PhD Thesis

Biochemical and molecular tools to monitor *Posidonia oceanica* (L.) Delile meadows

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PhD in Applied Biology

BIOCHEMICAL AND MOLECULAR TOOLS TO MONITOR *Posidonia oceanica* (L.) Delile MEADOWS

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STRUMENTI BIOCHIMICI E MOLECOLARI PER IL MONITORAGGIO DELLE PRATERIE DI *Posidonia oceanica* (L.) Delile

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Stretch'd on her mossy couch, in trackless deeps,
Queen of the coral groves, Zostera sleeps;
The silvery sea-weed matted round her bed,
And distant surges murmuring o'er her head.
High in the flood her azure dome ascends,
The crystal arch on crystal columns bends;
Roof'd with translucent shell the turrets blaze,
And far in ocean dart their colour'd rays;
O'er the white floor successive shadows move,
As rise and break the ruffled waves above.
Around the nymph her mermaid-trains repair,
And wave with orient pearl her radiant hair;
With rapid fins she cleaves the watery way,
Shoots like a silver meteor up to day;
Sounds a loud conch, convokes a scaly band,
Her sea-born lovers, and ascends the strand.

A stanza dedicated to seagrasses from the poem '*The Loves of the Plants*' written by Erasmus Darwin (1789), father of Charles Robert

To Lina, my grandmother

ABSTRACT

Posidonia oceanica (L.) Delile is the dominant endemic seagrass in the Mediterranean Sea, where it forms highly productive meadows. Worldwide seagrass monitoring is an issue of increasing interest in research, management and policies, due to the ecological role of these ecosystems, their global decline and their ecological indicator characteristics. The choice and combination of measurable, sensitive and integrative descriptors that adequately reflect the environmental alterations is a challenge for the whole scientific community.

This work has been devoted to the identification and development of different generation of "diagnostic" tools to be used in monitoring of the seagrass meadows conservation status. These tools are based on biochemical and molecular approaches that can provide early and reliable information on the ecophysiological status of plants (e.g. phenol determination, proteomics, RAPD genetic analysis, oxidative stress markers, etc.)

Results provide evidences of their convenient application as markers of the health status of *P. oceanica* and/or other seagrass meadows; some of them are early-warning indicators of plant stress responses. Furthermore, the proposed tools are standardizable, provide detailed information about physiological status of the plants and can be integrated to the traditional methods of investigation.

The approach proposed by this research project makes available several tools to establish the linkage between stressor and seagrass response and to better understand the effects of complex disturbances.

This PhD thesis is part of a research project developed in the Ecotoxicology laboratory at the University of Rome “Tor Vergata”

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A.R.

Contents

1. INTRODUCTION

Posidonia oceanica (L.) Delile

Posidonia oceanica (L.) Delile is the dominant endemic seagrass in the Mediterranean Sea (Procaccini et al., 2003). It is a monocotyledon belonging to the Posidoniaceae family.

Class	Liliopsida
Order	Alismatales
Family	Posidoniaceae
Genus	*Posidonia*
Species	*P. oceanica*

The genus *Posidonia* includes nine species, eight occurring in the waters of temperate Australia (Cambridge and Kuo, 1979; Kuo and Cambridge, 1984), and *P. oceanica* completely restricted to the Mediterranean Sea (Den Hartog, 1970). This pattern of distribution took place at a relatively early time in the history of the seagrasses, probably during the Late Eocene. The origins of the genus are postulated to have been in the Tethys Sea (Hemminga and Duarte, 2000).

Seagrasses possess the same organs and tissues as other terrestrial flowering plants (fig. 1.1) but they had to adapt to marine environments and this has profoundly influenced their morphology/anatomy and physiology (Kuo and den Hartog, 2006).

