P. oceanica forms dense meadows between the surface and depths of 30–40 m, constituting the *climax* stage of infralittoral zones (Ruiz *et al.*, 2009). These meadows are key habitats for the functioning of the Mediterranean coastal ecosystem, and their loss can result in complete devastation of the associated biological community and associated ecological services, entailing severe socio-economic consequences (Boudouresque *et al.*, 2009). *P. oceanica* colonizes, largely, environments with constant salinity values, the salinity range allowing the plant to adapt is more or less stable (36.5-38 PSU in the western basin and 38-39.5 PSU in the eastern basin, Klein and Roether 2001). This species is generally considered sthenoaline and highly sensitive to salinity changes, while the responses to saline variations include a variety of physiological and biochemical changes.

Early studies on *P. oceanica* provided experimental evidence of its reduced capacity to withstand chronic salinity increments (Fernández-Torquemada and Sánchez-Lizaso 2005; Sánchez-Lizaso *et al.*, 2008; Ruiz *et al.*, 2009a). When *P. oceanica* is exposed to a change in salinity it is vulnerable to osmotic stress, which results in alterations of the photosynthetic rate, metabolism, reproduction, and survival (Fernández-Torquemada *et al.*, 2005).

It is known that physiological processes in seagrasses are closely related to the saline environment in which they are found. One of the first physiological adjustments of *P. oceanica* in a hypersaline environment is regulation of water relations in order to cope with this type ofstress. In fact, *P. oceanica* is osmotically adapted (Tyerman 1989) and reduces the values of water potential (Ψw) and osmotic potential ($\Psi \pi$) of leaves compared to the same measured values of Ψw in seawater (Tyerman 1989; Kirst, 1989).

This difference represents a positive water balance for the tissues and consequently maintains optimal turgescence values (turgore pressure, Ψp), an essential condition for

maintaining the structural configuration, growth and metabolism of plant cells (Zimmermann 1978 ; Kramer and Boyer, 1995).

As salinity increases, the Ψw of seawater can decrease to near or even worse values than that of tissues. This new osmotic scenario involves reducing or interrupting the water balance, which can cause various metabolic abnormalities and the activation of specific physiological strategies to restore the original equilibrium. Under such circumstances, the main mechanism implemented by *P. oceanica* to restore the osmotic balance is the accumulation of additional solutions, such as proline (free amino acid), a process known as osmosis of regulation or osmoregulation.

In the short term (minutes, hours or days), osmotic adjustment can be easily achieved through cytosolic ion accumulation as Na^+ and Cl^- (Flowers *et al.*, 1977; Bisson and Kirst 1979; Tyerman 1982; Kirst 1989; Munns 2002; Touchette 2007). However, accumulation of these ions can have adverse effects on metabolic processes and, in the long term (days o weeks), must be replaced by organic osmotically active metabolites, termed osmolytes or compatible solutes (Wyn Jones and Gorham 1983; Kirst 1989; Hasegawa *et al.*, 2000; Marcum 2006).

The type of compatible solutes produced depends on the type and intensity of stress, and their functions are not restricted to osmotic adjustment, but also include protection of biochemical structures against ionic stress (Kramer and Boyer 1995). Soluble carbohydrates and proline amino acids are among the most common types of osmolytes that have been identified in seagrasses (Brock 1981; Tyerman 1989; Adams and Bate 1994; Murphy *et al.*, 2003; Touchette 2007).

This increase in salinity implies that the response of water relations and proline content to changes in the salinity of the medium, forms the basis of the ecological and metabolic strategies that plants can adopt to withstand stress conditions caused by hypersalinity, costituing a basic tool for understanding the specific strategies that each species employs to tolerate changes in external salinity (Kramer and Boyer 1995; Ashraf and Athar 2009; Kahn and Weber 2006; Verslues *et al.*, 2006). Other effects of this hypersaline stress that Fernández-Torquemada *et al.* (2004), and subsequently Marin-Guirao *et al.* (2013), have highlighted are a significant reduction in leaf growth and an increase in senescence, mortality, and necrosis. These effects are more marked at high salinity levels (40-50 PSU), although significant effects are also observed when an increase in salinity of 1 PSU is achieved. The osmotic stress caused by salinity changes reduces plant growth and is

considered an adaptive function for survival under stress. Reduction of the growth rate in these stressful situations could be caused by a decrease in photosynthetic capacity.

As regards *P. oceanica*, most of the aforementioned studies were carried out in mesocosms. It is noted that evidence on salinity stress observed *in situ* is scarce, since this species has only been found growing under salinity levels exceeding normal threshold of tolerance at certain sites (Marin-Guirao et al., 2017). Mesocosms provide a tool for performing ecological experiments under replicated, controlled, and repeatable conditions at relatively low cost. However, they are intrinsically limited as regards the reproduction of the complexity of the interactions occurring in natural ecosystems, and full extrapolation from mesocosms to the natural environment is questionable (Changwoo and Mitsch, 2002). Therefore, research projects using experimental design, combined with observed field data, can help to identify and interpret the true relationships between a factor and its biotic response, as recently underlined by Nelson (2017).

The aim of the reaserch was to verify the existence of a growing adaptation gradient in different salinity conditions, from the Mediterranean Sea, a stable environment, to an enviroment with seasonal hypersalinity, a clear phenomenon that occurs during the summer in a semi-enclosed lagoon like the Stagnone of Marsala, situated on the western coast of Sicily (Calvo & Fradà Orestano 1986). This environment can be defined as "extreme"; it is characterized by extreme temperature and salinity variations, with seasonal periods of hypersalinity, in particular; that can represent for the plants a natural type of impact.

In particular, the physiological traits and growth *performance* demonstrated by *P. oceanica* in stressful environments for plants, such as Stagnone of Marsala (Sicily), was evaluated by morpho-physiological variations, such as osmolarity of leaf tissue, proline content, respiratory and photosynthetic rates, photochemistry (Diving PAM), pigment content (chlorophyll and carotenoids) and vegetative variables.

For this purpose, it was decided to adopt two different approaches for evaluating the physiological traits and the growth *performance* of *P. oceanica* in this “extreme” environment. The first approach was an assessment of hyper-saline stress adaptation *in situ* (with natural salinity variations), dividing the year into two periods based on the increase in salinity within the Stagnone of Marsala; a period of homogeneous salinity (spring) between the external environment and the interior of the Stagnone of Marsala, and a period of heterogeneous salinity (summer) characterized by a high evaporation rate that leads to a hypersalinity level inside the semi-enclosed lagoon. The second approach was the

evaluation of the hyper-saline stress adaptation *ex situ*, a short-term mesocosm experiment (salinity conditions imposed by the experimental design). Three different sites of *P. oceanica* wereidentified within the Stagnone of Marsala, along a gradient of increasing salinity, between spring and summer, namely, Atolls (38 to 50 PSU), *Recif Barriere* (38 to 40 PSU) and *Plateau Recifale*, characterised by a constant value of salinity (38 PSU). It was decided to carry out this type of manipulative experiment *ex situ* with the physiological variations detected *in situ*, in order to isolate the combined effect of salinity and temperature, which cannot be isolated in the field, while maintaining a constant temperature throughout the experimental period. The *ex situ* experiment only allowed to change the salinity concentration, thus enabling to discriminate the single effect that it has on plants inside the Stagnone of Marsala and compare them with plants growing outside the lagoon and along this salinity gradient, without extreme variations. Finally, it was thus possible to verify different osmo-acclimative adaptation strategies under this condition of hypersalinity in the natural environment.

Despite numerous studies on marine seagrasses, knowledge about this specific physiological capacity, i.e. to tolerate or resist saline changes in natural environments, is still limited. In this study, growth *performance* and variations in the physiological traits of *P. oceanica* meadows were analysed along a natural hydrodynamic gradient, where significant salinity increases have been observed (Tomasello *et al.*, 2009). Moreower, *P. oceanica* collected along this gradient was also exposed to pure hypersaline short term stress in a mesocosm environment, in order to assess whether the shoots coming from natural different salinity levels exhibited a different ability to adapt and tolerate this kind of stress.

2. Plant biology

2.1 Seagrasses meadows: *Posidonia oceanica*

Marine Angiosperms (Alismastide Subclasses) are cloned plants, probably descendants of aquatic hydrophytes and coastal environment plants (Larkum and den Hartog 1989), which have colonized the infralitoral environment of temperate and tropical coasts all over the world, forming extensive marine meadows (Green and Short 2003).

Marine Angiosperms represent an ecological group of plants that have evolved and adapted to live their entire life cycle completely submerged in the marine environment (Hemminga and Duarte 200, Kuo and Hartog 2000, Hartog and Kuo 2006).

From a taxonomic point of view, it is a polyphytic group consisting of about 60 species, whose evolution converges into a particular adaptive series, related to the ecological niche they occupy (Spalding *et al.*, 2003). The important difference that distinguishes marine angiosperms from other aquatic plants is the adaptation to marine life (Arber 1920).

The species of Angiosperme considered marine are all those belonging to the families Zostraceae, Cymodoceae and Posidoniaceae and three genera of the family Hydrocaritaceae.

Marine angiosperms have similar characteristics to other aquatic plant groups, such as hydrophilic pollination and a developed radical system that allows anchoring on the seabed, and as a consequence the ability to complete its fully submerged life cycle.

They also have unique characteristics that distinguish such species as the greater or less tolerance to salinity variations, and its variations on both temporal and spatial scale.

Indeed, such physiological characteristics (such as a very negative osmotic potential) at the level of ultrastructures (splitting of the cuticle for the interchange of water and particular solutes) and morphology, seem to be related to the different adaptive capacity of the various species of seagrass (Jagels 1983; Tyerman 1989; Jagels and Barnabas 1989; Iver and Barnabas 1993; Kuo e den Hartog 2006).

The different species of marine angiosperms occupy a high diversity of coastal environments characterized by different salinity regimes; from open environments where salinity is constant over the year, to hypersaline coastal lagoons characterized by strong variations in seasonal and annual salinity, such as the Stagnone of Marsala, or estuaries with very variable salinity.

This suggests, the existence of different adaptive capacities on the part of the different species of seagrasses, on the one hand and on the other hand that salinity represents, along with other environmental parameters (such as light and nutrients), the determining factors explaining the different inter-and intraspecific adaptive capacities (Larkum *et al.*, 2006b; Ruiz *et al.*, 2009a).

These meadows represent one of the most important marine ecosystems characterised by high primary production, and a set of ecological functions and services considered fundamental to the functioning and current structure of the entire marine ecosystem.

Examples of such ecological importance include the following: maintenance of the biodiversity of both fauna and associated flora; a trophic network that finds the base for seagrass meadows; protection of coastal areas from erosion; carbon accumulation and nutrients (biogeochemical cycles); sedimentation of particulate matter and, consequently, maintenance of transparency and water quality.

Some studies have allowed estimating the ecological value, i.e. the level of ecosystem services and functions that can be converted into socio-economic value directly transferred to the human population (Costanza *et al.*, 1997; Orth *et al.*, 2006, Vassallo *et al.*, 2013).

2.2 Morphology and growth cycle

Posidonia oceanica is an endemic marine seagrass of the Mediterranean Sea that forms extensive submerged meadows between the surface and 40 meters in depth (Boudouresque and Meinesz, 1982). It has roots, stems (called hypogeous habitat rhizomes) and the typical leaves of terrestrial plants (Fig.2.2.1).

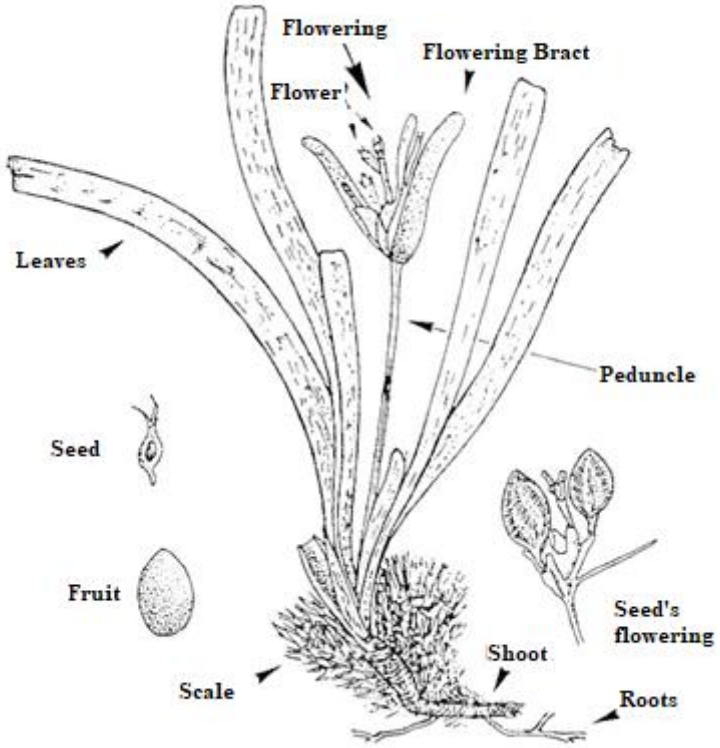

Figure 2.2.1 Drawing of *P. oceanica* shoot

Roots are formed on the ventral side of the rhizome and have a dual function: anchoring in the substrate and assimilation of nutrients that, through the vascular system (xilema), are then transported to the leaves.

The outermost part of the roots and rhizomes is subject to a gradual lignifications process that stops the degradation phenomena of the plant.

Rice typically grows both horizontally (plagiotropically) and vertically (orthotropic) (Cognetti *et al*., 1999).

Plagiotropic rhizomes have the function of anchoring the plant in the substrate, due to the presence of roots at the bottom, and allowing colonization of available space (the growth rate is about 5-10 cm/year). When the density of shoots becomes high and light and space become limiting factors, the plant uses the capacity of orthotropic growth that also allows it to counteract continuous sedimentation and prevent digestion. For orthotropic rhizomes, the rate of growth depends on the sediment accumulation rate; if sedimentation is rapid, growth increases slightly by ifit is not sufficient to compensate for the elongation of rhizomes, they are broken or removed (Bouderesque and Meinesz, 1982).

The interlacing of plagiotropic and orthotropic rhizomes traps enormous amounts of sediment, leading to the construction of typical, extremely compact terrace formations, called *matte* by french (Fig.2.2.2). These rise from the seabed about 1m/100 years. In the Mediterranean, reaches heights 6m, resulting from the effect of constructive and demolishing phases that alternated in time (Moliner and Picard, 1952).

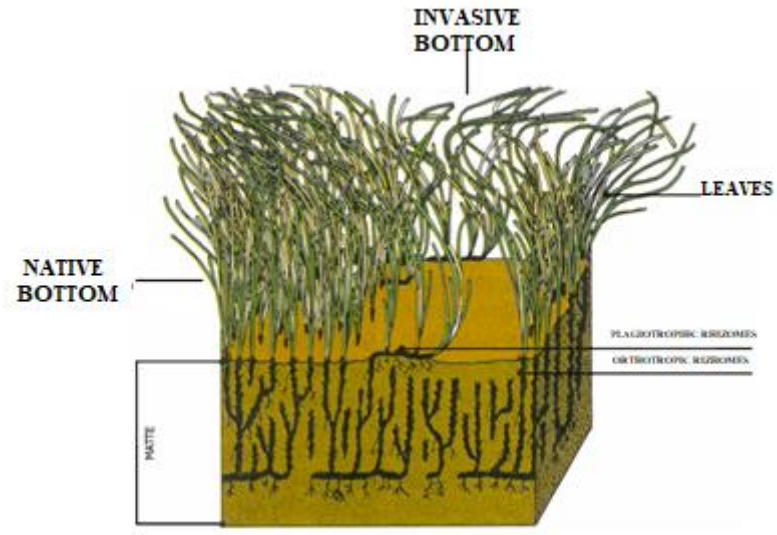

Figure 2.2.2 *Matte* growth pattern

The vertical growth of these formations is conditioned not only by the sedimentation rate, but also by exposure of the meadows to wave motion and the currents. In highly hydrodynamic areas, the matte may be eroded or scaled, resulting in regression of the meadows and formation of inter-channel channels (erosion channels) or circular erosion areas called "erosion mufflers".

In the more sheltered areas, where sedimentation is greater, in order to resist abstraction the meadows rise up to emerge with the leaves, forming a natural barrier called "*Recif Barriere*" (Cinelli, 1995).

The leaves have a basal growth, i.e. they stretch to form new tissue at the base, where the meristema is present. The presence of the basal meristem allows the growth of leaf foil even when the peak, which becomes the oldest part, first meets degenerative phenomena (Giraud, 1977). The leaves have a ribbon-like appearance, bright green colour and end with a rounded peak; the average width is about 1 cm and the length can exceed one meter. They are organized in tufts or files, each generally containing 6 or 7, with a fan arrangement, where the leaves are older and longer externally, while inside are the younger ones of smaller size (Panayotidis and Giraud, 1981). The leaves are then renewed from the inside to the outside.
Leaves are divided into:

- Adult, characterized by the photosynthetic active leaf and a base separated from the flap by a concave line called the "ligula";
- Intermediate, without "ligula";
- Youthful, which by convention does not exceed 50 mm in length (Giraud, 1977).

The juvenile leaves appear in the autumn; in winter they become intermediate leaves and reach maximum development in the spring-summer period. In autumn, the more exogenous adult leaves, aging, turn into senescent leaves characterized by photosynthetically inactive brown colour fabric. Following the storms, these leaves disintegrate at the ligula (the base remains tied to the rhizome and is called a scaffold) and, in the direction of the currents, are stranded and stacked on the heaps of heaps called *banquettes* (Fig. 2.2.3) . Dead leaves can also be transported and end up in benthic systems, or they can remain in the meadow of origin causing auto-pollution phenomena.

Figure 2.2.3 *Banquettes*, leaves laid and stacked on the shore (ph A. Tomasello)

2.3 Reproductive cycle

Posidonia oceanica has two reproduction modes, asexual and sexual reproduction (Fig.2.3.1). The first, however, seems to have a high incidence in the conservation and spread of the meadows (Balestri, *et al.*, 2017) contrary to what was believed previously (Meinesz *et al.*, 1993; Balestri *et al.*,2017).

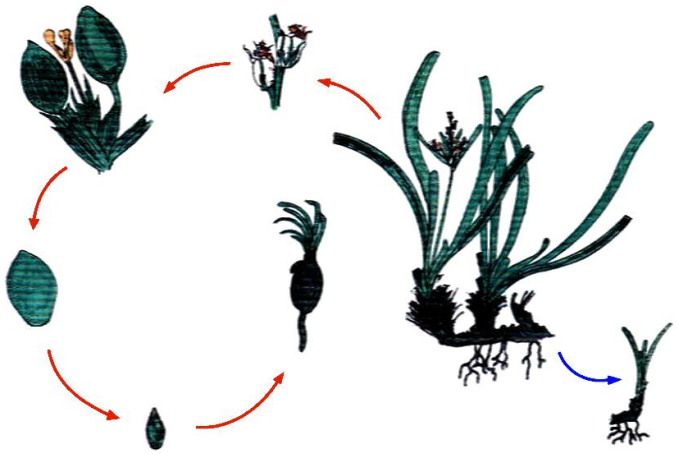

Figure 2.3.1 Reproductive cycle of *P. oceanica*. The red arrows indicate sexual reproduction, the blue the stolonization process

Asexual or vegetative reproduction is characterized by the "stolonization" mechanism. Some terminal rhizomes may be detached from the shoot, necrosis or hydrodynamism (Molinier and Picard, 1952; Boudouresque and Meinesz, 1982), and give rise to a new plant (clone). This strategy would primarily promote local colonization (Meinesz *et al.*, 1982). It has been noted that orthotropic rhizomes have slower growth and rarely divide plagiotropic rhizomes, while maintaining the typical vertical development (Wittmann, 1984).

Sexual reproduction occurs through the formation of flowers and fruits. The flowers (4 to 10), free of petals, are gathered in a greenish-coloured inflorescence; this is wrapped in floral brattees and is inserted into the center of the foliage by a pedicle. The flowers are hermaphrodite; the male part consists of stamens containing pollen, while the female part is formed by the egg holding egg cells. Pollen granules released by longitudinally

extending stamens have a filamentous, curved appearance that facilitates the attack of the same granules on the surface of the stigma.

After fertilization, fruit development begins and reaches maturation after about six months. The fruit, a fleshy drupa, due to its form is commonly called "sea olive" (Fig. 2.3.2).

Figure 2.3.2 Fruits of *P. oceanica*

Once matured, it separates from the mother plant and rises on the surface thanks to the oily substances contained in the pericarp; once on the surface, the waves and the wind transport the fruit from the meadows of origin to other areas.

The subsequent opening of the pericarp and the consequent release of the seed will allow implantation of a new individual and, if the conditions are favourable, the colonization of free substrates. Sometimes, the breeding cycle may be interrupted at an intermediate stage because it does not reach the formation or ripening of the fruit or because the seed does not find the appropriate substrate for implantation. Fruits that do not mature can degenerate and take on abrownish-blackish colour while remaining attached to the rhizome for several months.

Flowering takes place at different times of the year depending on the depth at which the meadows is located. In surface meadows, it is normally between September and October, while in deep meadows there is a phase-out of about two months (Mazzella *et al.*, 1984).

Flowering is a stressful phenomenon for the plant; it has been estimated that the plant spends about one-third of its energy on the reproductive activity. In particular, it has been shown that processes relating to flowering and fruiting in *P. oceanica* are at the expense of intermediate leaves (Panayotidis, 1986; Pergent and Pergent, 1988; Tomasello *et al.*, 1995). Moreover, the development of inflorescence inhibits leaf growth from the early

stages of the cycle (Tomasello *et al.*, 1994) and rhizome production in the year of flowering and for two years after (Calvo *et al.*, 2006). When flowering occurs for two subsequent years at the same site, the rhizomes involved are always different (Pergent *et al.*, 1989). The interval between two blooms on the same rhizome is at least 10 years.

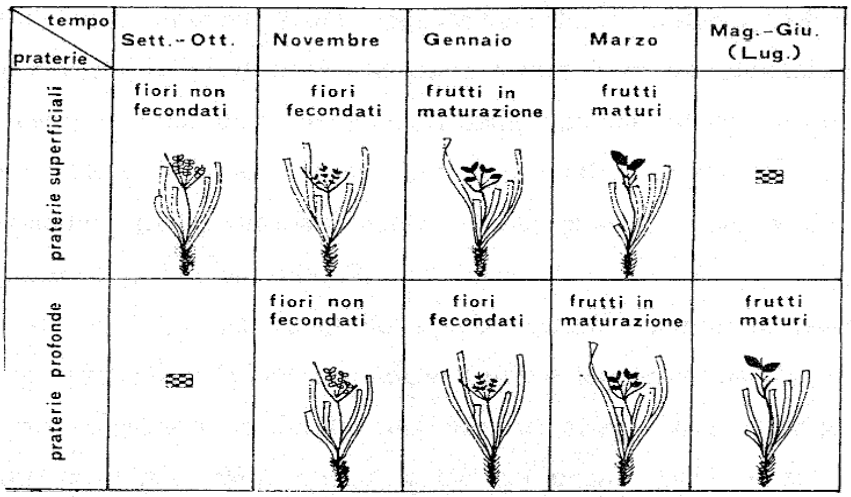

Figure 2.3.3 Time-lapse of the reproductive cycle between surface and deep meadows

It is important to emphasize that the bathymetric gradient, conditioned by fundamental ecological factors such as light and hydrodynamism, plays a predominant role in the reproductive phenomena of plants. On the surface, high light intensity and hydrodynamism have an effect on flowering. In deep areas, however, light constitutes a limiting factor for the ripening of fruit, which is poor in relation to the intense flowering.
Hence, the reproductive success of intermediate meadows where ecological factors display positive interactaction (Tomasello *et al.*, 1994).

2.4 The *Posidonia oceanica* ecosystem

The extension of the meadows, high productivity, but especially the phenological characteristics, growth dynamics and biomass distribution, make *P. oceanica* a trophic support for the plant and animal communities associated with the *P. oceanica* (Mazzella *et al.*, 1991). Meadows do not constitute a single biocenotic entity but are formed by two stratocenoses; one associated with the fronds and the other with rhizomes and the substrate.

The leaves host the photofile community, the substrate and the rhizomes, but due to the reduced penetration of light, they host a biocenosis typical of pre-coralligen or even coralline (Cognetti *et al.*, 1999). An important role is played by animal and plant organisms (epiphytes) that use the leaves as a substrate for settlement. The algal epiphytes colonize the leaves of *P. oceanica* according to a space-time succession based on the leaf gradient age of the leaves, regardless of season and depth. On young tissues and near the meristematic area, bacteria and diatomaceous cells dominate.

In the central part of the leaf, a layer of red macroalgae (Pneophyllum and Hydrolyton) and brown (*Myrionema orbiculare*) is observed. In the apical part, however, a layer of filamentous macroalgae is observed mainly brown algae, genera Castagnea, Giraudia and Dyctiota, and red of the genus Ceramium and Polysiphonia.

Unlike the insulating layer, the erect epiphytic layer has a different composition and abundance, depending on the depth and the season.

The investigation of the structure of the epiphytic community of leaves allows evaluating more rapidly the natural and anthropic alterations of the environmental parameters of the meadows as a whole (Chessa *et al.*, 1995). Environmental factors play a fundamental role, as variables related to depth and season affect the structure of the epiphytic community (Mazzella *et al.*, 1991).

Also on the rhizomes, there is a large number of algal epiphyte species; in fact, the overlying leaf layer reduces both the movements of water and light so that rhizomes become a stable substrate for epiphytic communities that are homogeneous at all depths.

The algal species that most frequently colonize the rhizomes are: Peyssonnelia and Ceranium, among the red algae; Halopteris and Dyctiopteris, among the brown algae; Cladophora and Udotea among the green ones.

P. oceanica meadows also host a rich fauna both quantitatively and qualitatively; fauna, in relation to its distribution along the vertical axis within the meadows, can be divided into four main categories:

• mobile and fixed organisms that live on the leaf layer;

• mobile organisms in the water column between the leaves;

• mobile and fixed organisms that live on rhizomes, based on foliar leaves or sediment;

• organisms living inside the *matte* (in fauna).

The fauna that lives on the leaves consists of sessile species, such as algae, erects (Hydroid and Attinie) and encrusting (Briozoi and Ascidie), and both microscopic (Protozoi) and macroscopic species (molluscs, crustaceans and echinoderms).

The fish population of the meadows is highly diversified; the Labridis represent the dominant group. Among the species of commercial importance, there are Salpe (*Sarpa salpa*), Menole (*Spicara maena*) and Serranidi (*Serranus scriba*); among the camouflage species, the needlefish (*Sygnatus typhle*). Other groups present among the leaves are cephalopod molluscs, including cuttlefish (*Sepia officinalis*), decapod shellfish (*Processa eduli*) and miscidiacei (*Siriella clausii*).

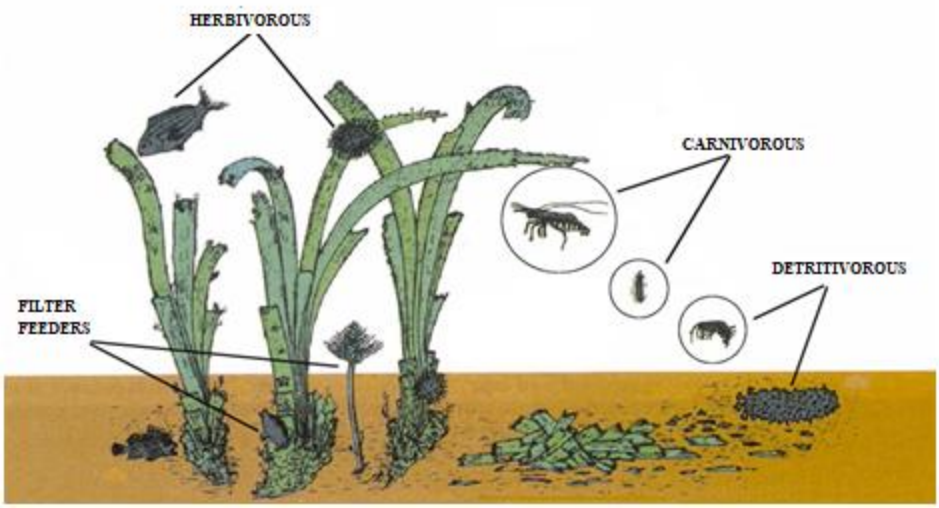

Figure 2.4.1 Foodweb inside *P. oceanica* meadows

2.5 Importance of the seagrass *P. oceanica*

P.oceanica meadows are very important ecosystems because they have many roles, both from a structural point of view and from a biological point of view:

- they attenuate the wave motion by dampening action of the *matte* and the leaf layer resulting in maintaining the balance of the ribs;
- they protect the shores from erosion by means of *banquettes* (Boudouresque and Meinesz, 1982; Mazzella *et al.*, 1986);
- they contribute to water oxygenation;
- they stabilize the sea bottom with the intermingling of rhizomes by compacting mobile substrates;
- they are a source of direct (*Paracentrotus lividus, Hidotea hectica, Sarpa salpa*) and indirect (through epiphytes) power and are the starting point of a complex trophic network (Mazzella *et al.*, 1995);
- they are the habitat of choice for many species (sponges, briots, nudibranchs, gasteropods, fish, algae, etc.);
- they provide a favourable substrate for the establishment, development and shelter of several marine communities;

They represent an ideal nursery area for innumerable organisms, even of considerable economic importance (Kikuchi, 1974; Bell, 1980). For these reasons, it is necessary to protect this important ecosystem that may regress due to both natural and man-made causes.

Large areas of the Mediterranean have been observed and described as extensive regression areas; the natural causes that trigger the regression processes of the meadows are related to phenomena such as:

- progressive global climate change;
- the mechanical role of currents;
- self-pollution;
- ageing of the species and individuals.

Unfortunately, the regression phenomenon is increasing with time in parallel with rising anthropic pressure along the coast. The main anthropic causes of regression of the meadows are:

massive coastal development (dam construction, beach restoration, modification of river basins);
urban and industrial sewage disposal, andtrawling.
The disappearance of the *P. oceanica* ecosystem causes damage not only from an ecological point of view (reduced amount of dissolved oxygen, disappearance of shelters for fauna, suitable substrates for laying eggs) but also erosion of sandy shores, the disappearance of plant debris that forms banquets and destabilization of mobile bottom.

3. Main environmental factors and adaptations of *P. oceanica*

Seagrasses, widely distributed along the Mediterranean coastline, are extremely sensitive ecosystems both to low and high levels of disorder often associated with high human impacts (Procaccini *et al.*, 2003; Boudouresque *et al.*, 2006; Ralph *et al.*, 2006; Marbà, 2009), but also changes in various environmental parameters such as temperature, salinity, light, pH, and are thus a valuable tool for the study of environmental management and conservation strategies. Due to the increase in human activity in coastal areas (Short & Wyllie-Echeverria 1996), there has been a consistent regression of seagrass meadows (Boudouresque *et al.*, 2009) with a consequent alteration of the natural distribution and composition of these communities (Short and Neckles 1999; Bjork *et al*., 2008; Waycott *et al.*, 2009; Marbà and Duarte, 2010). Anthropic impacts in some areas of the Mediterranean also overlap with natural impacts such as elevated salinity and temperature in some lagoon environments, high concentrations of CO_2 and consequent reduction in water pH as in surface hydrothermal fields. The physical and ecological characteristics of the environment influence the distribution and colonization of the territory of the seagrass by conditioning the relationship between shoot recruitment and clonal propagation; the balance between these two reproductive typologies determines the genetic structure of the meadows

3.1 Influence of salinity on the physiology and growth *performance* of seagrasses

Variations in salinity concentration induce physiological mechanisms that can create stress conditions for marine seagrasses. The concept of stress in terrestrial plants is well-developed, and can be defined as the capacity of cultivated plants to withstand water or salt stress (Kahn and Weber 2006).

The physiological characteristics and tolerance of the various species allow plants to acclimatize and adapt to the environmental conditions of the habitat that they occupy, maintaining a physiological optimum such as to guarantee the growth and survival of the plant itself.

In this physiological state, a plant can respond, with effective adjustments in the metabolic flow, to the natural daily variations in its the environment, such as reduction of light intensity, due to the passing of a cloud, decrease in temperature (caused by cold waterupwelling) and salinity variations such as high fresh water supply due to very intense storms.

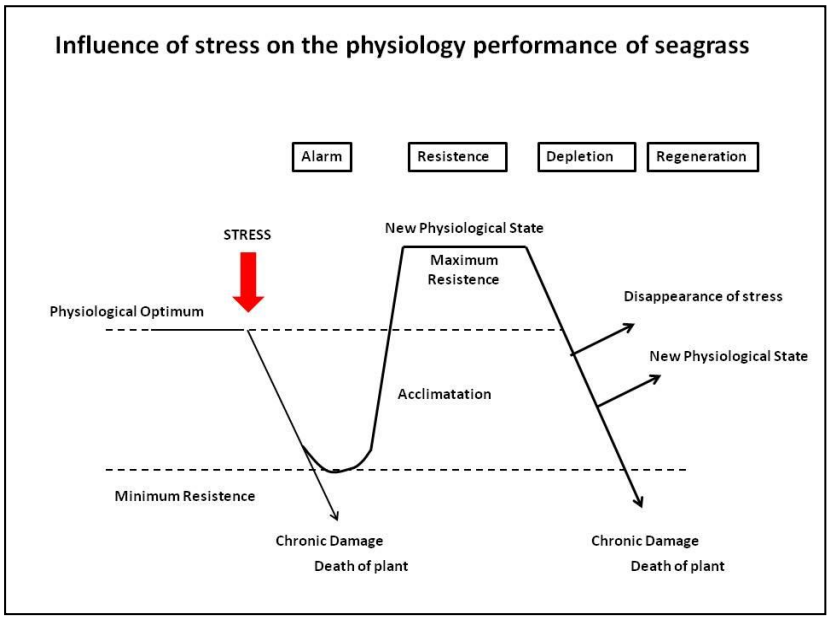

Figure 3.1.1 The sequential model consists of different steps in the general response of plants to the emergence of a certain stress condition. After reaching optimum physiological status and reduced stress, acclimation mechanisms are activated in the plant that can reach a new physiological state of resistance. If stress persists or increases in intensity, the ability of the plant to resist such stress can reduce plant resources, but sometimes can cause deleterious effects on the physiological state of the plant until chronic damage. (Modified by: Lichtenthaler1996, Taddeo e Gomez-cadenas 2008).

Following the general conceptual model illustrated in Figure 3.2.1, instances of more or less persistent salinity increase, beyond its natural variation limits, can cause a stressful situation that in turn triggers the activation of different types of acclimatization responses

in the plant. During the initial "alarm phase" after exposure to stressful conditions, plants react by slowing down their most basic metabolic functions, resulting in a reduction in the vitality of such functions. During this phase, physiological acclimatization mechanisms are also activated under the new osmotic conditions of the external environment that, depending on the inherent capacities of each species, or genotype, allow the plant to establish a new physiological "optimum" (equal or better), which is known as "resistance phase". If the stress persists in time or even increases in intensity, the metabolic resources and vitality of the plant can be drastically reduced. This intense stress phase, known as "depletion or depletion phase", can lead to an increase in cell senescence and cell death if the stress factor persists.

If, on the other hand, stress disappears at this stage, restoration of the original state is unlikely, even if the plant can reach a new physiological state or "regeneration phase." It should also be noted how the clonal nature of marine fenugreek can complicate or confuse the phases illustrated in this general model. For example, the distribution of resources between the shoots (Ramet) or tissues of the same clone (Marbà *et al.*, 2002; Rosmarino *et al.*, 2006) could cause simultaneous acclimatization, resistance, and exhaustion (senescence and death) mechanisms among different individuals, linked by the same clonal structure. Precisely this ability to integrate clonal stress response has been suggested as the main acclimatization mechanism for changes in key environmental parameters (e.g. light reduction with depth) and explains the low physiological plasticity of individual bundles, as observed in some case studies (Olesen *et al.*, 2002, Collier *et al.*, 2008). As with water stress in terrestrial plants, hypersaline stress in marine benthic macrophytes (marine macroalgae angiosperms) can lead to substantial adverse effects due to two types of factors: osmotic and ionic factor (Kramer & Boyer 1995; Bisson & Kirst 1995; Tyerman 1989). In marine fenugreek, the osmotic factor is mainly related to the difficulty of maintaining a positive water balance in the tissues. The ionic factor, on the other hand, is related to the increase in the concentration of the ions responsible for morpholysis, which in turn would lead to toxicity in the various metabolic compartments (Kirst 1989). Both factors, major components of a hypersalidal stress condition, can induce a number of alterations both physiological and metabolic, whose expression is closely related to the tolerance and ability of each individual species (Touchette 2007). However, with respect to the remarkable progress made in the field of ecophysiology (water stress and salt on terrestrial plants, Pirro and Das 2005, Ferri *et al.*, 2009), current knowledge of

physiological responses to hypersaline stress is relatively poor and limited to certain species and taxonomic groups.

3.2 Water Relation Alterations

Marine angiosperms are submerged plants whose physiological processes are closely related to the saline environment in which they are found. This implies that the response of water relations to changes in salinity of the medium form the basis of the ecological and metabolic strategies that plants can adopt to withstand stress conditions caused by hypersalinity. Consequently, it is crucial to know the water relations of these plants and their behaviour under conditions of variable salinity. However, the water relations of a plant and their alterations caused by a change in salinity are among the less studied aspects (Tyerman *et al.*, 1984; Tyerman 1989; Murphy *et al.*, 2003; Koch *et al.*, 2007b).

In general, macroalgae (Kirst 1989) and marine angiosperms (Tyerman 1989) are osmotically adapted (Kirst 1989) to the salinity regime of their environment, reducing the values of the water (Ψw) and osmotic potential (Ψπ) of leaves compared to the same values mezsured in the external environment (Tyerman 1989; Kirst, 1989).

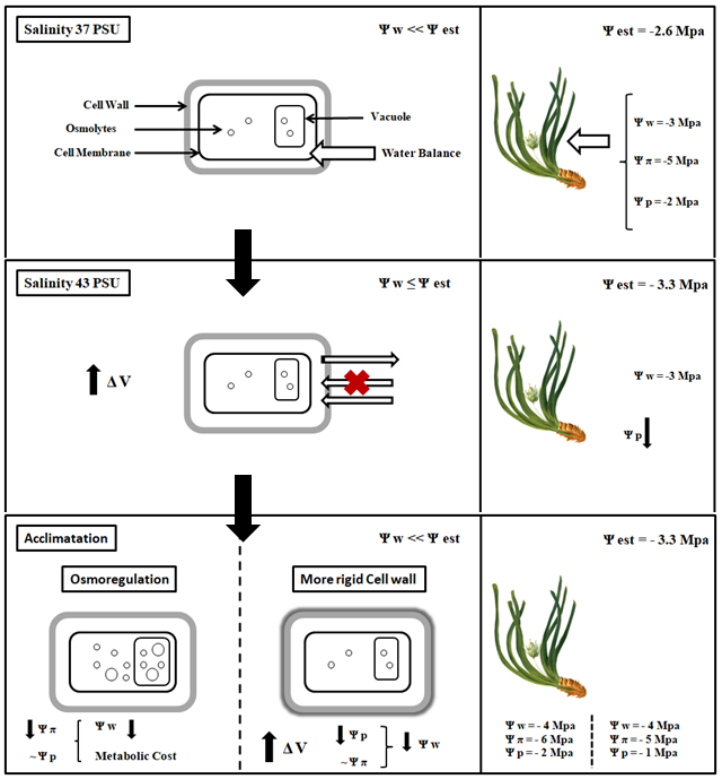

Figure 3.2.1 Representative pattern of the different known phases and strategies of acclimatization, such as "avoid dehydration" (i.e. osmoregulation, cell wall hardening processes) that could be applied by sea lanterns to rising in salinity. The left panels represent the succession of these processes at the cellular level of leaf tissue (Ψext = mean water potential; Ψw = leaf water potential; Ψπ =osmotic potential; Ψp = turgor pressure; ΔV = cell volume variation). In addition to the right panels, it is a hypothetical example of the development of both strategies in response to a hypothetical salinity increase of 37-43 PSU (practical salinity units), with the numerical values of the different potentials expressed in Megapascal (Sandoval-Gil,PhD Thesis)

This difference represents a positive water balance for the tissues and, consequently, it maintains optimal turgescence values (turgor pressure, Ψp), an essential condition for maintaining the structural configuration, growth and metabolism of plant cells (Zimmermann 1978; Kramer and Boyer, 1995). As the salinity increases, the Ψw of seawater can decrease to near or even worse values than that of tissues. This new osmotic scenario involves reducing or interrupting the water balance, which can cause various metabolic abnormalities and the activation of certain physiological strategies to restore the original equilibrium. These strategies are illustrated and schematized in Figure 3.3.1.