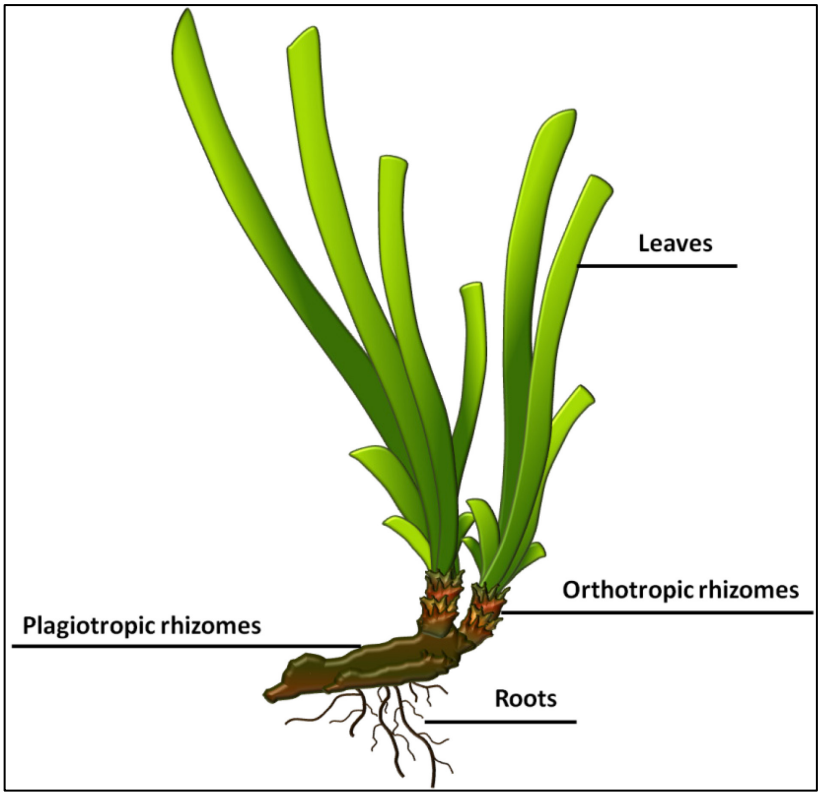

Fig. 1.1 *P. oceanica* (L.) Delile.

P. oceanica requires stable environmental conditions, preferring a coarse-grained sandy substratum but ranging from soft substrata (but not muddy sediment) to rocks. It is stenohaline and is absent when salinity is below 33‰; it does not resist to desiccation, but is able to tolerate a wide range of temperatures, *i.e.* 9-29 °C (Boudouresque et al., 2006).

P. oceanica shows both generative and vegetative growth, although the vegetative propagation of rhizomes has greater importance in the maintenance and spreading than seed production (Procaccini and Mazzella, 1998). It has inflorescences with bisexual flowers (hermaphrodite) and floating fruits. Flowering shows high spatial and temporal variability: is rare in cold waters along North-Western shores, while is frequent or even annual in the Southern and Eastern regions of the Mediterranean basin (Molinier and Picard, 1952; Den Hartog, 1970). Time of flowering (April-June) is coincident in both hemispheres (the same in all species of the genus *Posidonia;* Gobert et al., 2006).

Strap-shaped leaves are 10 mm width and up to 1 m long, with a life-span varying between 7 and 12 months. The old adult leaves got lost throughout the year

but storm events induce further leaf breakage and fall. When the leaves fall down, the leaf sheaths remains attached to the rhizomes constituting a persistent and fibrous cover. Furthermore, the finally detached sheath fibers are roller by wave action to form 'marine balls', found on Mediterranean beach since ancient times and called *Egagropile* [from Greek *αἴγαγρος* «goat» e *πῖλος* « stacked hairs»] because they look like those found in ruminant stomach. The foliage forms a unit of 6-7 leaves (called shoot), containing different developmental stages (with older leaves on the outside). Rhizomes grow either vertically to avoid burial (orthotropic), or horizontally to enable wider colonization (plagiotropic). The main functions of the rhizome are mechanical support, nutrient storage and regulation/maintenance of vegetative growth. The rhizome biomass shows low seasonal variability. The roots are adventitious and arise from the lower surface of the rhizomes. They have functions of both anchoring and nutrients acquisition, even if leaf tissues may play a much important role in nutrients uptake according to their availability (Kuo and den Hartog, 2006).

The leaves act as sediment-traps allowing the accumulation of inorganic and organic particulate matter on the sea bottom. The progressive silting and the two types of rhizome growth result in a typical terraced formation called '*matte*' consisting of the intertwining of various strata of rhizomes, roots, and sediment, with an average accretion rate of 0.175 cm/year (Gobert et al., 2006).

Seagrass meadows: ecological role and threats

Seagrass meadows constitute an engineering ecosystem playing a major ecological, geological and economic role in shallow coastal waters around the world except Antarctica (Spalding et al., 2003)

In the Mediterranean Sea, *P. oceanica* forms monospecific meadows, between the surface and 35-40 m depth, with different types of coverage pattern and shoot density ranging from 150-300 shoots/m^2 (very sparse bed) to more than 700 shoots/m^2 (very dense bed), according to Giraud (1977) classification.