3.3 Accumulation of osmolytes

During the osmoregolatory process, the concentration of active osmotic solute active (also known as osmolyte) within the major intracellular compartments (cytoplasm and vacuol), is determined by the nature of the solute (inorganic/organic) and other factors such as exposure time to stress or physiological and nutritional status of the plant (Hsiao 1973, Wyn Jones and Gorham 1983). In general, after a saline increase, there is immediate build up of salts and ions (Na + and Cl-) in the cytoplasm, mainly to counterbalance the gradient in favour of the external environment. This process represents the fastest osmoregolatory response (minutes/hour) that takes place in order to restore the intracellular water balance (Hasegawa *et al.*, 2000). If hypersaline conditions persist or increase, accumulation of these inorganic osmolins may be harmful to intracellular metabolism. In fact, high concentrations of inorganic ions can interfere with cytoplasmic enzymatic activity, alter membrane potential (both cellular and vacuolary) or even damage the structure of the different protein complexes, such as electron transport chains in chloroplasts and mitochondria (Flowers *et al.*, 1977; Hasegawa *et al.*, 2000; Zhu, 2003; Munns, 2002). To avoid excessive accumulation, these inorganic osmolites can be ejected in the outer environment, transported to other tissues, or compartmented into specialized organs (such as vacuoles) (Niu *et al.*, 1995; Zhu, 2003), but at the same time their function is also replaced by osmotic active cytoplasmic organic solutes. The accumulation of such solutes has evolved as a slower process that takes place in the post-osmoregulation response (i.e. days-months), making these osmolytics excellent indicators of osmotic regulation processes in prolonged or chronic cases of hypersensitivity conditions. The importance of these solutes lies not only in the osmotic function just described but also in the role of

osmoprotection (e.g. stabilization of membranes and enzymatic complex). Low metabolic toxicity at high concentrations within the cell allows considering these osmolites as compatible solutes (Munns, 2002). Increased concentration of organic solutes in leaf tissue such as non-structural carbohydrates and free amino acids such as proline has been demonstrated in some species of marine angiosperms (Stewart and Lee, 1974; Brock, 1981; Pulich, 1986; Tyerman, 1989; Murphy *et al.*, 2003; Adams and Bates 1994a; Koch *et al.*, 2007b). However, direct evidence of ion accumulation during saline experimental treatments was found only at the end of the 1980s by Tyerman and collaborators (Tyerman *et al.*, 1984; Tyerman, 1989).

3.4 Variations in the photosynthetic rate and concentration of photosynthetic pigments

Photosynthesis is a key physiological process of plant growth and productivity. In fact, at the metabolic rate, the reduction of the photosynthetic rate due to a stressful condition can be considered as one of the most significant negative effects (Kramer and Boyer 1995, Huchzermeyer and Koyro 2005). In hypersaline stress conditions, a reduction of photosynthesis in both terrestrial plants and seagrasses was observed (Kirst 1989; (Huchzermeyer and Koyro 2005), caused by several factors:

- reduction of Ψw and physiological consequences (energy consumption, accumulation of solute toxic);
- alterations in the ultrastructure, number and disposition of chloroplasts;
- modification of the photosynthetic apparatus at tilacoid level;
- inhibition of the metabolism of enzymes-mediated carbon reactions;
- reductions in the concentration of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids).

According to the literature, hypersaline stress can cause at least one of these physiological alterations. However, knowledge in this field is very limited by reducing stress response in: reduction of the maximum photosynthetic rate (Ogata and Matsui 1964, Biebl and Cross 1971, Drew 1978a, Kerr and Strother 1985, Koch and Dawes 1991, Berns 2003, Fernandez (Koch and Dawes 1991, Berns 2003, Fernandez-Torquemada *et al.*, 2005, Kahn and Durako 2006), and a decrease in or increase in photosynthetic efficiency (McMillan

Moseley, 1999), a reduction in photochimic efficiency of photosynthesis pigment (Fv / Fm, ΦPSII, Ralph 1998, 1999, Kamermans *et al.*, 1999; Koch *et al.*, 2007b; Beer *et al.*, 1980a) and inhibition of the activity of different enzymes such as RuBisCo and PEPcarboxylase (Beer *et al.*, 1980a). These variations, while highlighting an alteration in the structure and functioning of the photosynthetic machine, do not provide an insight into the mechanisms involved, the intensity of stress and the relationships between them.

3.5 Respiratory Rate Alterations

Another important effect of saline stress is the result of mitochondrial respiration response, and the extent to which alterations affect the photosynthetic (i.e. NET) andproductivity rate of plants. The respiratory rhythms of photosynthetic tissues are generally lower than the photosynthetic rate, but their alteration can have significant functional consequences due to carbon balance imbalances, resulting in a reduction of the resources available for the growth and survival of a plant (Atkin and Macherel, 2009). In marine angiosperms, breathing does not display a consistent pattern of response to hypersaline stress. In fact, it has been observed that respiration may increase, decrease, or remain unchanged (or even present a bimodal pattern) between species within the same experimental conditions and salinity values (Ogata and Takada 1968; Biebl and Cross 1971; Drew 1978a; Fernandez-torquemada *et al.*, 2005b; Kahn and Durako 2006). This absence of general response is consistent with the one described for terrestrial plants and attributed to various factors such as:

- Possible changes in the activity of some enzymes involved in different respiratory processes (e.g. glycolysis, peak (TCA cycle, electron mitochondrial transport chain);
- Changes in the concentration of the respiratory substrate (e.g. due to reduction in photosynthesis rates and, therefore, in the synthesis of photosynthetic products);
- Increases metabolic energy demand (e.g. for the supply of osmodulatory processes, ionic homeostasis, etc.) (Kramer and Boyer 1995; Munns, 2002; Atkin and Macherel, 2009).

4. Materials and methods

4.1 Study Area

The Stagnone of Marsala, a nature reserve since 1984, is a vast coastal lagoon overlooking the western coast of Sicily (Trapani - 37° 52' N; 12°28' E), extending for 12 km along the North-South axis and covering a total area of about 20 km^2. From a geomorphologic point of view, the Stagnone of Marsala is divided into two sub-basins, the north (14 km^2, average depth about 1 m) and the south (6 km^2, average depth about 2 m). The first basin is open to the north with the Mouth of Tramontana between Punta Tramontana and Punta S. Teodoro (about 400 m wide), and is characterized by a sandy bottom covered by a water layer of only 20-30 cm; in some stretches, however, the channel is deep enough to navigate in small boats. The sub-basin is bordered by the Great Island and houses the three islets of S. Maria, S. Pantaleo (Mothia) and La Scuola that, together with the low backdrops, favour the reduction of hydrodynamism.

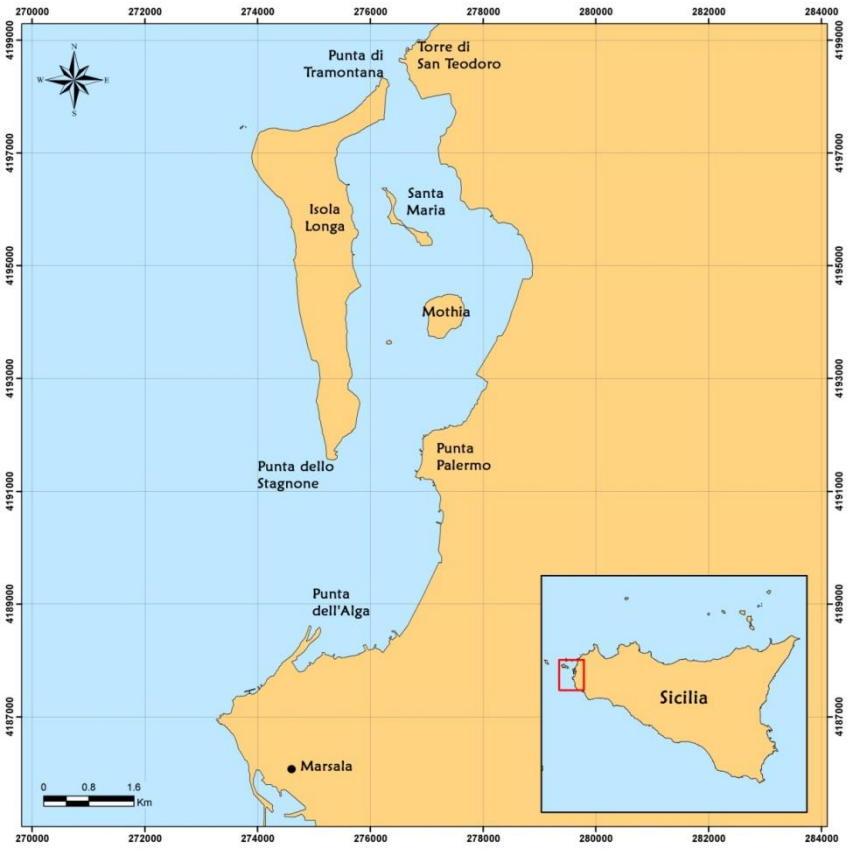

Figure 4.1.1 Study area: Stagnone of Marsala.

The southern sub-basin, however, is characterized by higher hydrological, due to the greater width of the southern opening, between Punta dello Stagnone and Punta dell'Alga (Great Bocca, about 1200 m wide), connecting the southern sub-basin to the sea open. This opening is discontinuous due to erosion channels that determine, in a distance from the same mouth, the breaking and damping of the waves coming from the far side. The depth in the southern basin reaches 3 m at some points; the bottom is predominantly muddy, with typical black coloured sludge resulting from reduction processes. It is probable that the Stagnone of Marsala had not yet formed at the beginning of the Pliocene. According to Leo Pardi (D'Ancona, 1976), Sicily was far less extensive. In the Pleistocene, the Earth was subject to one of the most intense glacial phases (Wurm) *(*Agosta, 1976*)*, which resulted in a decrease in the sea level to the -110 m isobath. At this stage, the Aegadian islands were attached to Sicily and the Stagnone of Marsala appeared as a dip, crossed by one or more streams. The next interglacial period was a very hot period with intense rainfall, melting of the polar ice caps and retreat of the glaciers that had invaded Europe as well. This led to a rise in the sea level to today's limits and consequently the separation of the Aegadians from the mainland and most likely the entry of water into the Stagnone of Marsala (Agosta, 1976*)*. The latter, therefore, originates from an ancient alluvial plateau, today submerged by the rising of marine waters that have created the four islands.

The coastal strip stretching from Trapani to Marsala consists of an extensive sedimentation platform (Agnesi *et al.*, 1993) (Fig. 4.1.2).

At a depth of 30 m, the coast and the submerged morphology are characterized by the presence of two arches connecting Trapani with Favignana and the latter, following the Isola Grande Island, with Capo Lilibeo. In these two areas, at a depth of 20-25 m, a lagunary depression or retrograde stagnant sediment with fine sediments is identified. Moreover, at a depth of -15 m, a threshold of sediments, both fine and coarse, reveals the presence of a tombolo connecting Favignana with Isola Grande.

The hydrogeological conditions in the past differed to the present ones, due to the larger open-sea exchanges by means of two broad channels (fretumintraboream and fretumextraboream) dividing Isola Grande into three parts: Borron, Favilla, and Insula Longa (Fig. 4.1.3).

The morphology of the lagoon has undergone major changes over the centuries *(*Di Girolamo, 1898*)* due to interference in sedimentation and internal hydrodynamics. The northern basin is considered the real "Stagnone", featuring lagoon characteristics, in

particular. In fact, it features high annual variability in water temperature and salinitycompared to the surrounding sea *(*Sarà *et al.*, 1999*)*.

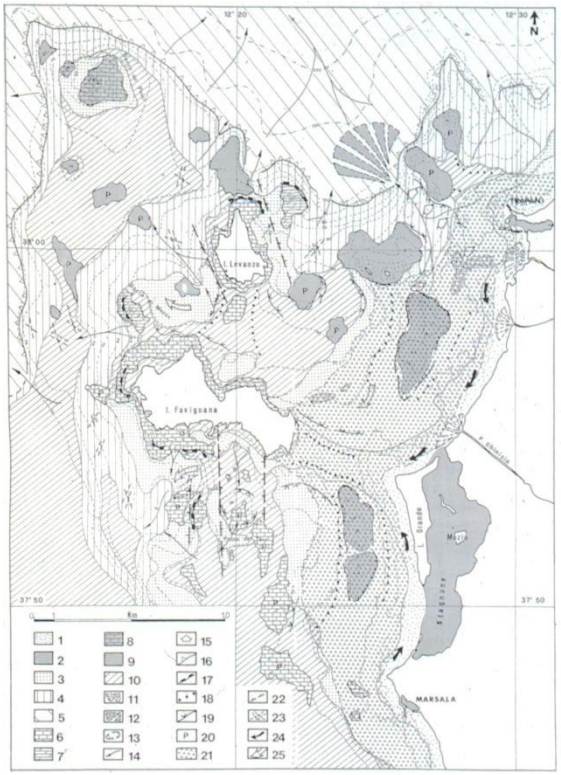

Figure 4.1.2 Geomorphological diagram of Stagnone of Marsala *(*Agnesi *et al.*, 1993*)*.

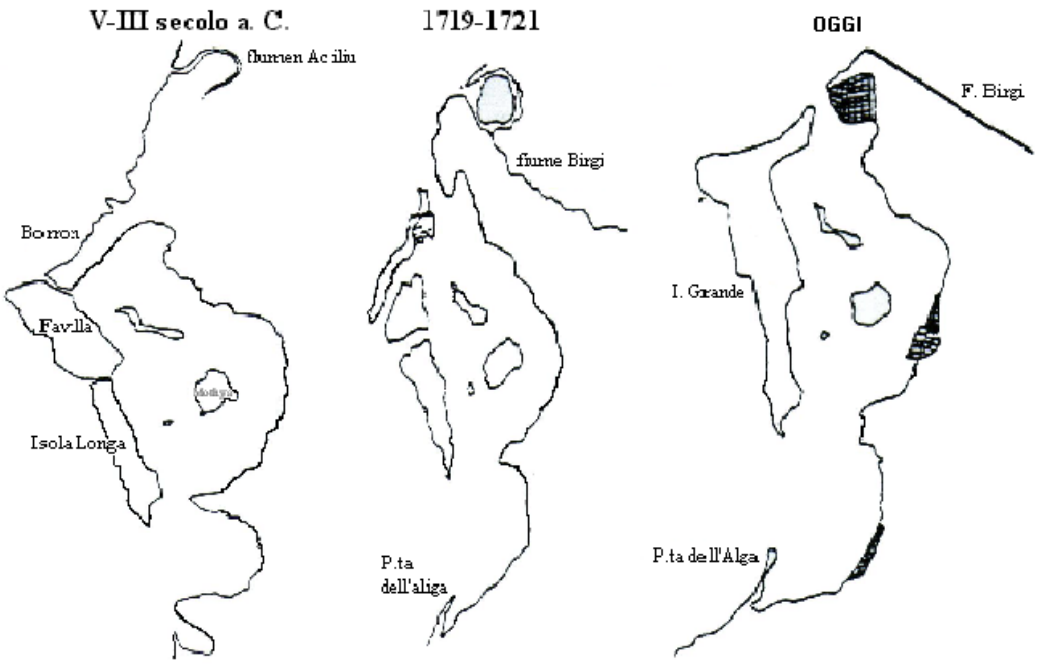

Figure 4.1.3 Morphological condition of Stagnone of Marsala at different historical times (dal V-III sec a.C. al 1984)

Nutrients are present in low concentrations (Maimonti *et al.*, 1998) and trophic response in terms of chlorophyll concentration (about 1.0 μg l^{-1}) identifies the lagoon as oligotrophic *(*Sarà *et al.*, 1999*)*. Overall, it is greatly affected by the open sea and the predominantly marine vegetation communities that inhabit it are considered, in all respects, as belonging to the evolutionary series of populations that colonize the sea-front in front of the lagoon. The main biotic component is represented by discontinuous meadows of *P. oceanica*, present in the central and southern part of the lagoon, which play a fundamental role in the evolutionary dynamics of the natural (Stagnone) and artificial (saline) lagoon ecosystems that characterize the area. The *P. oceanica* meadows cover about 12% of the total area. In the western sector, in particular, coverage is 21%, compared to 11.7% in the south and 5.9% in the central-eastern sector, where the highest regression was observed (Fig. 4.1.6) (Calvo *et al.*, 1996; 2003). The meadows form platforms (*Plateau Récifale* Fig. 4.1.4), 2-3 m wide emerging barriers (*Récif-Barrièr*, Fig. 4.1.5) and more or less circular Atolls approximately 10-20 m in diameter (Fig. 4.1.7).

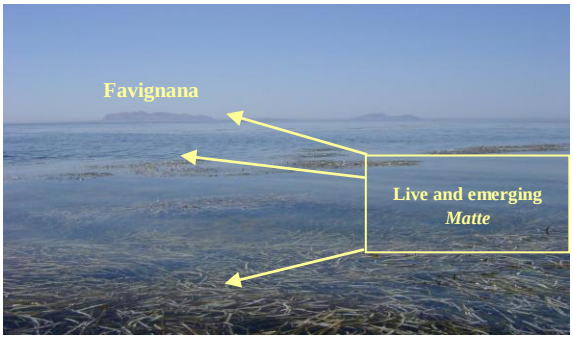

Figure 4.1.4 *Plateau récifale* of Stagnone of Marsala

Figure 4.1.5 *Récif-barrière* of Stagnone of Marsala

The *Plateau Recifale* present in the southern part of the lagoon, between P.ta Alga and P.ta dello Stagnone, limits external exchange by dampening the erosive effects of the wave motion and extends along a NS direction with a length of 2500 m and a maximum width of 1400 m. This formation, described for the first time in the Mediterranean, is an impressive example of the construction work of *P. oceanica* meadows.

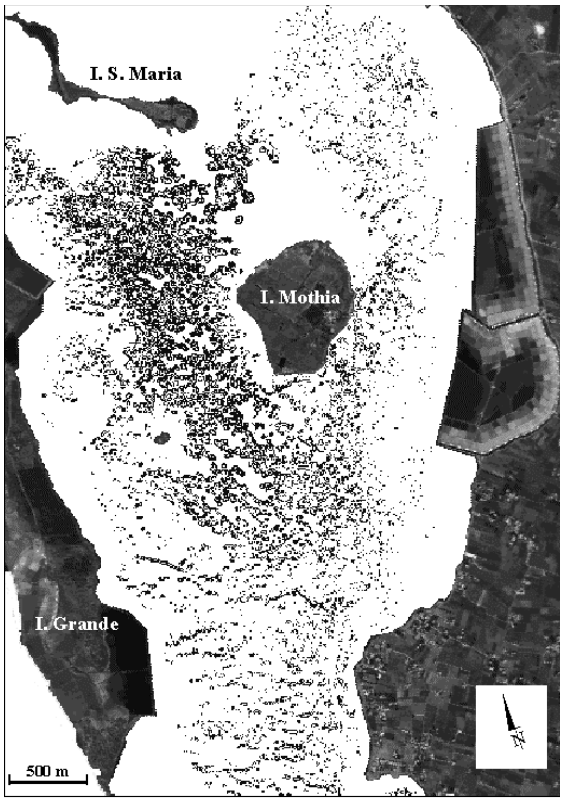

Figure 4.1.6 Distribution of the *P. oceanica* meadow in the northern sub-basin of the Stagnone of Marsala in 1980.

The *Plateau Recifale* has a different appearance and includes the following:

- dead *matte* of *P. oceanica*, more than 3 m thick, on the outer face of the plateau, rising to 40-50 cm from the surface of the sea, limiting the exchanges with the outside and dampening the hydrodynamic effects of the storms caused by the Ponente wind;
- *mattes* of *P. oceanica* living with domed profile (the result of intense surface hydrodynamism);
- sandy inter-*mattes*.

The meadow is heavily deep grooved along its edges, due to erosion phenomena, ditches, grottoes and siphons that resemble formations on rocky substratum as regards the quality and quantity of fauna. Inside the *Plateau Recifale*, where the action of waves is attenuated, meadows come close to the surface and form cords that emerge at low tide. During the autumn period, the older leaves detach themselves from the plant and, driven by the current, drift and accumulate on the shore, forming what the French call *banquettes*. Comparison with the data of a bathymetric paper, whose surveys date back to the end of the eighteenth century, allowed verifying that *P. oceanica* meadow building is still

ongoing. In fact, the plateau in a century has shifted to the wide for several hundred meters, expanding the area occupied by the lagoon.

The *Récife-Barrière* (the outcrop of *P. oceanica*), which is found within the lagoon, has a length of 800 m and a width of 126 m, extending in a O-NO / E-SE direction by communicating the two sub-basins due to two erosion channels, one at Punta Palermo and the other at Punta dello Stagnone. This training is the first reported in Italy and is the most extensive described for the Mediterranean. The observations made on the *Récif Barrière* have highlighted several emerging lines and large areas occupied by dead *matte*.

Figure 4.1.7 Atolls of *P. oceanica* inside the Stagnone of Marsala.

Finally, there are the Atolls, the particular formations of *P. oceanica* meadows probably due to the synergistic effect of hydrodynamics and temperature. In fact, cordon structures gradually acquire circular shapes of different sizes (about 20-30 m in diameter) showing intermediate evolutionary phases. Similar formations, but on a much larger scale, have been described for the Gulf of Gabès along the northern coast of Tunisia.

Currently, *P. oceanica* living in the Stagnone of Marsala is at the limits of its thermal and salinity tolerance (Calvo *et al.*, 2000) while conditions for settlement and development are not optimum due to reduced water replacement and the nature and composition of the substrate. In particular, observations have shown greater presence of *P. oceanica* in the central and southern areas of the lagoon, and a shortage in the northern part due to the introduction of fresh water *(*Calvo *et al.*, 1996*)*. In these conditions, *P. oceanica* is partially

subdivided by lawns of *Cymodocea nodosa* (Ucria) Asch. and the green algae *Caulerpa prolifera* (Forssk) Lam. The two species generally populate the sandy bottoms of the harbours or repair bays and, under equilibrium conditions, supplant *P. oceanica* when reduced water exchange does not allow it to be maintained. *C. nodosa*, in fact, prevails in the areas of the lagoon subject to higher water supply, while *C. prolifera* prefers the most stagnant areas. The presence of the latter in these areas can be an indicator of the environmental stress situation to which the Stagnone is subject. In fact, current *Cymodocea nodosa* and *Caulerpa prolifera* formations can be considered as the expression of the modifications that the hydrodynamic regime and the nature of the substrates have undergone over time (Calvo *et al.*, 1982).

The geomorphological configuration, the shallow water and the outlying vegetation, together with the high temperatures and the intense evaporation phenomena that occur during the hottest months, account for the marked spatial diversification along the horizontal gradient of the water masses in the lagoon in the summer and autumn months. In summer, variations in temperature and salinity are observed, including 29.1° C and 47.1 PSU (Sarà *et al.*, 1999); the O.D. shows values between 82.2% and 131% saturation. In the winter, however, due to the conditions that favour a greater water exchange inside, the Stagnone di Marsala displays greater homogeneity, with average temperature and salinity values close to those found offshore. Chlorophyll concentration measured is between 0.1 and 1.3 mg/m^3. The data highlights the reduced production of the planktonic compartment, whose main ticopelagic component is benthic diatoms that are suspended or sedimented in relation to the receptor's body dynamics. The concentration of nitrogen and phosphorus compounds is always very low, typical of oligotrophic environments. Nitrogen compounds display diversification between seasons. In particular, the average values of ammoniacal nitrogen and nitric nitrogen are 82.4 and 73.8 µg/l respectively, while nitrous ions are always present in very low concentrations (average values of 2.2 µg/l). The fluctuations in the concentration of nutrients, albeit mild in absolute values, should be related to the prevalence of photosynthetic and heterotrophic bacterial activity. However, the influence exerted by the anthropogenic component appears virtually absent. Phosphorus exhibits low concentrations in the analyzed time interval. Nitrogen is the limiting factor (N/P average = 4.9). The ecological state of the lagoon system, assessed on the basis of the TRIX Trophy Index, places the Stagnone of Marsala in Class 2 (state = good according to Legislative Decree 152/99) (AA. VV., 2003).

4.2 Mesocosm Setup

The mesocosm system consisted of six 250 l glass aquaria, each with their own light source provided by a Led PhytoLED 280W (PHYTOLITE, Fig. 4.2.1). This light source created a highly homogenous field of irradiance across the cross section of each aquarium (measured just underneath the seawater surface), with only small deviations from the mean irradiance selected for the experiment (i.e. 300 ± 30 mmol quantam^{-2} s^{-1}).

Figure 4.2.1 Led Phytoled 280 W

Each 250 l aquarium was integrated into a closed circuit feed with seawater from a 50 l reservoir diverted to each aquarium and back again (see seawater circuit in Fig. 4.2.2). Seawater was circulated using a 1000 l/h self-priming pump, allowing complete replacement of water in the system 124 times per day. Within each aquarium, incoming seawater was spread through a diffuser in order to create a homogenous water movement.

Figure 4.2.2 Mesocosm Sytem with 50 l reservoirs

Water temperature was controlled by a highly precise (0.1°C) system. Seawater quality was controlled through continuous chemical and mechanical filtration, with nitrate and phosphate concentrations analysed every 15 days using standard colorimetric tests

(Merck). Special care was taken with the pH of the seawater, as this has been found to be a critical factor in seagrass photosynthesis (Invers *et al.*, 1997).

The pH and salinity of the seawater in the aquaria was recorded and monitored continuously using a Multiparameter pH/ORP/EC/TDS/Salinity/DO/Pressure/Temperature Waterproof Meter, Hanna Instruments (HI98194), and kept constant by adding osmotic water. Natural seawater from a nearby unpolluted area was used to fill the mesocosm circuit. It is widely known that keeping large, slow-growing seagrass species such as *P. oceanica* in aquarium conditions is particularly difficult. However, results obtained in previous trials have indicated that this system can maintain healthy plants, with 100% survival rates, for more than two months, long enough to achieve the objectives of this experiment.

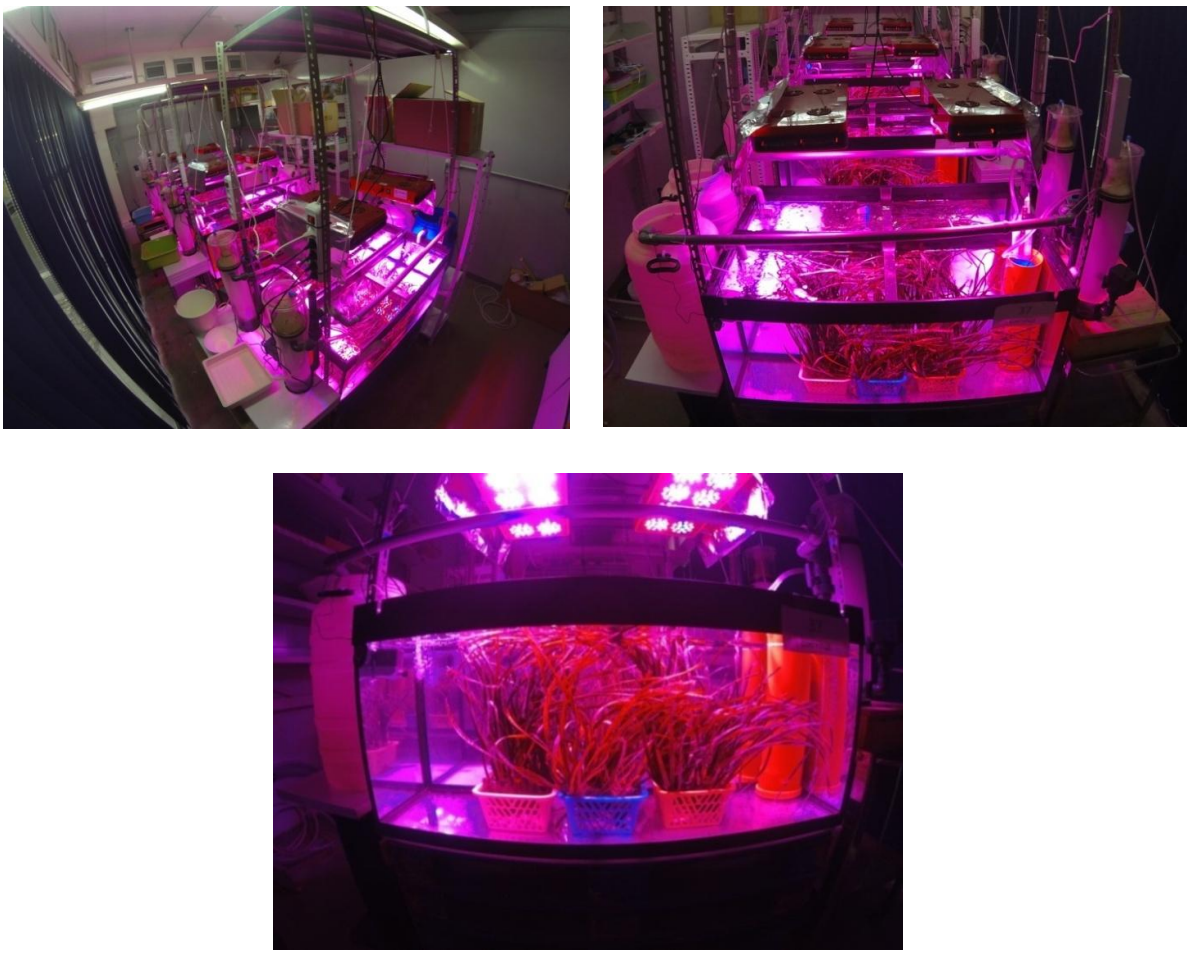

Figure 4.2.3 Mesocosm System

4.2.1 Sampling and experimental designs

***In situ* experiment**

Physiological traits were measured at three sites: Atoll, *Plateau Recifale*, and *Recif Barriere* (Table 4.2.1.1):

- Atoll is located inside the Stagnone of Marsala, which is characterized by growth under stress (51 PSU, unpublished data), forming with the plants the characteristic circular beads called Atolls (shoot density 197.9 ± 15.1 shoot/m^2; Fig. 4.2.1.1);
- *Recif Barriere* is located between the southern mouth of the Stagnone of Marsala and the Mediterranean Sea (42 PSU; shoot density 337.5 ± 30.8 shoot/m^2 ; Table.4.2.1.2);
- *Plateau Recifale* is located in the Mediterranean Sea with salinity values that never exceed the standard values (37/38 PSU) (extending in a N-S direction, 2500 m long and 1400 m large; Calvo *et al.*, 1984).

		"atolls" sector	*Récif-barrière sector*
Shoot density (n°/m^2)		197.9 ± 15.1	337.5 ± 30.8
Meadow height (cm)	Spring	43.2	64.2
	Summer	62.3	68.0
Total leaves (n°)	Spring	4.9 ± 0.1	6.3 ± 0.1
	Summer	4 ± 0.3	5.7 ± 0.3
Shoot surface (cm^2)	Spring	74 ± 4.1	148.6 ± 7.7
	Summer	64 ± 5.8	90.6 ± 6.4
LAI (m^2/m^2)	Spring	1.5	5
	Summer	1.3	3.1
Leaf production (n° y^{-1})		6.5 ± 0.0	7.3 ± 0.1
Growth rate (mm y^{-1})		5.3 ± 0.1	9.5 ± 0.2
Rhizome production (g dw y^{-1})		0.044 ± 0.001	0.115 ± 0.004

Table 4.2.1.1 Main phenological and lepidochronological variables of a *Posidonia oceanica* meadow in the Stagnone of Marsala (Calvo *et al*., 2009)

Water depths at all the sites were similar; *P. oceanica* shoots (plagiotrophic and ortotrophic) were sampled at each site, at 1 m depth, during the following two sampling periods: March 2017 (T0 salinity 37 PSU) and July 2017 (T0 salinity 51 PSU). For each sampling period and each site, a number of samples were randomly collected within a

circular area 20 m in diameter for subsequent analyses. For each sample, shoots were collected and transported (<2 h) in large coolers filled with ambient seawater collected at the time of sampling to reduce light and temperature stress. Once in the laboratory, some shoots were kept in the dark and aerated in their ambient seawater at sampling temperature until the following day, while other shoots were immediately processed and/or stored for subsequent analysis.

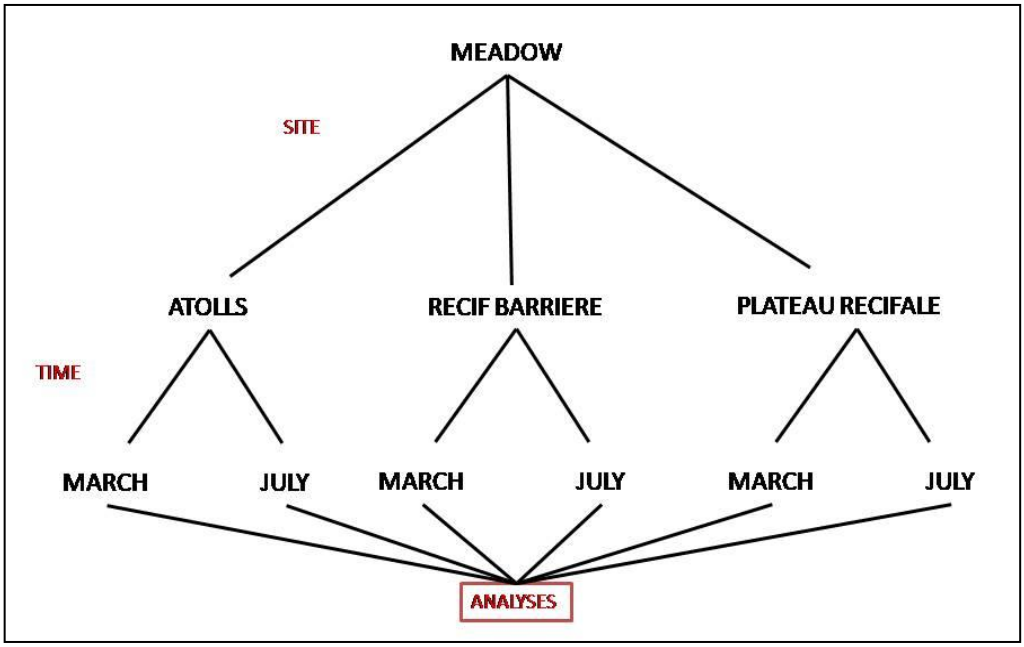

Figure 4.2.1.2 *In situ* sampling design. (T0=March, T1=July)

***Ex situ* experiment**

In April 2017, *P. oceanica* shoots from the three different sites were collected according to the experimental design (Fig. 4.2.1.2):

- Atoll: inside the Stagnone of Marsala;
- *Recif Barriere*: transition site, between the southern mouth of the Stagnone of Marsala and the Mediterranean Sea;
- *Plateau Reciffale*: Mediterranean Sea site, with salinity values that never exceed the standard values.

Sampling was performed simultaneously at an average depth of 1 meter. A number of samples were randomly collected at each site from small *matt*e portions, in order to maintain the interconnections between the shoots and and thus reduce cutting stress. For each sample, shoots were transported (<2 h) in large coolers filled with ambient seawater collected at the time of sampling, to reduce light and temperature stress. Once in the laboratory the units were transplanted immediately in aquariums for subsequent acclimatization. Each transplantation unit contained 30-40 shoots of *P. oceanica* connected to each other to reduce transplant stress.

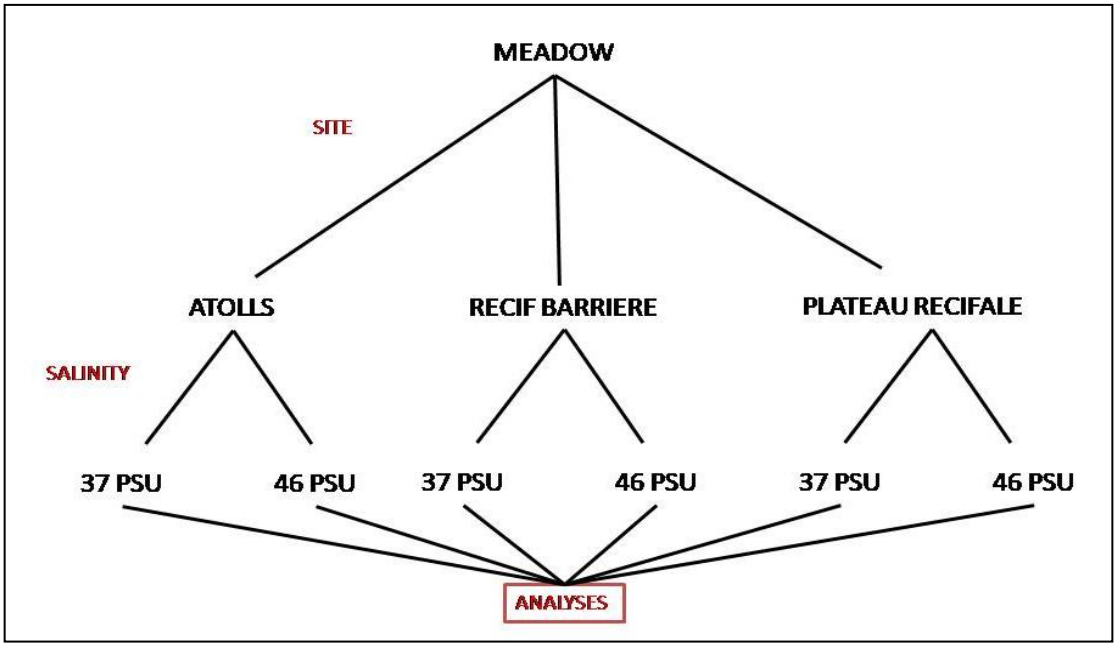

Figure 4.2.1.3 *Ex situ* experimental design

Figure 4.2.1.4 Transplantation units

Three transplantation units were placed in each aquarium, one of each Atoll, *Recif Barriere* and *Plateau Recifale* (Fig. 4.2.1.4); the units were distinguished by colours: pink, green and blue respectively.

For a week prior to starting the experimental treatments, the plants were acclimatised to the mean environmental conditions at the plant collection site during the season in which the experiment was performed, i.e. at a temperature of 20° C, salinity of 37 PSU, and a saturating irradiance of c.a. 300 µmol quantam^{-2} s^{-1} measured on the leaf tips on a 12 h:12 h light:dark cycle (i.e. 12.96 mol quantam^{-2} day^{-1}).