P. oceanica meadows are highly productive ecosystems, one of the most important in the Mediterranean coastal waters (fig. 1.2 and 1.3; Boudouresque et al., 2006). They cover large areas, a global surface of 37,000 km^2, corresponding to about 1–2% of the Mediterranean Sea bottom (Pasqualini et al. 1998; Boudouresque

et al., 2006). Similar to a forest on land, the *P. oceanica* meadow is the "climax" community in the coastal sea-bed, *i.e.* the final event of a very long ecological succession (Molinier and Picard, 1952).

Seagrass meadows play an important role in ecosystems of shallow coastal waters in several ways by (as reported in Boudouresque et al., 2006):

(1) producing high amount of oxygen and organic matter;

(2) sustaining complex food nets and providing habitat for a highly diverse biota;

(3) playing a crucial role in coastal preservation, by stabilizing sediments and reducing hydrodynamics effects (see fig. 1.4);

(4) offering a nursery/refuge sites for many fish and invertebrate species.

Fig. 1.2. *Posidonia oceanica* meadow (courtesy of Valentina Martini).

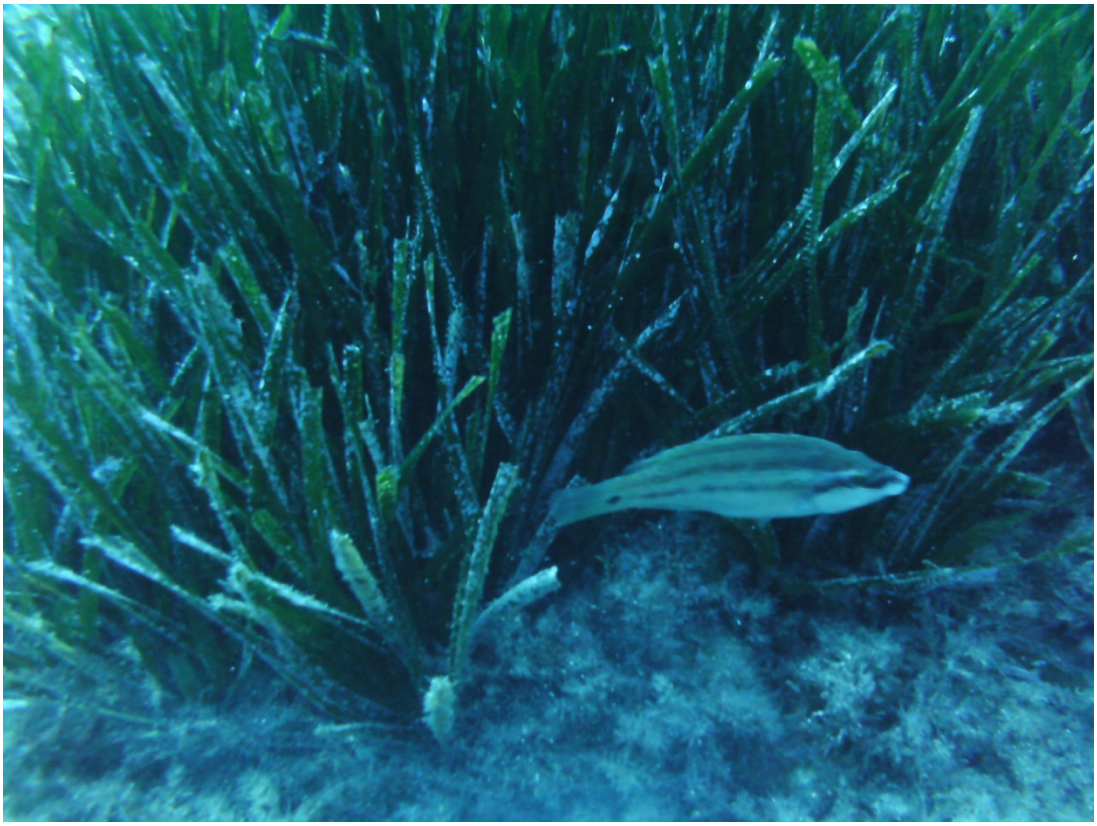

Fig. 1.3 A particular of *Posidonia oceanica* meadow and one of its common inhabitant, (courtesy of Valentina Martini).