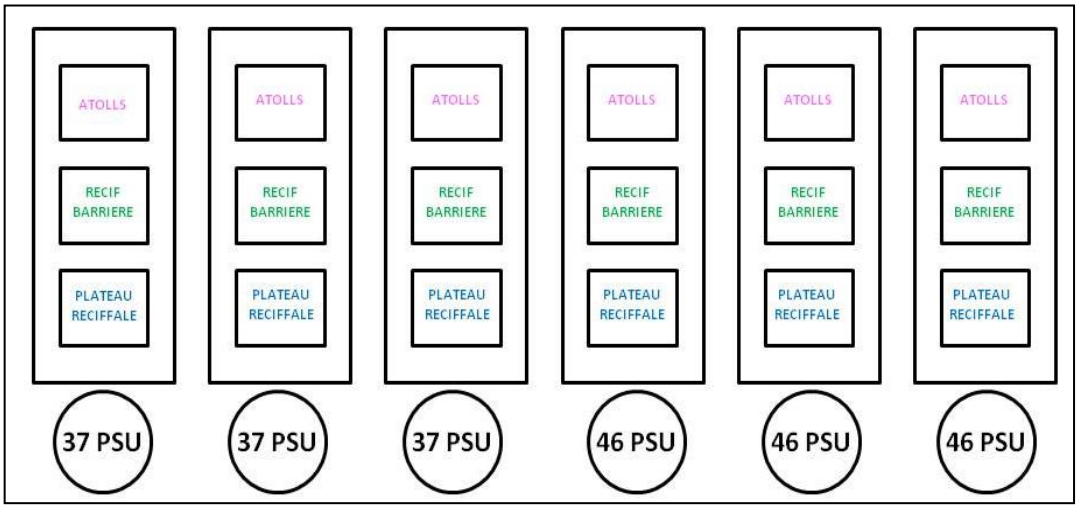

Figure 4.2.1.5 Transplantation units

Environmental data had been obtained during the previous year using underwater continuous CT and PAR irradiance recorders (Hobo Pedant Onset Temperature and Light datalogger). After the acclimation period, three aquariums were maintained with ambient conditions (control, 37 PSU) while salinity was increased in the other three aquaria to produce the following experimental hypersaline conditions: 46PSU. This salinity was selected based on the increased salinity threshold levels of *P. oceanica* established in previous analyses of the Stagnone of Marsala.

Each experimental treatment was randomly assigned to three of the six 300 l aquaria. In order to achieve these salinity levels, high quality artificial marine salt (Seachem Reef Salt) was dissolved in 1000 l auxiliary tanks before the solution was transferred to the reservoir tank of the aquaria (Fig. 4.2.1.5). This hypersaline solution was mixed with the aquarium seawater at a slow rate until the desired salinity level was reached. Adjusting the salinity levels took about 4 h, with these levels then maintained for a total experimental period of 30 days, long enough to observe early physiological responses to hypersaline stress before the occurrence of more severe lethal effects. As explained above, the environmental parameters were monitored daily, with the tanks, aquaria and filters cleaned every day in order to prevent the appearance of epiphytes and macroalgal blooms. Additionally, the transplantation units were periodically repositioned within the sub-aquaria to avoid possible confounding effects due to micro gradients. The response variables were measured at the end of the experimental period, using an individual shoot as the sampling unit (replicates) for all measurements. All shoots of a sample were randomly collected from the experimental population (Atolls, *Recif Barriere* and *Plateau Recifale*) formed by the three transplantation units contained in each large aquarium (i.e. treatment level). For each variable, a strategy was followed that allowed homogenous distribution of the sampling effort throughout the aquarium (see below). Shoots less than 2/3 years old were excluded from sampling in order to avoid or minimise possible dependence effects caused by the influence of internal resources allocation gradients, which are characteristic of these clonal plants (Marbá *et al.*, 2002). For similar reasons, the collection of neighbouring shoots was also avoided.

4.3 Cholorophill-a fluorescence (Diving-PAM)

In recent years, many studies on the eco-physiology of marine seagrasses, aimed at evaluating their photosynthetic rate, have been conducted using fluorescent chlorophyll technology and the introduction of some fluorometers including pulse-amplitude modulated (PAM) fluorometry.The use of these tools has allowed estimates of the photosynthetic activity of these ecosystems, both daily and seasonally, in response to environmental factors. Recently, some studies have emphasized the use of this technique within the framework of a reforestation intervention aimed at the recovery and restoration of degraded meadows. In particular, the use of PAM has made it possible to compare the values of the photosynthetic variables measured before, during and after the intervention. The photosynthetic characteristics of a seagrass can vary in relation to light conditions or climate change. It has been observed that leaves adapted to high luminous intensity have low chlorophyll content, a high capacity and photo-protective photosynthetic mechanisms (e.g. xanthophyll), while leaves adapted to low luminous intensity display opposite characteristics (Demmig-Adams *et al.*, 1999). In the course of time, different methods have been developed for the assessment of plant photosynthesis and primary production rates. These techniques include the assessment of the exchange of CO_2 (Vollenweider (1974; Bittaker and Iverson's, 1976; Gilbert *et al.*, 2000a), exchanges of O_2 (Strickland and Parsons, 1972; Umbreit *et al.*, 1972; Häder and Schäfer, 1994; Longstaff *et al.*, 2002; Carr and Björk, 2003), plant growth (Zieman 1974; West and Larkum, 1979; Kowalski *et al.*, 2001) and chlorophyll fluorescence (Schreiber *et al.*, 1995; White and Critchley, 1999; Gilbert *et al.*, 2000b; Ralph *et al.*, 2005).

4.3.1 Rapid Light Curve

RLC brightness curves provide important information on the characteristics of electron transport and photosynthetic *performance* of a plant (Ralph and Gademann, 2005).
Compared to traditional light intensity curves (*e.g*, photosynthesis-irradiation PE curves, based on oxygen or carbon dioxide), an RLC curve measures the actual brightness (ΔF/F'm, *effective quantum yeld*) as a light irradiance function. In particular, an RLC curve provides an assessment of the photosynthetic activity of a plant, allowing

integration in light intensity variations (Schreiber *et al.*, 1997; White and Critchley, 1999).

Graphically represented, RLC curves are similar to PE curves (photosynthesis-irradiance) and have been used in several eco-physiological studies of segrasses to illustrate the photosynthetic situation associated with daytime and diurnal cycles (Beer *et al.*, 1998; Ralph and Gademann, 1999; Ralph *et al.*, 2002b). Compared to P-E curves, RLC curves do not require stable conditions during each light stage. Normally, an RLC curve uses only 10 s of actinic light in each of the eight light stages and, therefore, the parameters ΔF/F'm and ETR indicate the current state of photosynthesis (Ralph and Gademann, 2005).

RLCs curves are described by photosynthetic variables: photosynthetic efficiency (α), saturation irradiance (Ek) and electron transport rate (ETR). These parameters have been widely used to compare, from a quantitative point of view. RLCs curves can also be used as indicators of the physiological stress of a plant (Wen-Tao *et al.*, 2010).

Along with the parameters derived from RLCs curves, two more important chlorophyll fluorescence parameters are also considered in the evaluation of the photosynthetic rate of marine fenugreek. These parameters are the *maximum* (or *potential*) *quantum yield*, Fv/Fm, and the *effective quantum yield*, ΔF/F'm.

The maximum quantum yield (Fv/Fm) is one of the parameters from which more information about chlorophyll fluorescence can be obtained. Although, theoretically, it does not depend onchlorophyll concentration, this parameter can be considered as an indicator of photoinhibition or damage to the photosystem II complex (PSII) (Rohaccek and Bartk 1999). Fv/Fm has been used as a stress indicator in plant eco-physiology studies to diagnose thermal stress, hypersensitivity, drying and other environmental stresses (Campbell *et al.*, 2006; Koch *et al.*, 2007; Kahn and Durako 2008; Biber *et al.*, 2009; Kahn e Durako 2009).

For a thorough determination of this ratio, a complete relaxation of competitive mechanisms with photochemical conversion of energy (*non photochemical quenching*) is required. Moreover, a long period of adaptation to darkness of the affected tissues is required (about 6-7 hours, Enriquez *et al.*, 2002).

Fv/Fm is a constant ratio for most terrestrial plants and ecotypes. Under non-stress, Fv/Fm is approximately 0.83 (Björkman and Demmig, 1987); in stressed or damaged plants, this ratio is greatly reduced, indicating a possible damage to PSII.

The effective quantum yield (ΔF/F'm) parameter is used to measure the efficiency of photochemical processes in PSII. Therefore, it constitutes a measure of the photosynthetic efficiency of a plant. Genty *et al.* (1989) described a linear relationship between the fluorescence parameter of chlorophyll a and the *effective quantum yield*; the *effective quantum yield* of a photochemical conversion of energy can be expressed by the following equation:

$$\text{Yield} = (F_m' - F) / F_m' = \mathbf{\Delta F/F'_m} \quad \textbf{(Equation 1)}$$

Where Fm'= maximum light-acclimatized fluorescence and F = fluorescence value for a given light state before the saturation pulse is sent.

Since this variable describes the efficiency of the entire photosynthetic process, any functional-level changes will be reflected in this parameter. The accuracy of this measurement is very high and can be acquired quickly. More detailed information on the photosynthetic process can be obtained by considering the environmental and physiological conditions to which the plant is subjected.

For a correct estimate of the parameter, it is also important to evaluate some environmental parameters such as light intensity and temperature. For example, if the ΔF/Fm value of a sample A is lower than the value of sample B, this may be due to sample A being exposed to high luminous intensity or lower temperature than sample B. The Diving Pam Fluorometers offers the ability to measure the luminous intensity (PAR) and temperature of the site where fluorescence is measured.

The two parameters mentioned above are used together with a third photosynthetic variable, obtained from RLCs curves, namely, the rate of electron transport (ETR). This parameter can be used to draw conclusions about the activity and photosynthetic capacity of the leaves (Lichtenthaler 1988, 1990). In particular, ETR is used to describe the photosynthesis of the plant in response to irradiance; similar to P-E curves.

ETR is evaluated by the following equation:

$$\text{ETR} = ([F_m' - F]) / F_m') \times \text{PAR} \times \text{AF} \times 0.5 \quad \textbf{(Equation 2)}$$

where Fm '= maximum light-acclimatized fluorescence and F = fluorescence value for a given light state, before the saturation pulse is sent, PAR = intensity of

photosynthesis-active radiation (400-700 nm), AF = average absorption of light (absorbance) by the leaves of a seagrass and 0.5 is the correction factor for two photons that must be absorbed by the PSI and PSII for each electron carried. The widely accepted value of the ratio of PSII to PSI is 0.5 (Major and Dunton, 2002).

In order to determine the correct value of ETR, it is also necessary to know the absorbance value of marine fenugreek, since it is first necessary to ascertain how much of the light that reaches the leaves is actually absorbed and used in photosynthesis.
For terrestrial plants, an absorbance value of 0.84 is used, which is inappropriate for sea lanterns. In recent studies, other values regarding absorbance factors for individual species of seagrasses ranged from 0.44 ± 0.02 for *Zostera marina*, 0.50 ± 0.03 for *Halophila stipulatcea*, 0.72 ± 0.11 for *Cymodocia nodosa*, and up to 0.78 ± 0.04 for *Thalassia testudinum* (Beer *et al.*, 1998; Durako and Kunzelman, 2002). In addition, Horn (2009) determined three absorbance factors for three other species of seagrass: 0.64 ± 0.04 for *P. sinuosa*, 0.59 ± 0.02 for *P. australis* and 0.55 ± 0.02 for *H. ovalis*, and 0.84 for *P. oceanica* (Marin-Guirao *et al.*, 2011).
Another important factor to be considered in order to obtain accurate ETR measurements using a Diving Pam is the conditions under which RLCs curves are determined. These are estimated according to the following conditions:

• the leaves are adapted in the dark;

• the leaves should not be moved within the Universal Sample Holder;

• the light coming from the Diving Pam must fall completely on the leaves;

• the distance between the end of the fibrotic tube and the leaves must remain constant;

• the leaves should not show traces of epiphytes.

A limitation on the use of RLC curves, identified by Enriquez and Borowitzka (2011), relates to their determination by brief actinic illumination; under this condition, RLC curves cannot provide an estimate of ETR and, consequently, cannot be considered as a tool to evaluate the photosynthetic response of a plant to light. Furthermore, this type of determination cannot be used to estimate the descriptors that are equivalent to the photosynthetic process obtained from the classical photosynthetic response curve to

irradiance (P-E curve), such as Pmax, α and Ek, identified by Ralph and Gademann (2005).

Another key aspect of the ETR parameter is its use as a descriptor of gross photosynthesis.

A first linear correlation between these two variables was found at low light intensities in some previous studies on marine and macro-algae ferns (Beer *et al.*, 1998; Beer and Bjork, 2000; Longstaff *et al.*, 2002; Cabello-Pasini and Figueroa, 2005; Colombo-Pallotta *et al.*, 2006; Enriquez and Rodríguez-Romàn, 2006), although the results are controversial. Possible deviations from the linearity of the relationship depend on the species of plant considered, its light history, irradiance and other factors, including CO_2 and nitrate levels. A common observation of the numerous studies carried out in this field is that, when light saturation intensity, when carbon availability is low and/or oxygen production is limited by another process, ETR values tend to overestimate the evolution rates of O_2. In general, deviations from a theoretical value of 4 for the ETR and O_2 gradient were estimated.

In conclusion, the variations in the Fv/Fm, ΔF/F'm and ETR parameters are effective measures to detect any stress in marine fenugreek, while the operation of a Diving Pam is also based on these important variables. In addition to the parameters mentioned, some studies have also evaluated other significant variables that allow acquiring complete information on the photosynthetic efficiency of a seagrass. It is known that the absorbed light could follow three different pathways; most of it is used in the photosynthetic process while excess energy can be dissipated as heat or re-emitted as fluorescence (Walker, 1987). During fluorescence measurement, the two alternative energy dissipation pathways are known as *photochemical quenching* (qP), *non-photochemical quenching* (coefficients qN and NPQ) mechanisms. *Photochemical quenching* (qP) refers to the transport of electrons while *non-photochemical quenching* (qN) refers to the thermal dissipation of energy, in the form of heat.

To determine the qP and NPQ parameters, it is necessary to estimate the maximum (Fm) and minimum fluorescence (F0) values that can be obtained following an adaptation of the plant to darkness. It is important that the various photosynthetic parameters considered are estimated under stable environmental conditions in order to avoid potential errors in the acquisition of RLC curves. Chlorophyll fluorescence emission measurements were performed following the protocol described in Marín-Guirao *et al.* (2013a, b), using a portable Diving-PAM fluorimeter (Walz, Germany).

All measurements were carried out in the morning, on dark-matched plants all night, to ensure complete oxidation of all reaction centres and primary electron acceptors. For the Fv/Fm calculation, minimum fluorescence (Fo) and maximum fluorescence (Fm) were measured on the pre-illuminated leaves with red modulated light, and then with the exposure of the photosystems to a saturated impulse (0.8 s) of white light. To measure fluorescence, each single leaf was placed in a DCL-8 leaf clip holder so as to maintain a constant distance between the leaf and the fiber optic.

The fluorescence parameters of *P. oceanica* leaves were measured, confirming the typical pattern of variation according to the age of the leaf already described for other species of fanerogame (*Thalassia testudinum*, Durako and Kunzelman, 2002; Enríquez *et al.*, 2002).

In order to prevent this variation source from masking the possible effects of treatment on Fv/Fm, fluorescence was measured at regular intervals of 4 cm from the base of the leaf to the apex in all the leaves of each shoot. The maximum value obtained was selected as the Fv/Fm value of a single shoot. The same measurement was taken on 3 randomly selected bundles for each site of each single treatment (i.e. replica n = 9 for treatment and for site).

The Rapid light curves were measured in the same area of the sheet after being exposed for 2 hours to a homogeneous radius of 100-130 μmol m^{-2} s^{-1}. *Photochemical quencing* (qP) and its maximum electronic transport rate (r-ETRmax) were obtained from the PAM WinControl program (Walz, Germany), and *non-photochemical quencing* (NPQ) was calculated according to the Maxwell method (Johnson,(2000). Finally, the absolute ETRmax was calculated by multiplying the r-ETRmax for the absorption by the leaves (the fraction of light absorbed by the leaf pigments). Since fluorescence measurement methods allow measurements without altering the state of plants in the aquarium, these measurements were carried out at three different times (T0, T1 and T2) in order to evaluate any change in Fv/Fm values throughout the experimental period.

4.4 Photosyntetic and Respiratory Rates

The photosynthesis-irradiance curves for the leaves of *P. oceanica* were determined according to the method proposed by Walker (1985) and Cayabyab and Enríquez (2007), by randomly selecting 3 shoots for each site, then 9 replicas for each site and treatment. The photosynthetic and respiratory rate was obtained through the polarographic method, using a DW3 respirometric incubation chamber connected to a Clark-type O_2 electrode, all connected to a room temperature control system.

The incubation chamber was filled with filtered seawater and maintained at the same aquarium temperature.

For the photosynthetic rate measurement, small portions of about 0.6 cm^2 of the central part were used starting from the first mature leaf of each shoot.

This leaf portion represents the point where the maximum value of Fv / Fm was recorded.

Initially, the portions were incubated in the dark for 15 minutes to determine the initial breathing rate (Rd), and then exposed to different light intensity (intensity 50 for 4 minutes and 300 µmoli $quanta^1$ s^{-1} for 6 minutes) using a tungsten halogen lamp (LS2; Hansatech, UK), and calibrating through the quantum sensor of a Diving-PAM Fluorimeter.

Water with 5 mM $NaHCO_3$ was added to the incubation chamber to prevent the reduction of carbon due to respiration, and gaseous N_2 was added to maintain the constant oxygen concentration between 20 and 80%.

Oxygen reading was continuously performed using a transduction unit (Oxygraph; Hansatech, UK) connected to a PC and the electrode.

Variations in oxygen concentration were determined for each incubation interval in mmol O_2 cm^2/h, and plotted with the corresponding irradiance value to construct the PE Curve, which showed the typical kinetic energy of saturation in relation to the irradiance of saturation Ek.

Gross photosynthesis was calculated as the sum of the net Pmax and the final Rd.

Photosynthetic efficiency (a, mmol O_2 cm^2 h^1 / mmol as m^2 s^1) was calculated as the slope of the linear regression corresponding to the initial part of the P-E curve.

Ec compensation irradiance was obtained from the intercept of this linear regression with the x axis.

Ek was calculated as the Pmax/a ratio. The P: Rd ratio was used as a daily carbon balance proxy; P was calculated by multiplying the gross-Pmax by the number of hours of light (12

h), Rd being total respiration over a period of 24 h, with final-Rd for the light period (12 h) and initial-Rd for the dark period (12 h).

4.5 Pigment Content (Chlorophill-a, b and total Carotenoids)

To determine the pigment content of the leaves of *P. oceanica*, 4 samples from each aquarium (for a total of 12 replicates for each treatment and each site) were analyzed following the methodology described by Marin-Guirao *et al.* (2013a) and Sandoval-Gil *et al.* (2014a). In particular, small segments (about 1 cm^2) were homogenized in acetone (80%) by adding a few drops of $MgCO_3$ to minimize degradation of chlorophyll content.
Subsequently, using a spectrophotometer and setting the wavelengths at 470, 646, 663 and 725 nm, the absorbance of each sample was measured.
The concentrations of chlorophyll a, b and total carotenoids were calculated using the equazone proposed by Lichtenthaler and Wellburn (1983).

4.6 Organic osmolytes: Proline

As described above, Proline plays an essential role in the morphological regulation of terrestrial plants (Stewart and Lee 1974) and Brock 1981, Tyerman 1989). The concentration of Proline was detected by applying the colorimetric method of Ninhydrin Acid (Bates *et al.*, 1973).
To determine the proline content of the leaves of *P. oceanica*, 4 samples for each bath (for a total of 12 replicates for each treatment and each site) were analyzed following the methodology described by Marin-Guirao *et al.* (2013a).
First of all, the leaf tissue portions were frozen (-80ºC, 24 h) in 3% sulfosalicylic acid and then homogenized and centrifuged. A supernatant aliquot was incubated at 100ºC for 1 hour in a solution of acetic acid, distilled water and orthophosphoric acid.
Finally, toluene was used to extract the organic phase with a chromophore and the readings were carried out at a wavelength of 520 nm. Proline concentrations were determined using a standard calibration curve and the concentration was expressed in μmol g^{-1} FW.

4.7 Leaf Water Relation of *P. oceanica*

For the determination of water relations, the osmolarity of the leaf tissue (mmol kg-1) of 4 shoots for each aquarium (for a total of 12 measurements for each treatment and for each site) was measured using a Wescor Vapor PRessure 5600 Osmometer.

For each shoot, the osmolarity was measured both on fresh and frozen tissue (at -80 ° for 4 h) to obtain water (Ψw) and osmotic (Ψπ) potential, following the method described by Sandoval-Gil (2012a) and in agreement with Tyerman (1982) and Boyer (1995). In the method described by Tyerman *et al.* (1984), the existence of an osmotic pressure gradient along the leaves is highlighted, showing the minimum value in the basal area of the leaf and the maximum value along the leaf (unpublished data).

This distance has been used as a criterion for cutting leaf segments to measure osmolarity, and is about 20 to 25 cm for the leaves of *P. oceanica*.

The leaf segments had to cover the entire base of the osmometer, so small leaf disks of 6.5/7 mm in diameter were cut.

For measurements of the water relation of fresh tissue, each sample was placed inside the osmometer chamber for 10 minutes to allow the sample to stabilize within the reading chamber, according to the protocol described by Tyerman (1982) and Murphy *et al.* (2003).

As for frozen tissue, the measurements were taken instantly, as sample stabilization is immediate.

In order to minimize the evaporation of the water content of the plant, the individual leaf disks have were cut while keeping the leaves completely submerged in their treatment water bath (Tyerman 1982, Wullschleger and Oosterhuis, 1986; Murphy *et al.*, 2003).

The osmolarity measurements made were expressed in mega-Pascales (MPa), using Van't Hoff's report (Tyerman 1982; Nobel 2009), and leaf tissue turgor pressure was finally calculated as the difference between Ww and Wp measured in individual samples (Kramer and Boyer, 1995).

The water osmolarity of each treatment was measured using the 6.5 mm disks according to the standard protrusion proposed by Wescor Inc.

4.8 Vegetative variables

When samples were taken to the laboratory, shoots for unit of treatment and site were counted, for each site 4 randomly selected for a total of 12 replicates for ech treatment and each site, marking each single shoot using the method used by Zieman (1974), a method adapted to *P. oceanica* by Romero (1989). At the end of the experimental period, all the previously labeled shoots were harvested and all morphometric variables were considered in the laboratory, including:

- the number of leaves per shoot;
- the length and width of each leaf, to calculate shoot size (cm^2 $shoot^{-1}$);
- the length of the new leaf portions (those identified by the Zieman mark) to calculate daily growth throughout the experimental period (cm^2 $shoot^{-1}$ day^{-1});
- the leaf growth rate was also calculated for younger adults, i.e. exits during the trial period (non-marked youthful leaves)
- the size of brown tissue (i.e. tissue no longer photosynthetically active) was calculated using the ratio of necrotized tissue of a single shoot.

These parameters and shoot counts were carried out at the end of the experimental period to estimate the survival rates of plants both within the mesocosm (under control conditions: 37 PSU) and under hysteresis conditions (46 PSU). The difference between the initial and final counts allowed us to estimate the percentage of shoot variation (i.e. survival and mortality); negative values indicate a marked reduction in the number of shoots due to an increase in the mortality rate, while positive values indicate an increase in the number of shoots (Ruiz *et al.*, 2009).

4.9 Statistical analyses

4.9.1 Univariate analysis

Statistical analysis of each variable was performed using the SPSS 14 statistical package. Two-way analysis of variance (ANOVA) (Underwood, 1997) was used to test the effects of two fixed factors on response variables in *EX SITU* experiments. The factors were: 'Treatment' (with two levels: 37 and 46 psu) and 'Site' (with three levels: Atolls, *Recif Barriere* and *Plateau Recifale*). For *IN SITU* study, one-way ANOVA was applied in which the fixed factor was 'Site ()' (with three levels: Atolls, *Recif Barriere* and *Plateau Recifale*). In this case, the ANOVA was performed separately, in March and July. A post-hoc mean comparison test (Student Newman Keuls, SNK) was performed when significant differences were found ($p < 0.05$). Prior to the analysis, data were checked for normality and homocedasticity, and transformed when necessary.

5. Results

5.1 *In situ* experiment

P. oceanica leaves from the Atoll sites showed significantly lower osmotic potential (Ψπ) ($p<0.01$) (Fig. 5.1.1) than those from the *Recif Barriere* and *Plateau Recifale* sites during both samplings, although greater differences were found during the July sampling, characterized by higher hypersaline waters. Also, turgor pressure (Ψp) (Fig. 5.1.1) was significantly lower ($p<0.01$) in the leaves from the Atoll sites in July, while no differences were observed in the leaf water potential (Ψw) (5.1.1.1) of leaves in March but a significant difference ($p<0.01$) was found for July.

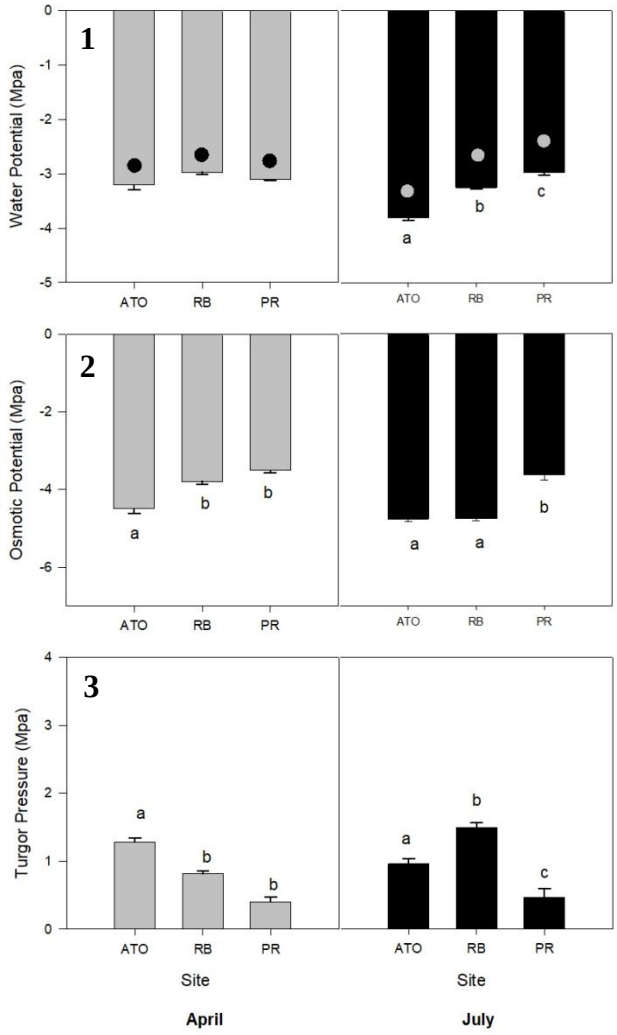

Figure 5.1.1 Leaf tissue water relations (expressed in MPa) of *P. oceanica* shoots from different sites and different sampling times. 1: Water potential March/July 2017, dots inside the bars represent seawater osmolality (expressed in pressure units, MPa); 2: Osmotic Potential March/July 2017; 3: Turgor Pressure March/July 2017. (ATO:Atolls; RB:*Recif Barriere*; PR:*Plateau Recifale*). (a, b and c indicate a homogeneous group checked by SNK test). Bars represent the mean values and standard errors.

P. oceanica leaves from the Atolls site in March ($p<0.01$) showed twofold higher proline concentrations than *Recif Barriere* and *Plateau Recifale* leaves, for both sampling periods (Fig. 5.1.2). The leaves from the *Recif Barriere* showed a significant increase in proline concentrations in July, due to a high salinity level at this site at this time, while no difference was observed for *Plateau Recifale* for both samplings.

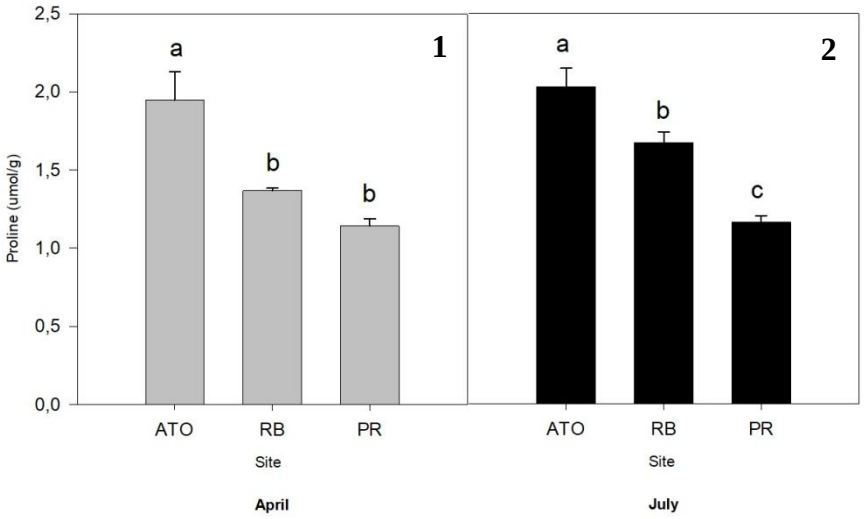

Figure 5.1.2 Proline content (expressed in µmol/g) of *P. oceanica* shoots from different sites and different samplings.
1: March 2017; 2: July 2017.
(ATO:Atolls; RB:*Recif Barriere*; PR:*Plateau Recifale*).
Letters indicate the groups of homogeneous means obtained in the post hoc SNK test ($p < 0.05$)
Bars represent the mean values and standard errors.

Plants from both sampling times showed high and significant differences in their leaf morphology. Compared to the *Plateau Recifale* sites, shoots from the Atoll sites were twofold smaller and their leaves were significantly narrower (20%) and three times shorter in July (Fig. 5.1.3). These leaves also showed a significant increment in the percentage of necrotic leaf marks (Fig. 5.1.3), while the number of leaves per shoot was similar at the three sites (Fig. 5.1.3).

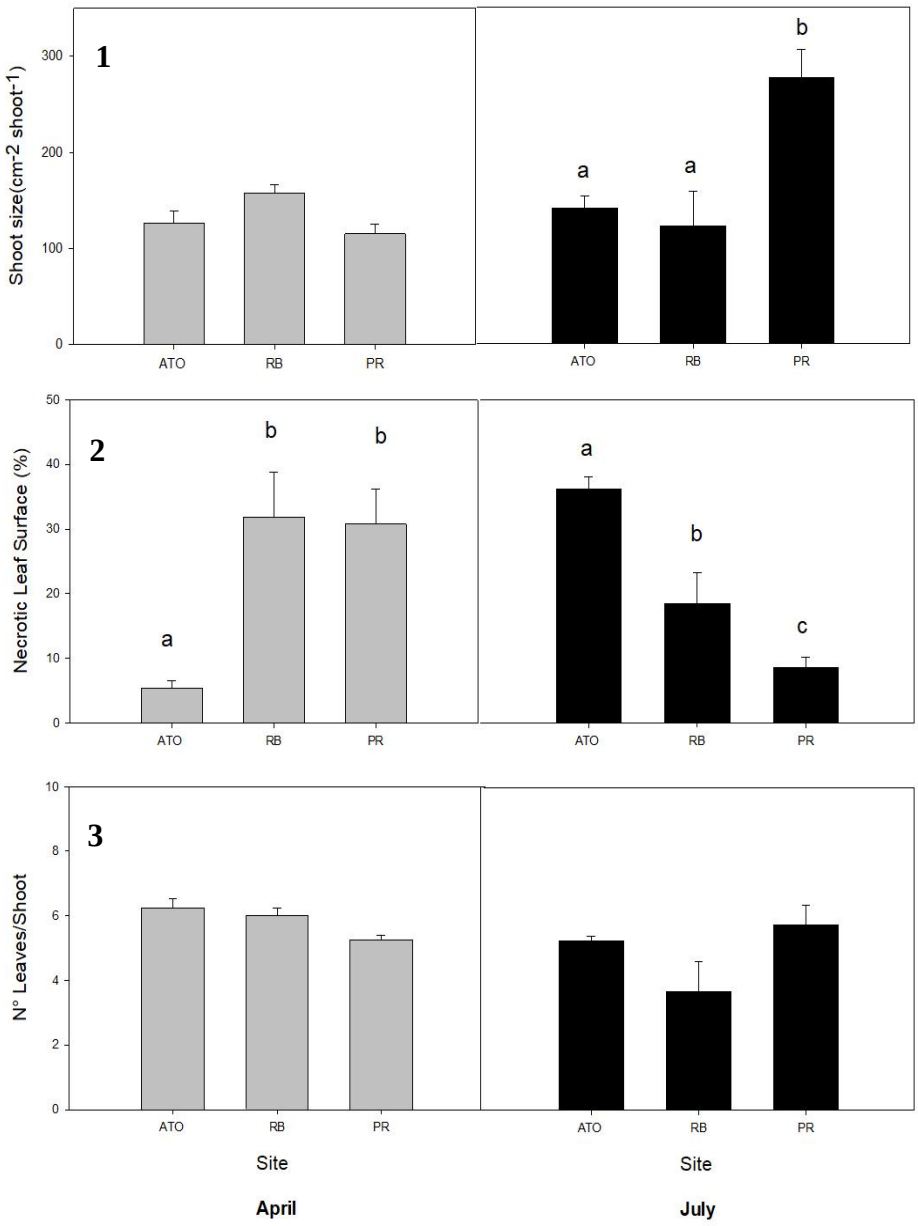

Figure 5.1.3 Vegetative variables (ATO:Atolls; RB:*Recif Barriere*; PR:*Plateau Recifale*).
1: Necrotic Surface March/July 2017; 2: Number of Leaves March/July 2017; 3: Shoot size March/July 2017.
Letters indicate the groups of homogeneous means obtained in the post hoc SNK test ($p < 0.05$).
Bars represent the mean values and standard errors.

5.2 *Ex situ* experiment

P. oceanica leaves from all sites showed significantly lower Ψw and Ψπ in high salinity treatment than those exposed to natural conditions ($p<0.01$ and $p<0.01$ respectively), while no significant differences were observed in the Turgor pressure but there is a non significant reduction in the stressed Atoll (ATO 46). Furthermore, the values of Ψw and Ψπ of all three sites showed a significant interaction between site and treatments ($p<0.05$). (Fig 5.2.1, Table 5, see below).

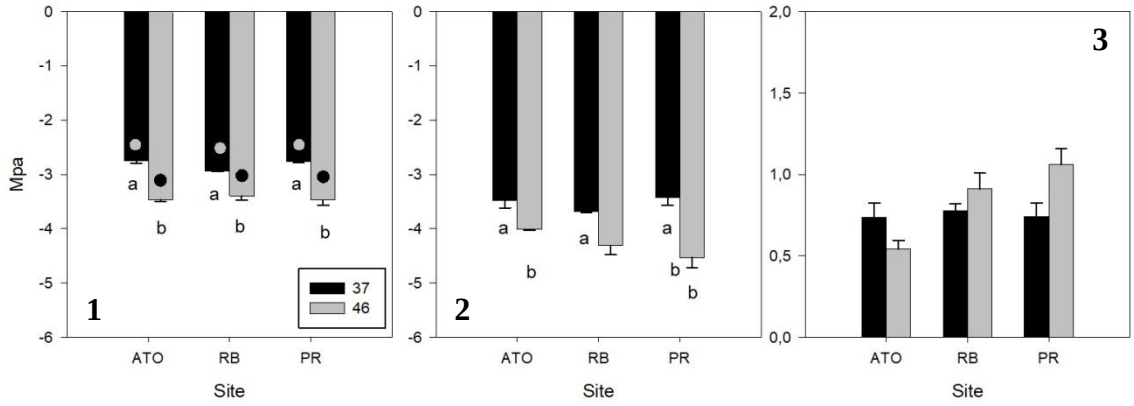

Figure 5.2.1: Leaf tissue water relations (expressed in MPa) of *P. oceanica* shoots from different sites (ATO:Atolls; RB:*Recif Barriere*; PR:*Plateau Recifale*) exposed to experimental treatments (37 and 46 psu) throughout the experimental period.
1: Water potential (Ψw), dots inside the bars represent seawater osmolality (expressed in pressure units, MPa). 2: Osmotic potential (Ψπ). 3: Turgor pressure (P). Letters indicate the groups of homogeneous means obtained in the post hoc SNK test ($p < 0.05$). Bars represent the mean values and standard errors.

P. oceanica leaves from stressed Atolls (ATO 46) showed twofold higher proline concentrations than both the Control Atolls (ATO 37), than the other two sites, *Recif Barriere* and *Plateau Recifale*, Control and Stress. Two-way ANOVA showed a significant interaction between s and treatments, for the Atolls site in particular ($p<0.05$) (Fig. 5.2.2).

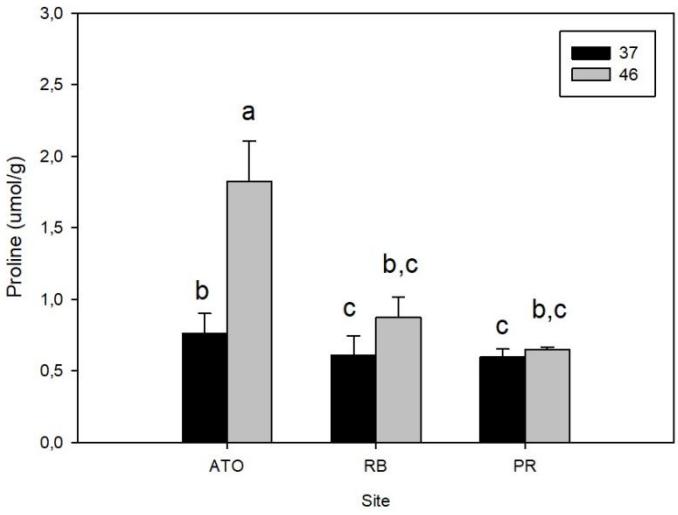

Figure 5.2.2: Proline content (expressed in µmol/g) of *P. oceanica* shoots from different sites.
(ATO:Atolls; RB:*Recif Barriere*; PR:*Plateau Recifale*) exposed to experimental treatments (37 and 46 psu) throughout the experimental period.
Different letters indicate the groups of homogeneous means obtained in the post hoc SNK test ($p < 0.05$)
Bars represent the mean values and standard errors.

Fv/Fm values for each experimental treatment throughout the experimental period are shown in Figure (5.2.3). The salinity treatments for each site did not show a significant effect on mean Fv/Fm values ($p<0.01$). There was a difference between sites but there was no difference between sites and treatments. The Fv/Fm values were much higher in *Recif Barriere* and *Plateau Recifale* than Atolls.

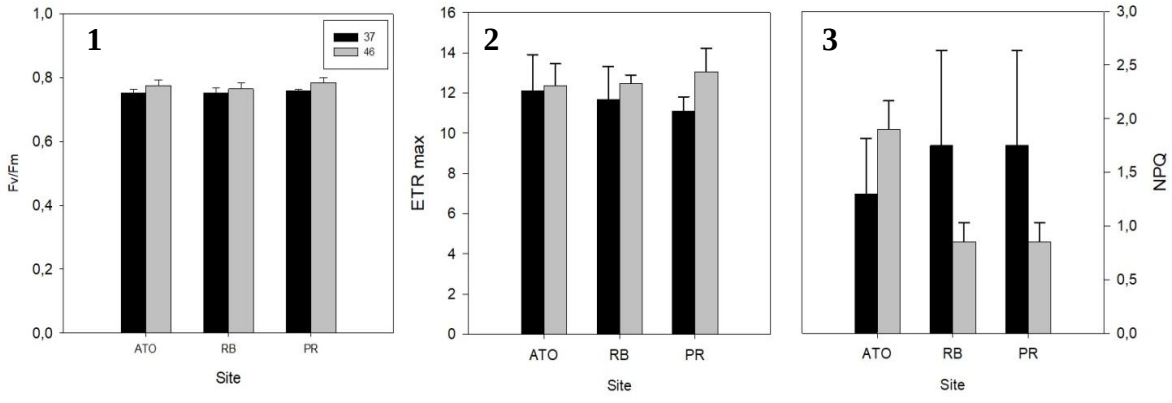

Figure 5.2.3: The Fv/Fm, ETR-max and NPQ-max values of *P. oceanica* shoots from different sites.
(ATO:Atolls; RB:*Recif Barriere*; PR:*Plateau Recifale*) exposed to experimental treatments (37 and 46 psu) throughout the experimental period. 1:Fv/Fm; 2: Etrmax; 3: Non-photechemical quencing.
Bars represent the mean values and standard errors

Non-photochemical quenching (NPQ) of salt-stressed plants increased with respect to control mean values after the hypersaline exposure of the Atolls site (Fig. 5.2.3). The ANOVA showed a significant difference between sites ($p<0.05$) but not for the interaction between site and treatments. Etr-max did not show any significant difference between sites; only an increase of this value for each treatment was observed.