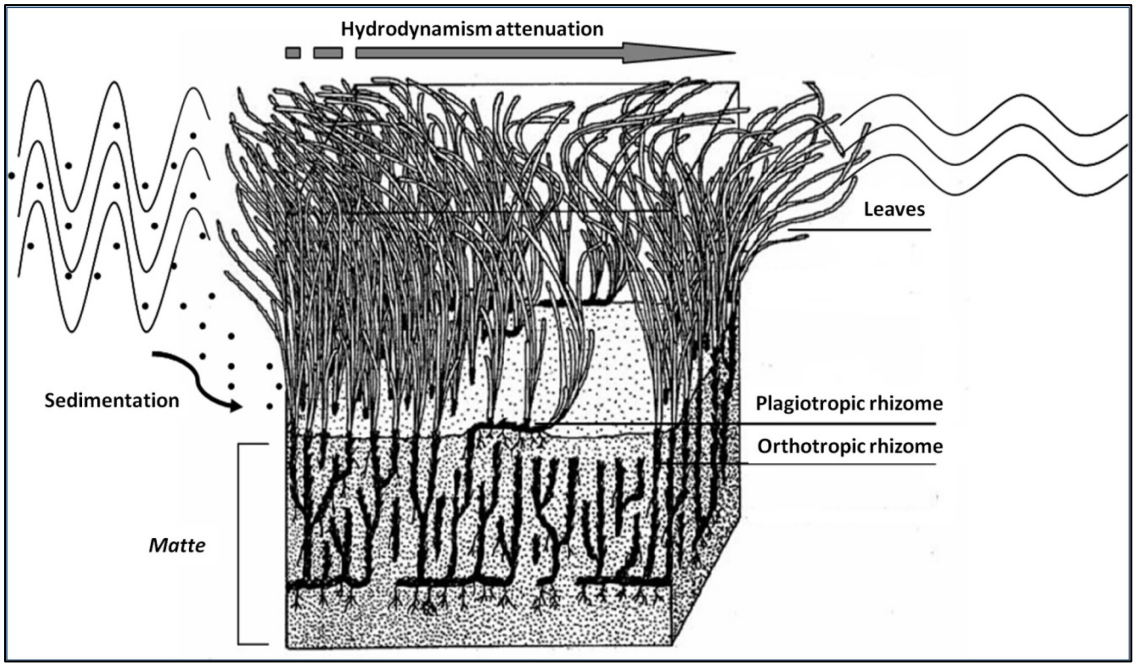

Fig. 1.4. Schematic representation of *P. oceanica* meadow (*matte*, rhizomes and leaves) and its effect on sediment stabilization and reduction of hydrodynamism (from Migliore et al., 2007)

All of these reasons have led to the identification of seagrass meadows as a benchmark of environmental health for aquatic systems by several governments and institutions worldwide (Council of Australian Governments Water Reform Framework of 1994, in Australia and New Zealand; Water Framework Directive 2000/60/EC, in the European Union; Clean Water Act of 1972 and Endangered Species Act of 1973, in the United States).

Despite the ecological and economical importance of seagrass beds, an increasing number of reports document the ongoing loss or regression of seagrasses in all countries, with global decline rates estimated at 2–5% per year (Duarte, 2002; Orth et al., 2006).

The decline of *P. oceanica* meadows is mainly due to human-induced disturbances, such as modified hydrogeological regime and littoral transport (Guidetti and Fabiano, 2000; Ruiz and Romero, 2003), pollution (Balestri et al., 2004), aquaculture (Pergent-Martini et al., 2006; Apostolaki et al., 2009), trawling (Dayton et al., 1995; Sanchez-Jerez and Ramos-Espla, 1996), anchoring (Francour et al., 1999; Milazzo et al., 2004; Montefalcone et al., 2008), placing of cables/pipes or damping (Savini et al., 1999). There is also growing evidence that seagrass meadows are vulnerable to the climate change (e.g. Duarte et al., 2008; Short & Neckles, 1998),

particularly through the impact of higher temperature on shoot survival (Duarte, 2002; Marbà and Duarte, 2010).

Hence, due to the ecological role, the global decline and the bioindicator capability, seagrass monitoring, including *P. oceanica*, is worldwide an issue of increasing interest in research, management and policies.

P. oceanica monitoring: traditional descriptors, Indices and innovative approaches

To protect and conserve *P. oceanica* meadows is necessary to establish their ecological status. Because of the complexity of these biological systems, their inherent high variability, and the influence of multiple environmental factors or stressors, the search for indicators have not been confined to one level of biological organization, ranging from the metabolism of a single organism to complex communities (Niemi et al., 2004).

The indicators utilised for *P. oceanica* work at three levels: the "individual" level, where the functional descriptors of the plant (e.g. leaf biometry) provide information about its status and growth conditions; the "population" level, where the structural descriptors of the meadow (e.g. shoot density) represent a characteristic imprint of environmental conditions; the "community" level, where the associate flora and fauna are considered as similarly susceptible to environmental alterations (Montefalcone, 2009).

Among the standardized methods usually adopted for this purpose, ''destructive'' and ''not destructive'' techniques can be recognized, linked with the necessity or not, respectively, to collect *P. oceanica* shoots samples. In table 1.1 the most employed analyses by the Mediterranean research laboratories have been listed (Buia et al., 2004; Pergent-Martini et al., 2005; Boudouresque et al., 2006)

Table 1.1. The most employed monitoring techniques for *P. oceanica*, separated in destructive and not destructive techniques (from Montefalcone, 2009, modified).

DESTRUCTIVE TECHNIQUES	NOT DESTRUCTIVE TECHNIQUES
Leaf biometry and related descriptors: • Type of leaves (adult, intermediate or juvenile) • N° of leaves per shoot • Length and width of leaves • Leaf surface area per shoot and per square metre (*i.e.* ''Leaf Area Index'') • Presence of dead brown tissue • % of broken leaves (Coefficient A) and the cause of their damage (water movement or grazing) **Epiphytic assemblages of leaves and rhizomes:** • Quantitative analysis of biomass and coverage • Qualitative analysis of specie composition **Indirect estimation of the past primary production of leaves and rhizomes, throughout:** • Lepidochronology • Internodal length • Plastochrone interval	**Shoot density (shoots/m^2) and its classification** **Upper and lower limits of the meadow:** • Bathymetric position of limits • Typology of the lower limit • Monitoring the position of limits over time, by fixed marks (''balise''), in situ photographs, aerial diachronic photographs **Structure of the *matte*:** • Presence of inter-*matte* channels and of dead *matte* • Measuring the baring of the rhizomes • Evaluating the homogeneity and the compactness of the *matte* and measuring the % of plagiotropic rhizomes and the thickness of the *matte* **% of bottom covered by living *P. oceanica*** **Mobile fauna associated to the meadow and the presence of other macrophytes**

The need of standardized methodologies to be applied by both researchers and administrators, to the proper management of the *P. oceanica* meadows lead to the introduction of a new class of ecological indicators. These ecological indicators are projected to be (Dale et al., 2001): (i) sensitive to disturbance and to respond to improvements or deterioration of environmental conditions within the required time frame, (ii) able to synthesize complex information in a simple and reliable way, (iii) simple to apply and cost-effective, (iv) applicable to wide geographical areas, (v) relevant to policy and management requirements, in particular to WFD requirements.

Many ecological indices have been introduced since the '90; they are still in use for the seagrass monitoring plans, e.g. the biomass of leaves and rhizomes, the Epiphytic Index (Morri, 1991), the Leaf Area Index (Buia et al., 2004), the bottom cover, etc. A second generation of multiparametric indices based on a number of structural and functional descriptors that utilise *P. oceanica* to evaluate the status of

coastal waters, includes: PosWare (system for coastal water classification according to WFD; Buia et al., 2005); POMI (multivariate index based on *P. oceanica*; Romero et al., 2007); Valencian CS (Fernandez Torquemada et al., 2008); BiPO (Biotic index; Lopez y Royo, 2008); PREI (*P. oceanica* Rapid Easy Index, Gobert et al., 2009). Synthetic ecological indices based on not destructive evaluations were also introduced, e.g. CI (Conservation Index; Moreno et al., 2001), SI (Substitution Index; Montefalcone et al., 2006), PSI (Phase Shift Index; Montefalcone et al., 2007).

Furthermore, remote sensing demonstrated to be a useful method for seagrass monitoring. With the development in recent years of satellite remote sensing, especially high resolution satellite images, such as Landsat MSS, TM, ETM+, SPOT, IKONOS and aerial photography, many research projects concerning the distribution and living status of seagrass have been conducted using high resolution satellite remote sensing data (Dekker et al., 2006).