Maximum net and gross photosynthetic rates (net-Pmax and gross-Pmax) were significant and higher in Stressed Atolls than the Control but consistently lower in the Stressed *Recif Barriere* and *Plateau Recifale* than the respective Control.

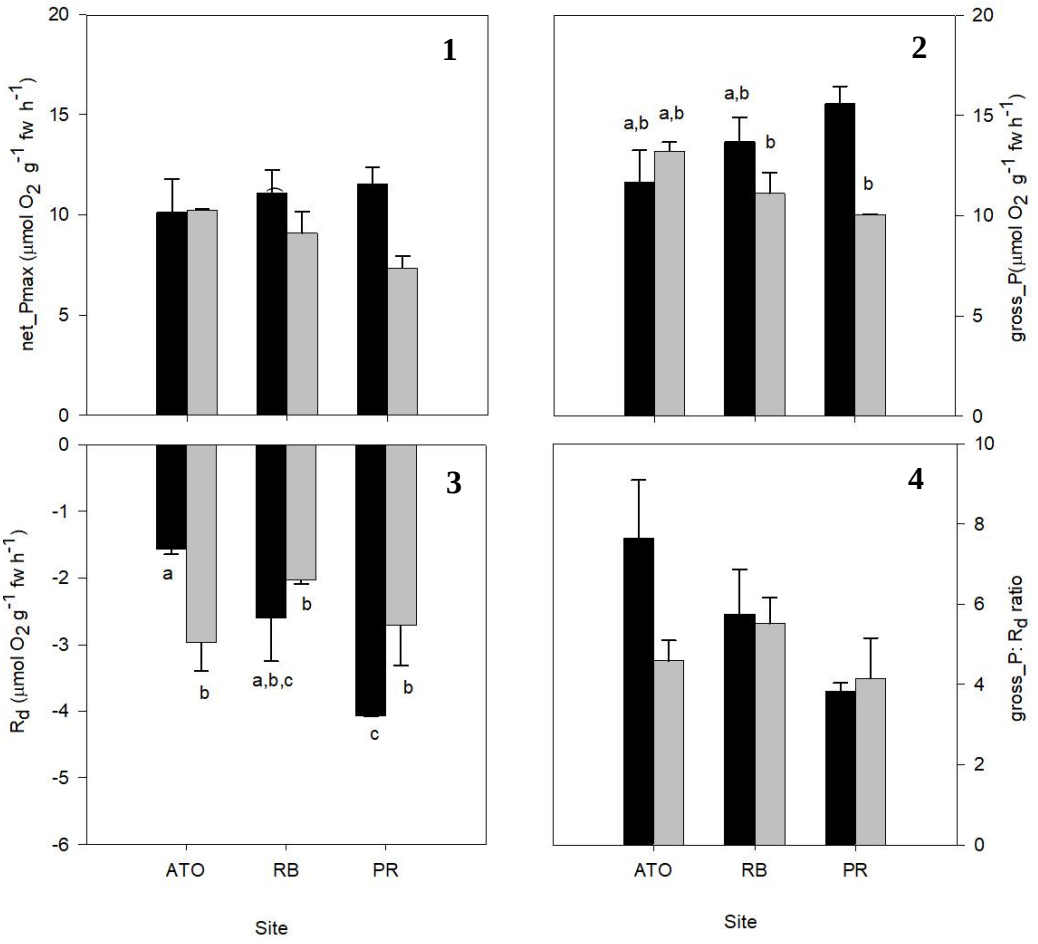

Figure 5.2.4: Photosynthetic and respiratory rates of *P. oceanica* shoots from different sites.
(ATO:Atolls; RB:*Recif Barriere*; PR:*Plateau Recifale*) exposed to experimental treatments (37 and 46 psu) throughout the experimental period.1: Net Pmax; 2: Gross Pmax; 3: Rd; 4: P:Rd Ratio.
Bars represent the mean values and standard errors.

GrossP-max differed in *Plateau Recifale* only; more negative in the 46 PSU treatments.

As regard the Respiration rates, differences were found for the hypersaline treatments only; very positive in Stressed Atolls and the opposite in Stressed *Plateau Recifale* (more negative than the Control).

The P:Rd ratio decreased for stressed Atolls compared to Control Atolls. In fact, the ANOVA highlights the differences between the hypersaline treatments (more negative in 46 PSU), but the pairwise comparison showed that these differences only applied to Atolls.

Total pigment and chlorophyll a concentrations varied in control plants during the whole experimental period between 688 and 711 mg cm^{-2} for the Atolls site, between 1117 and 1401 mg cm^{-2} for *Recif Barriere* and between 583 and 690 mg cm^{-2} for *Plateau Recifale*, treatments 37 and 46 respectively. No significant differences in leaf pigment content were observed between salt-stressed and control plants in any experimental treatment. ANOVA showed the interaction between sites for chlorophyll a and b, whith higher values in *Recif Barriere* but hypersaline treatments did not display a pattern.

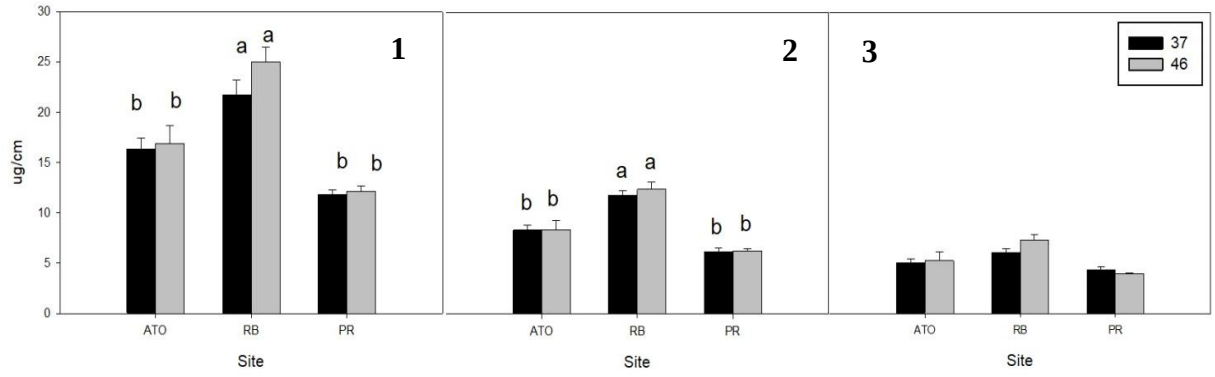

Figure 5.2.5: Pigment content rates of *P. oceanica* shoots from different sites.
(ATO:Atolls; RB:*Recif Barriere*; PR:*Plateau Recifale*) exposed to experimental treatments (37 and 46 psu) throughout the experimental period. 1: Chlorophyll a; 2: Clhorophyll b; 3: Carotenoids.
Bars represent the mean values and standard errors.

The vegetative responses of *P. oceanica* plants exposed to experimental increases in salinity are shown in Figure 6.2.7 and Figure 6.2.8. Net shoot change was significantly affected by the salinity treatments ($p<0.001$; table 5, see below) showing an average percentage loss of - 4.2 shoots at 46 PSU compared to to 37 PSU in which an increase of 1,29 shoot was recorded. However, the ANOVA interaction showed that the differences between net shoot change at different salinities depend on the maximum loss at *Recif Barriere* ($p<0.05$)

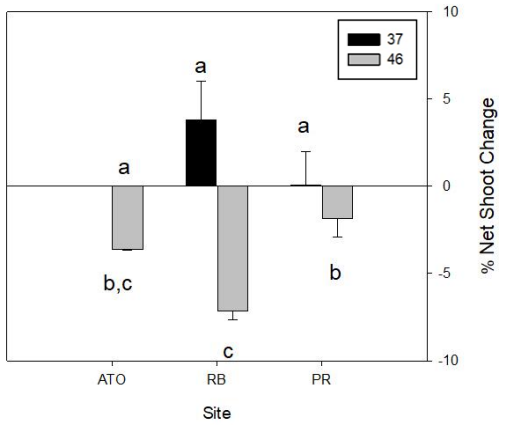

Figure 5.2.6: Net Shoot change (ATO:Atolls; RB:Recif Barriere; PR:Plateau Recifale).
Bars represent the mean values and standard errors.

However, growth rates of new leaf tissue that had formed during the experimental period (i.e. unmarked leaves) showed a significant and consistent negative interaction with treatment salinity; the ANOVA interaction showed that the differences between net shoot change at different salinities depends on site ($p < 0.05$), and was significantly lower for plants originating from the Atolls. In particular, the value decreased from 0.47 $cm^2\ d^{-1}$ for the Control Atolls to 0.34 $cm^2\ d^{-1}$ for the Stressed Atolls. The same was observed for another two stressed treatments of *Recif Barriere* and *Palteau Recifale* but with a lower reduction.

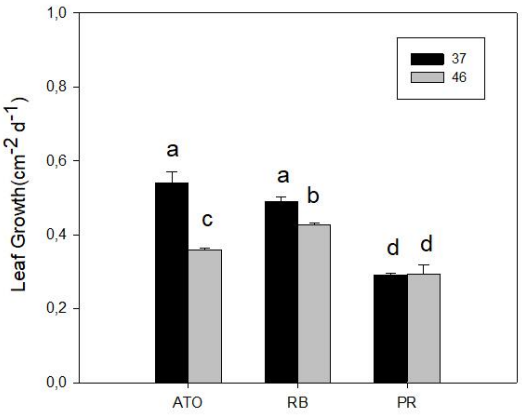

Figure 5.2.7: Leaf Growth. (ATO:Atolls; RB:Recif Barriere; PR:Plateau Recifale).
Bars represent the mean values and standard errors.

Mean values of the surface area of the necrotic leaf tissue were less than or equal to 10% and always observed at the apical senescent part of the leaves, indicating normal values for this variable obtained in natural meadows at similar depths and during the same season.

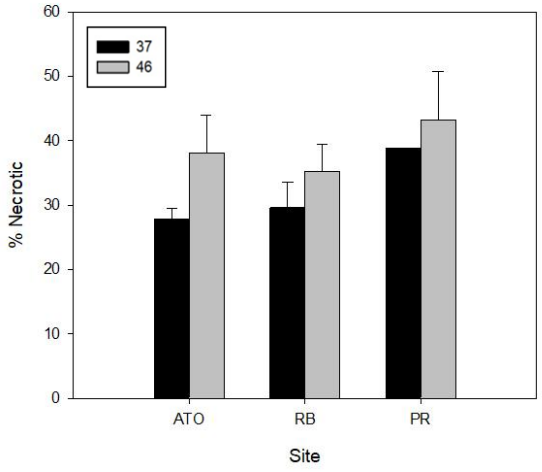

Figure 5.2.8: Necrotic leaf surface. (ATO:Atolls; RB:*Recif Barriere*; PR:*Plateau Recifale*).
Bars represent the mean values and standard errors.

Both the shoot size and number of leaves were affected by the salinity treatments. But for Atolls a consistent decrease from 163.21 to 121 cm^2 $shoot^{-1}$ for shoot size and from 6.83 to 5.83 for the number of leaves was observed.

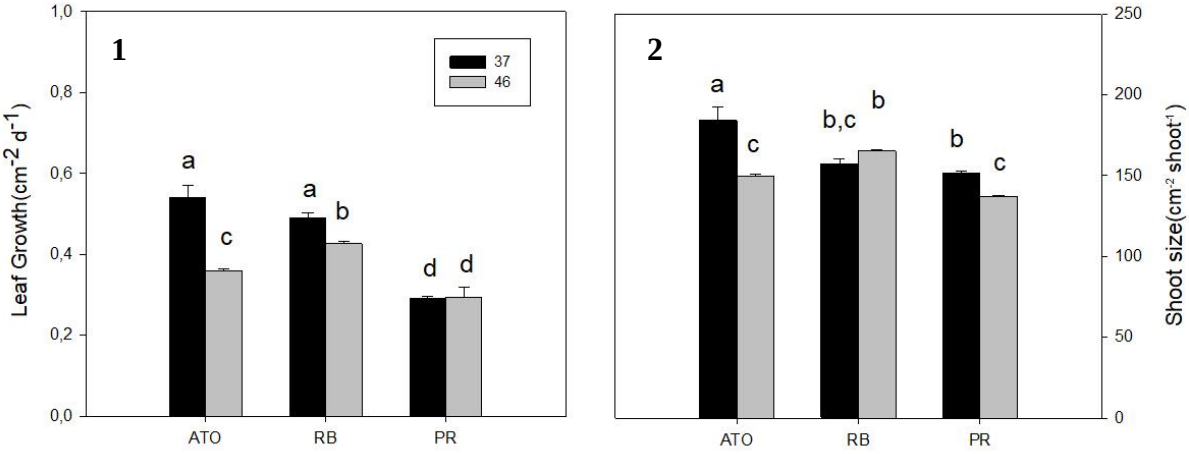

Figure 5.2.9: 1.Shoot size; 2. Number of leaves. (ATO:Atolls; RB:*Recif Barriere*; PR:*Plateau Recifale*)
Bars represent the mean values and standard errors.

6. Discussion and conclusions

The overall aim of this reasearch was to expand current knowledge on the growth *performance* and physiological traits of *P. oceanica* in this hypersaline environment.

In particular, through the two experiments it was possible to evaluate the adaptation to a natural gradient of salinity *in situ* and then isolate the salinity as the only parameter in the short-term experiment *ex situ*, in salinity conditions imposed by the experimental design.

By integrating the two responses, efforts were made to discriminate salinity as the only parameter affecting the physiological and morphological traits of the plants that are able to resist in such hypersaline environments.

P. oceanica plants growing in the Center of the Stagnone of Marsala (Atolls) are exposed to outflows of hypersaline waters due to high evaporation in the summer, and "fresh-water" from the Mediterranean Sea in the winter. Plants at these sites that are influenced by hypersaline waters also experience higher temperatures than plants from the same meadow without such influence (i.e. the *Plateau Recifale*). However, compared to these factors, salinity was the environmental parameter that showed the greatest deviations from normal conditions. At the Atoll sites, the salinity observations (on an annual basis) exceeded salinity levels up to 46 PSU; these values were well above those measured at the *Plateau Recifale* sites and clearly exceeded the critical levels established previously for this seagrass species under chronic hypersaline conditions (Sánchez-Lizaso *et al.*, 2008; Fernández-Torquemada and Sánchez-Lizaso 2005; Ruiz *et al.*, 2009a; Marín-Guirao *et al.*, 2011; Sandoval-Gil *et al.*, 2012a, b, 2014a). In fact, some of the observed effects on the Atolls site are consistent with effects previously reported for the species under hypersaline stress (Ruiz *et al.*, 2009a; Marín-Guirao *et al.*, 2011, 2013a; Sandoval-Gil *et al.*, 2012a, b, 2014a; Garrote-Moreno *et al.*, 2015). The results suggest that plants have developed acclimative homeostatic mechanisms to cope with the huge salinity fluctuations at the Atolls, although interactions with other factors (i.e. temperature) are involved. In spring, Ψw was constant across sites, while in summer *P. oceanica* shoots at the Atolls site, exhibited reduced (more negative) mean values of leaf Ψw compared to the shoots at *Recif Barriere* and *Plateau Recifale*, where the highest value was recorded. This pattern clearly reflects the salinity spatial gradients in this area, recorded during this analyses and previous studies carried out in the same area (Sarà *et al.*, 1999). In particular, non significant differences in salinity were recorded among sites in spring compared to the summer periods when Atoll, *Recife Barriere* and *Plateau Recifale* showed the highest values and

the intermediate values respectively. This degree of reduction of Ψw is a primary osmo-acclimative strategy for the plants growing at the Atolls site for coping with hypersaline stress, allowing to maintein their positive water balances under hypersaline conditions (Tyerman 1989; Murphy *et al.*, 2003; Koch *et al.*, 2007b; Sandoval-Gil *et al.*, 2012a). Previous experimental studies have demonstrated that the reduction in Ψw is mainly accomplished via osmo-regulation processes in *P. oceanica* leaves exposed to hypersaline stress, which implies active accumulation of osmolytes (ions and organic solutes) and a decrease in Ψπ (Sandoval-Gil *et al.*, 2012a; Marín-Guirao *et al.*, 2013a, 2014a).

Quite different patterns have been observed for Ψπ, expecially in March when the Atoll showed the highest and the lowest mean values respectively. Shoots at the Atolls site exhibited higher concentrations of organic osmolytes (proline) in their leaves in Marh compared to shoots from *Recif Barriere* and *Plateau Recifale*. However, since this higher accumulation was not reflected in more negative values of leaf Ψπ, it is very likely that these compatible solutes act not only as osmoticums but also as osmo-protectants, thereby helping to stabilize and protect intracellular structures and metabolic processes (Touchette, 2007; Marin-Guirao *et al.*, 2017), and being possible sinks for excess energy and reductants (Lambers *et al.*, 2006).

Another striking result was the reduced turgor pressure Ψp in leaves from the Atolls site compared to the other sites. Although it is unfortunate that results cannot provide a conclusive explanation of this response, it is more likely to be the result of modifications of cell wall properties through the so-called cell wall hardening processes. This has been described for other seagrass species in mesocosm experiments with hypersaline stress (Sandoval-Gil *et al.*, 2012a, b) or an environment with fluctuating (Marin-Guirao *et al.*, 2017) saline increments. Leaves from the Atolls may have developed thicker or more rigid cell walls, one of the main dehydration avoidance mechanisms in plants (Verslues *et al.*, 2006). Rigid cell walls enable rapid adjustment of leaf Ψw as external salinity changes through the reduction in turgor pressure promoted by small intracellular water losses (Marin-Guirao *et al.*, 2017). This osmo-acclimative strategy is more energy-conservative than osmo-regulation and allows, at the same time, a more rapid response to changes in external salinity, which can be beneficial for *P. oceanica* plants living in a highly fluctuating saline regime. However, the fact that these leaves have displayed similar turgor pressures under two contrasting conditions of hypersaline influence (Atolls vs *Plateau Recifale*) may alternatively be suggesting that they have developed more elastic cell walls, as described for some macroalgae growing in estuaries with large saline fluctuations

(Bisson and Kirst 1995) and in *P. oceanica* growing with large saline fluctuations (Marin-Guirao *et al.*, 2017). Elastic walls lead to lower turgor pressures but enable to keep them constant under fluctuating salinities thanks to their rapid adjustment to potential changes in cell volume. Modifications of cell wall properties have not been previously evidenced in *P. oceanica* plants exposed to constant hypersaline levels during weeks and months (Sandoval-Gil *et al.*, 2012a, 2014a; Marín-Guirao *et al.*, 2013a; Garrote-Moreno *et al.*, 2014), and targeted experiments are needed to discern which of the two suggested alternatives (osmoregulation *vs* rigid cell wall) is the one adopted by the species. However, it is possible that the seasonal periodic stress produced, during decades by hypersaline lagoon waters could have facilitated the development of modified cell walls in the species most probably through environmentally induced epigenetic modifications (Marin-Guirao *et al.*, 2017). At the vegetative level, the reduced plant size together with increased leaf necrosis observed in the plants of the Atolls site are considered common symptoms of hypersaline stress in *P. oceanica* (Fernández-Torquemada and Sánchez-Lizaso, 2005; Gacia *et al.*, 2007; Ruiz *et al.*, 2009; Marín-Guirao *et al.*, 2013a). Thus, these undersized plants could potentially reduce their metabolic energy consumption for maintaining vegetative structures, and hence, it should be considered as a key morphological adaptation of this species in overcoming the stressful hypersaline environment. In summary, contrary to expectations based on other experiments that used short- and medium-term (Sandoval-Gil *et al.*, 2012, 2015) exposures to chronic hypersaline conditions, it was showen that this species of seagrass is able to cope with fluctuating hypersalinity stress, and persist over time, in a location influenced by the hypersaline waters of a coastal lagoon (Stagnone of Marsala). For the first time, the physiological basis of this particular study area and type of hypersalinity tolerance is revealed, which also involves key morphological adaptations. These are, for instance, the ability to accumulate higher concentrations of organic osmolytes that allow osmotic stability. Also, it was observed that these specific *P. oceanica* plants growing in the centre of the Stagnone of Marsala manage to survive in this long-term unfavourable environment by limiting their size, as typically documented for terrestrial plants subjected to long-term environmental stress (Lichtenthaler 1996). This notion is confirmed by a previous study of the undersized *P. oceanica* shoots growing at the Atoll sites in the centre of the Stagnone of Marsala (Tomasello *et al.*, 2009; Calvo *et al.*, 2009) and also consistent with the existence of undersized *P.oceanica* shoots in other potentially unfavourable environments, such as the lagoon of Mar Menor, Spain, where hypersalinity can reach levels of up to 42 PSU (Marin-Guirao *et al.*, 2017).