In recent years researchers focus on the use of early symptoms, or biomarkers, defined as cellular, molecular and biochemical changes induced by chemical pollutants, measurable in biological systems such tissues, cells and biological fluids (Mc Carthy and Shugart, 1990). Very few and exploratory studies have been developed on *P. oceanica* in this field and nowadays this kind of descriptors are underemployed for the diagnosis or prediction of the meadows' regression. As reported in a review by Ferrat et al. (2003), biomarkers tested on *P. oceanica* regards photosynthetic activity (such as photosynthetic pigment content), enzymatic processes of nutrition, secondary metabolite synthesis (such as phenol compounds) and/or oxidative stress (e.g. Catalase and GST activity, TBARS, MDA and GSH concentrations). More recently Sureda et al. (2008) measured antioxidant response and oxidative damage (e.g. MDA and GSH content; Catalase, GPX and SOD enzymes activity) in *P. oceanica* leaves epiphytized and Mazzuca et al. (2009) analysed the protein expression in *P. oceanica* leaves under different light regimes.

Aim of the study

This work has been devoted to the identification and development of different generation of "diagnostic" tools to be used in monitoring of the seagrass meadows conservation status.

These tools are based on biochemical and molecular approaches that can provide early and reliable information on the ecophysiological status of plants.

The experimental approaches have been selected by applying the criteria of reproducibility and cost-effectiveness in order to obtain ecologically relevant information useful for policy and management goals. The outcomes of this work represent the first step for future identification and validation of a suite of biomarkers devoted to:

- a better understanding of causes and dynamics of decline phenomena;
- an implementation of effective strategies to assess the marine systems quality and the development of tools for environmental recovery and restoration.

In the long run this approach could help to get unequivocal information about the quality of the environment and affordable standardized methodologies for marine protection policies.

2. MATERIALS AND METHODS

Standardizable methodologies for *P. oceanica* meadows were developed and tested to assess the health status of the meadows. These methods were selected to obtain suitable descriptors of the conservation status of *P. oceanica*. One test was also performed on *Zostera noltii*, which was a first attempt to utilize the same test on different seagrasses.

In the following table the list of all tests performed on each species/sampling site is reported.

Table 2.1. Sampling and analyses plan.

	Posidonia oceanica S. Marinella (Rome, Italy)					*Posidonia oceanica* Monterosso (La Spezia, Italy)	*Zostera noltii* Ria Formosa (Faro, Portugal)
	2005	2006	2008	2009	2010	2010	2010
Total phenol content	X	X	X	X	X	X	X
Protein pattern analysis		X	X	X			
Heavy metal content				X			
Genetic analysis by RAPD	X						
Oxidative stress markers					X	X	
Photosynthetic pigments					X	X	

The proposed tests are at different development step. The better developed analyses (total phenol concentration, protein pattern analyses, heavy metal content and RAPD) were performed on a number of samples high enough to allow statistical handling. The newer methods (oxidative stress markers) were performed on a lower number of plants and the development of this method is still in progress.

All reagents utilized in the experimental procedures and cited in this Chapter are listed in Annex A.

Study Site and Sampling

The *P. oceanica* plants utilized to perform the analyses were collected from two different meadows: S. Marinella and Monterosso.

The Santa Marinella meadow (Rome, Italy) is a Site of Community Importance (according to Habitat Directive 92/43/EEC). The meadow spanning the 13.5 km coastline from Capo Linaro to Santa Severa and covering a surface of 1,800 ha, shows patched distribution and regressive limit, and is characterized by the presence of dead *matte*. The lower limit is at -20 m depth; the great majority of the meadow lies on hard bottom; some limited areas lie on soft bottom, showing alive and/or dead *matte* (Diviacco et al., 2001). According to the classification proposed by Pergent et al. (1995) and modified by Buia et al. (2004), it can be considered a disturbed meadow, under anthropogenic pressure and turbid water condition.

P. oceanica plants from this meadow were sampled in late spring by SCUBA divers in 20 stations (fig. 2.1) covering the central area (50 ha) of the meadow (both on sand and matte, from 7.5 to 13.5 m depth), from 2005 to 2010 (excluding 2007 because of bad weather conditions). In each station 3 orthotropic shoots were collected at least.