This study allowed also setting up a Mesocosm enviroment that is very usefull for testing short-term haline stress in *P. oceanica*, and to isolate salinity as the only parameter, while keeping temperature constant.

The results regarding growth and physiological *performance* of shoots exposed to normal conditions (37 PSU) showed mean leaf growth rates and photosynthetic activity (Pmax) values within the ranges found in previous *in situ* studies on *P. oceanica* (Alcoverro *et al.*, 1998; Ruiz and Romero, 2001, 2003; Sandoval-Gil *et al.*, 2015; Marin.Guirao *et al.*, 2017). Fv/Fm values were also consistent with those recorded in healthy plants of other seagrass species (Ralph, 1999; Durako and Kunzelman, 2002; Ralph *et al.*, 2002; Cayabyab and Enríquez, 2007). Mean values of these plant variables obtained in the control treatment were also very close to those previously measured in plants from the same sampling site during the same season (unpublished data, characterization of the study area), indicating that the experimental manipulation caused low or negligible stress on plants. Moreover, the survival rates (ca. 100%) of the transpalted units of *P. oceanica* in the control aquaria, throughout the 4 week experimentatl period, are certainly worth highlighting. This is quite a notable success, considering that this species has been particularly problematic when used for laboratory experiments due to its low plasticity and strict ecological requirements. Significant shoot mortality has been reported for fragments of *P. oceanica* and other seagrass species kept under control conditions in laboratories for shorter experimental periods than the one of this study (e.g. Fernández-Torquemada and Sánchez-Lizaso, 2005; Cayabyab and Enríquez, 2007; Marin-Guirao *et al.*, 2011). What is interesting about these results is that the mesocosm system used in this study maintains an optimal physiological status and vitality of the plantsand also minimises further stress caused by experimental manipulation that could confuse the main effect of experimental treatments. This study has provided experimental evidence that the photosynthetic capacity of *P. oceanica* is highly sensitive to increases in salinity simulated in a mesocosm system. In fact, the photosynthetic rates (both net- and gross-Pmax) were significantly and consistently lower at higher salinities (46 PSU) compared to the control treatment (37 PSU) for *Recif Barriere* and *Plateau Recifale*, but higher at the Atolls. Partial inhibition of photosynthetic O_2 production has consistently been reported for temperate and tropical seagrass species exposed to experimental hypersaline conditions (Ogata and Matsui, 1964; Biebl and McRoy, 1971; Beer *et al.*, 1980; Kerr and Strother, 1985; Fernández-Torquemada *et al.*, 2005; Koch *et al.*, 2007b).

As regards the respiratory rate in plants originating from the Atoll site, an increase in the respiratory rate was observed, which can probably be explained by an increase in the demand for metabolic energy, while a completely opposite pattern was found at *Plateau Recifale*, probably caused by inhibition due to the increase in salinity. Such an increment in the photosynthetic rates at the Atolls can be explained by an increase in energy requirements for the production of organic osmolytics, which are responsible for osmoregoulation and osmoprotection. This increase in demand for metabolic energy can be explained by the increase in the concentration of organic osmolytes. In fact, it is possible that the values of proline concentration are the same in plants from all three sites with the same salinity conditions but, after high salinity exposure, Atoll plants showed an increase compared to the other two sites. On the other hand, shoots growing at the Atolls exhibited higher concentrations of organic osmolytes (proline and soluble sugars) in their leaves at the Control compared to shoots from *Recif Barriere* and *Plateau Recifale*. However, since this higher accumulation was reflected in more negative values of leaf $\Psi\pi$, it is very likely that these compatible solutes also act as osmo-protectants rather than osmoticums, by helping to stabilize and protect intracellular structures and metabolic processes (Touchette, 2007), and being possible sinks for excess energy and reductants (Lambers *et al.*, 2006).

These results suggest that *P. oceanica* plants growing in the center of the Stagnone of Marsala under the fluctuating influence of hypersaline waters for decades possess an osmo-acclimation strategy that differs from that documented in mesocosm experiments, where plants growing under natural salinity levels (37–38 PSu) were exposed to persistent hypersalinity (up to 46 PSU) during days or months (Sandoval-Gil *et al.*, 2012a; Marín-Guirao *et al.*, 2013a, 2014a). Thus, it can be inferred that, at longer time scales, the species has been able to develop physiological and/or structural properties to support this particular strategy, which allow the plants to resist fluctuating salinities in the long term. However, other factors (such as Temperature) are necessary to explain the metabolic and morphological adjustments adopted by *P. oceanica* to persist at the study site. Hence, caution should be applied in directly extrapolating the results of the current study to other situations. Therefore, while *P.oceanica* is certainly a stenohaline species, it also demonstrates a certain capacity to resist and survive under fluctuating hypersaline stress in natural environments. Finally, these particular populations could serve as an experimental model, at Mediterranean scale, for what might happen under the predicted environmental changes associated with global climate change.

7. Appendix

In situ experiment

Table 1 March T0 (Oneway ANOVA)

	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
PROLINA	Intercept	26.47	1	26.47	568.96	.000
		1.38	2	.691	14.85	.001
Water Potential	Intercept	113.23	1	113.23	15351.52	.000
		.062	2	.031	4.20	.051
Osmotic Potential	Intercept	178.49	1	178.49	9047.66	.000
		1.56	2	.778	39.45	.000
Turgor Pressure	Intercept	7.39	1	7.39	329.10	.000
		1.24	2	.62	27.72	.000
Chl a	Intercept	2.49E+07	1	2.492E+07	117.33	.000
		41473.35	2	20736.67	.098	.908
Chl b	Intercept	5169176.56	1	5169176.56	140.34	.000
		104.07	2	52.03	.001	.999
Carotenoids	Intercept	2279913.32	1	2279913.32	85.47	.000
		216.38	2	108.19	.004	.996
Ratio b/a	Intercept	2.46	1	2.46	2588.78	.000
		.005	2	.002	2.47	.140
Shoot size	Intercept	219143.02	1	219143.02	180.81	.000
		3097.80	2	1548.90	1.28	.325
Necrotic	Intercept	10170.45	1	10170.45	19.52	.002
		3488.72	2	1744.36	3.35	.082
New Leaves	Intercept	408.33	1	408.33	668.18	.000
		2.17	2	1.08	1.77	.224

Table 2 July T1 (Oneway ANOVA)

	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
PROLINA	Intercept	31.52	1	31.55	1170.30	.000
		1.51	2	.758	28.10	.000
Water Potential	Intercept	135.32	1	135.32	22814.49	.000
		1.33	2	.66	112.07	.000
Osmotic Potential	Intercept	230.28	1	230.28	7370.88	.000
		3.40	2	1.70	54.45	.000
Turgor Pressure	Intercept	12.54	1	12.54	400.52	.000
		1.56	2	.783	25.01	.000
Chl a	Intercept	5757941.59	1	5757941.59	60.49	.000
		420487.30	2	210243.65	2.20	.166
Chl b	Intercept	1577822.04	1	1577822.04	67.45	.000
		96181.03	2	48090.51	2.05	.184
Carotenoids	Intercept	473760.74	1	473760.74	54.83	.000
		28278.98	2	14139.49	1.63	.248
Ratio b/a	Intercept	3.35	1	3.35	1374.14	.000
		.009	2	.005	1.89	.206
Shoot size	Intercept	460611.83	1	460611.83	43.06	.000
		40770.61	2	20385.30	1.90	.204
Necrotic	Intercept	21051.40	1	21051.40	23.91	.001
		1850.60	2	925.30	1.05	.389
New Leaves	Intercept	320.33	1	320.33	67.83	.000
		3.16	2	1.58	.335	.724

Table 3 MarchT0 (Mean values of principal analyses)

	Prolina	Chla (µg/G)	Chlb (µg/g)	Carotenoids (µg/g)	chlb/chla ratio	Water pot (Mpa)	Osm pot (Mpa)	Turgor (Mpa)	Shoot size (cm^2 $shoot^{-1}$)	Necrotic leaf surface (%)	Number leaves shoot-1
1	1.46	1859.87	794.93	536.90	0.42	-3.25	-4.49	1.24	114.13	7.74	6.00
1	2.33	2091.55	909.13	706.07	0.43	-3.19	-4.33	1.13	70.40	3.36	5.00
1	2.05	1376.50	607.93	353.68	0.43	-3.05	-4.53	1.48	149.85	0.00	7.00
1	1.95	611.69	299.27	169.20	0.48	-3.06	-4.11	1.05	171.30	16.80	7.00
2	1.41	1830.33	807.56	564.34	0.43	-2.87	-3.78	0.91	164.13	0.00	6.00
2	1.36	1558.63	664.77	488.83	0.42	-2.91	-3.48	0.58	157.44	87.25	6.00
2	1.33	1025.87	462.54	264.03	0.44	-3.06	-3.66	0.60	119.30	51.40	5.00
2	1.37	1506.14	689.71	422.85	0.45	-3.05	-3.60	0.54	189.80	55.19	7.00
3	1.04	1059.69	503.50	294.96	0.47	-3.14	-3.69	0.55	155.80	48.05	5.00
3	1.23	1787.19	836.09	523.45	0.46	-3.02	-3.51	0.49	86.10	32.65	5.00
3	1.16	1366.19	624.72	485.42	0.45	-3.14	-3.58	0.44	128.00	33.95	5.00
3	1.14	1218.64	675.79	420.84	0.55	-3.12	-3.52	0.40	115.39	12.96	6.00

Table 4 July T1(Mean values of principal analyses)

	Prolina	Chla (µg/G)	Chlb (µg/g)	Carotenoids (µg/g)	Chlb/Chla ratio	Water pot (Mpa)	Osm pot (Mpa)	Turgor (Mpa)	Shoot size (cm^2 $shoot^{-1}$)	Necrotic leaf surface (%)	Number leaves shoot-1
1	1.88	955.93	499.83	274.38	0.51	-3.74	-4.64	0.90	169.98	54.00	6.00
1	2.36	464.21	239.49	132.43	0.51	-3.79	-4.77	0.98	105.75	46.44	5.00
1	1.85	1335.62	679.28	391.87	0.50	-3.79	-4.92	1.13	100.52	39.56	5.00
1	2.03	693.00	347.98	186.54	0.49	-3.91	-4.73	0.82	187.15	55.46	5.00
2	1.47	701.27	361.80	183.87	0.51	-3.28	-4.63	1.34	313.49	102.96	7.00
2	1.74	477.13	254.57	127.81	0.53	-3.26	-4.87	1.62	248.36	77.78	6.00
2	1.68	897.39	466.49	260.21	0.51	-3.19	-4.75	1.56	119.48	28.66	5.00
2	1.79	1060.47	552.14	299.02	0.51	-3.30	-4.75	1.45	120.00	26.00	0.00
3	1.17	237.60	132.16	64.33	0.55	-2.86	-3.47	0.60	237.78	27.36	4.00
3	1.27	267.86	187.67	106.06	0.69	-3.08	-3.42	0.34	147.65	8.40	4.00
3	1.09	853.42	428.06	256.20	0.49	-3.06	-4.00	0.94	364.35	52.18	7.00
3	1.12	368.47	201.83	101.63	0.54	-3.04	-3.63	0.59	356.52	9.81	8.00

Ex Situ experiment

Table 5 (Twoway ANOVA)

	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Prolina		1.521	2	.760	10.894	.002
	TratSal	.940	1	.940	13.465	.003
	* TratSal	.856	2	.428	6.133	.015
	Total	18.2	18			
Water Potential		.014	2	.007	.658	.536
	TratSal	1.8	1	1.8	176.3	.000
	* TratSal	.083	2	.041	3.9	.048
	Total	177.9	18			
Osmotic Potential		.193	2	.097	1.482	.266
	TratSal	2.3	1	2.327	35.7	.000
	* TratSal	.274	2	.137	2.1	.165
	Total	276.2	18			
Turgor Pressure		.231	2	.115	5.9	.016
	TratSal	.031	1	.031	1.6	.228
	* TratSal	.203	2	.101	5.2	.023
	Total	12.1	18			

	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
ETR-max		.097	2	.048	.010	.990
	TratSal	4.5	1	4.5	.984	.341
	* TratSal	2.3	2	1.1	.249	.783
	Total	2708.1	18			
Yield-max		.000	2	.000	3.0	.087
	TratSal	.002	1	.002	24.0	.000
	* TratSal	.000	2	.000	1.1	.375
	Total	10.5	18			
Yield-eff		.044	2	.022	11.1	.002
	TratSal	.002	1	.002	1.2	.292
	* TratSal	.002	2	.001	.556	.588
	Total	6.6	18			
NPQ		9.7	2	4.9	5.7	.018
	TratSal	.150	1	.150	.177	.682
	* TratSal	3.7	2	1.9	2.2	.153
	Total	98.6	18			

Table 6 (Twoway ANOVA)

	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Shoot Size		1705.5	2	852.8	20.0	.000
	TratSal	834.906	1	834.9	19.6	.001
	* TratSal	1341.0	2	670.5	15.7	.000
	Total	449730.4	18			
Necrotic Surface		413.0	2	206.5	3.2	.073
	TratSal	41.5	1	41.5	.660	.432
	* TratSal	61.202	2	30.6	.487	.626
	Total	23938.5	18			
Leaf Growth		.106	2	.053	82.1	.000
	TratSal	.029	1	.029	44.6	.000
	* TratSal	.026	2	.013	20.0	.000
	Total	3.1	18			
N° Leaves		2.1	2	1.0	2.9	.091
	TratSal	.926	1	.926	2.6	.131
	* TratSal	1.1	2	.568	1.6	.240
	Total	655.4	18			
Net Shoot Change		2.98	2	1.5	.296	.749
	TratSal	135.915	1	135.915	27.058	.000
	* TratSal	68.487	2	34.244	6.817	.01
	Total	305.865	18			

	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Rd		4,8	2	2,4	4,9	,027
	TratSal	.134	1	.134	.275	.609
	* TratSal	6.1	2	3.1	6.3	.014
	Total	144.1	18			
Pgross		.631	2	.316	.106	.900
	TratSal	21.6	1	21.6	7.2	.020
	* TratSal	37.8	2	18.9	6.3	.013
	Total	2933.9	18			
Pmax		1.9	2	.992	.311	.739
	TratSal	18.3	1	18.3	5.7	.034
	* TratSal	13.9	2	6.9	2.2	.157
	Total	1836.3	18			
P-Rd Ratio		14.8	2	7.4	2.9	.095
	TratSal	4.4	1	4.4	1.7	.215
	* TratSal	9.8	2	4.9	1.9	.192
	Total	554.8	18			

Table 7 (Mean values of principal analyses)

		Salinity Treatment	Proline	Chla (µg/G)	Chlb (µg/g)	Carotenoids (µg/g)	Chlb/Chla ratio	Water pot (Mpa)	Osm pot (Mpa)	Turgor (Mpa)	Leaf growth (cm^2 day^{-1})	Shoot size (cm^2 $shoot^{-1}$)	Necrotic leaf surface (%)	Number leaves $shoot^{-1}$
1: ATO	1	1	0.75	1054.23	522.23	305.50	0.49	-2.68	-3.30	0.62	0.60	200.44	31.02	6.50
2: RB	1	1	1.01	473.55	248.58	136.64	0.52	-2.73	-3.41	0.68	0.52	178.33	26.50	6.50
3: PR	1	1	0.52	538.06	271.42	165.62	0.50	-2.83	-3.74	0.91	0.50	173.12	25.97	7.50
	1	2	2.12	668.91	336.04	222.61	0.48	-3.41	-4.06	0.65	0.37	152.09	28.10	6.50
1: 37 psu	1	2	2.08	593.11	288.06	170.22	0.56	-3.50	-4.00	0.50	0.36	147.22	23.58	6.33
2: 46 psu	1	2	1.26	872.75	431.05	251.47	0.48	-3.49	-3.97	0.47	0.35	149.65	37.18	5.50
	2	1	0.50	1138.44	558.51	319.04	0.53	-2.95	-3.68	0.84	0.47	157.18	38.86	5.75
	2	1	0.88	1437.42	820.80	369.07	0.51	-2.95	-3.65	0.70	0.48	162.53	38.90	5.00
	2	1	0.45	777.20	385.11	238.42	0.50	-2.92	-3.72	0.80	0.52	151.82	38.82	6.00
	2	2	0.98	1465.91	729.95	443.14	0.49	-3.53	-4.64	1.10	0.44	164.06	33.54	6.75
	2	2	1.04	1522.21	756.17	447.21	0.48	-3.26	-4.09	0.83	0.42	166.19	31.16	5.25
	2	2	0.60	1216.22	605.85	338.40	0.49	-3.40	-4.20	0.80	0.42	165.12	49.72	5.50
	3	1	0.51	737.47	397.70	280.78	0.49	-2.81	-3.50	0.69	0.30	153.81	36.70	6.00
	3	1	0.59	496.49	256.54	208.27	0.49	-2.73	-3.16	0.63	0.29	151.17	27.30	6.00
	3	1	0.70	517.77	261.83	164.36	0.49	-2.72	-3.63	0.91	0.28	148.53	41.63	6.75
	3	2	0.68	687.99	357.85	236.79	0.52	-3.66	-4.90	1.24	0.29	135.17	47.74	4.75
	3	2	0.63	595.23	310.30	208.01	0.51	-3.37	-4.41	1.04	0.29	136.67	28.71	6.00
	3	2	0.63	789.00	399.83	225.52	0.49	-3.38	-4.28	0.90	0.30	138.17	53.33	5.33

Table 8 (Mean values of principal analyses)

		Salinity Treatment	rETRmax	Yieldmax	Yield-eff	NPQ	Netshoot change	Pmax	Rd	Pgross	P:Rd
1: ATO	1	1	9.07	0.76	0.69	0.87	0.00	13.42	1.41	14.83	10.50
2: RB	1	1	11.93	0.76	0.58	2.36	0.00	8.84	1.56	10.40	6.66
3: PR	1	1	15.30	0.74	0.66	0.72	0.00	8.10	1.71	9.80	5.74
	1	2	11.50	0.77	0.57	2.02	-3.70	10.35	2.97	13.32	4.48
1: 37 psu	1	2	11.03	0.77	0.61	1.44	-3.64	10.13	2.24	12.37	5.52
2: 46 psu	1	2	14.53	0.78	0.65	2.35	-3.57	10.24	3.71	13.94	3.76
	2	1	8.43	0.74	0.68	0.28	3.70	10.47	3.89	14.36	3.69
	2	1	12.83	0.75	0.66	1.68	7.69	13.34	2.03	15.37	7.56
	2	1	13.77	0.77	0.67	3.92	0.00	9.44	1.89	11.33	6.00
	2	2	11.83	0.76	0.57	0.87	-8.00	7.24	2.13	9.37	4.39
	2	2	12.30	0.76	0.60	1.18	-7.13	10.95	1.93	12.88	6.67
	2	2	13.27	0.77	0.71	0.57	-6.25	9.09	2.03	11.12	5.47
	3	1	9.97	0.75	0.55	1.78	3.45	10.05	4.06	14.11	3.48
	3	1	12.43	0.75	0.50	2.70	0.00	12.99	4.08	17.07	4.19
	3	1	10.87	0.77	0.54	3.21	-3.23	11.52	4.07	15.59	3.83
	3	2	10.83	0.78	0.54	3.42	0.00	6.27	3.76	10.03	2.67
	3	2	13.53	0.79	0.51	3.49	-3.70	8.42	1.66	10.08	6.08
	3	2	14.80	0.78	0.57	3.84	-1.85	7.34	2.71	10.05	3.71

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