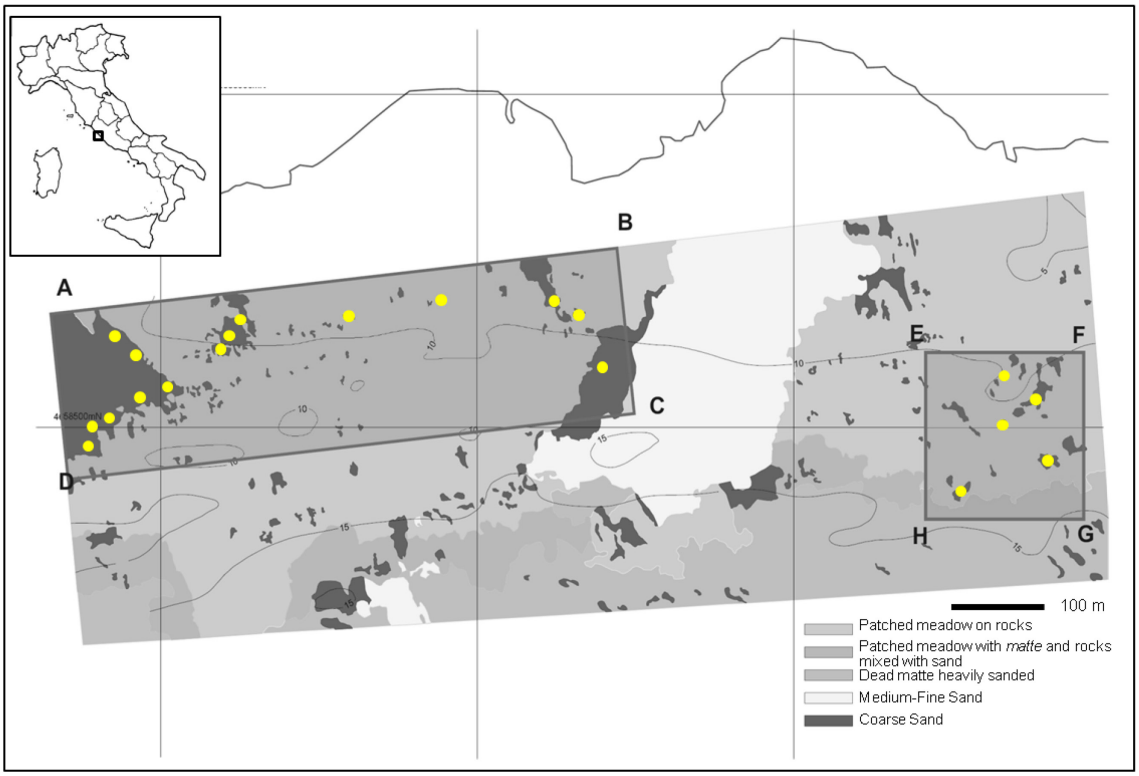

Fig. 2.1. The 2005-2010 sampling stations in Santa Marinella meadow.

The Monterosso meadow (La Spezia, Italy), spanning from Punta Mesco to Monterosso al Mare, covers a surface of 30 ha and is included in the ''Cinque Terre'' National Park (Ligurian Sea).

The meadow lies on soft bottom and the coverage ranges from 100% to 20%, with lower values in proximity to Monterosso a Mare proximity. The lower limit, at ca. 20 m depth, is a regressive limit (according to Pergent et al., 1995 classification), characterized by the presence of dead *matte*. The suffering condition of the meadow is due to intense human activities along the coast; in particular an embankment built in the '60s altered the hydrogeological and sedimentological regime in the area, burying part of the meadow. Samples were obtained by SCUBA diving in summer 2010. Three stations were chosen (St. 1: Punta Mesco; St. 2: Intermediate; St. 3: Embankment; fig. 2.2) all at 14 m depth. Sampling was carried out along a 50 m transect, at 7 sites for each station, taking 3 plants (orthotropic shoots) at random from each site.

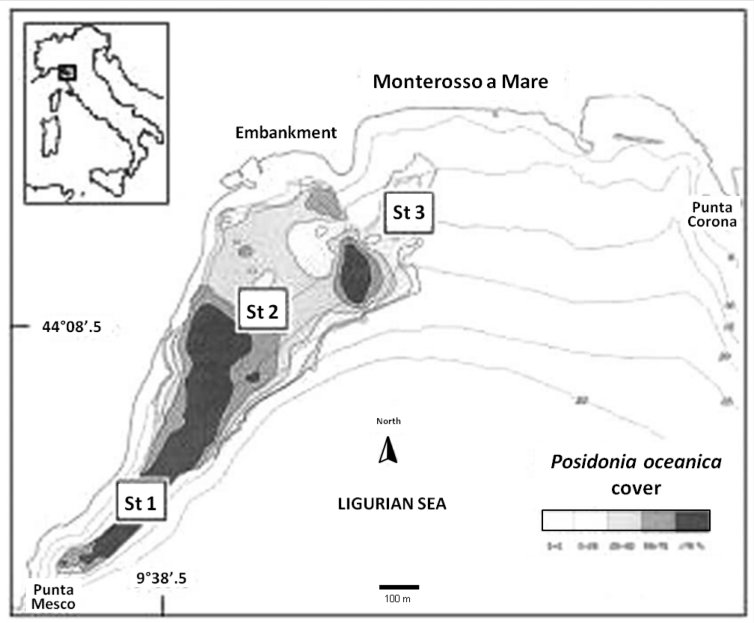

Fig. 2.2. The sampling stations in Monterosso a Mare meadow (from Micheli et al., 2005, modified).

Z. noltii plants were collected from a third sampling site in Ria Formosa (Faro, Portugal). *Z. noltii* samples were collected to optimize the determination of the total phenol content protocol and to test this descriptor on a different model plant.

Ria Formosa is a shallow mesotidal lagoon on the South coast of Portugal, (fig. 2.3) characterised by large intertidal flats with a salt marsh community that covers ca. 43% of the lagoon's area.

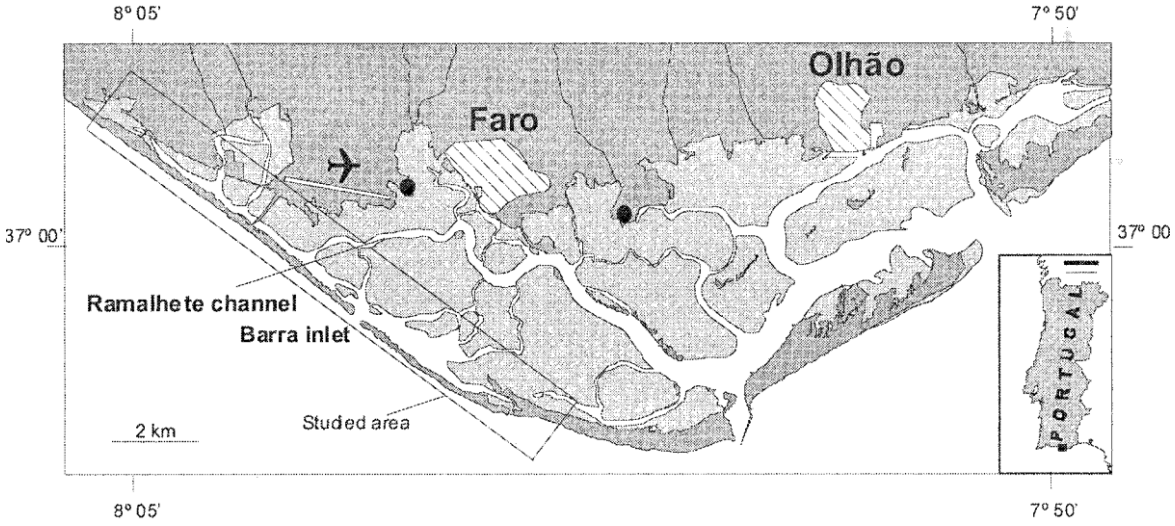

Fig. 2.3. Ria Formosa lagoon (from Santos et al., 2004, modified).

The average water depth is less than 2 m and the tidal height varies from a maximum of 3.7 m in spring tides to a minimum of 0.4 m in neap tides. This results in the flushing of most of the water volume in each tidal cycle (Andrade, 1990), thereby imposing an intense exchange of materials between the Ria and the adjacent coastal waters. The lower intertidal zone is dominated by meadows of *Z. noltii* distributed within a bathymetric range of about 2 m that alternate daily periods of immersion and air exposure (Santos et al., 2004).

Sample handling

Immediately after sampling, *P. oceanica* plants were washed in distilled water and rhizomes separated from leaf shoots. Rhizomes and leaves were cleaned with a blade to remove epiphytes, sheets and cortical tissues (to avoid metal contamination a ceramic knife was used to clean material for metal content analysis). Cleaned rhizomes and leaves were frozen in liquid nitrogen and then stored separately at -80 °C until chemical analyses. It was only in the last sampling campaign (2010) that has been possible to wash and immediately freeze the biological material in liquid N_2 after collection,. This is an important practice to better preserve biomolecules.

Z. noltii samples were washed in distilled water and immediately frozen in liquid nitrogen after what were stored at -80 °C until processed.

The ratio fresh/dry weight was determined when necessary, using 150 mg (fresh weight) of leaves or rhizomes.

Total phenols

Posidonia oceanica

Total phenol determination was carried out in duplicate on 3 different rhizomes for each sampling site, following the protocol by Migliore et al. (2007). Phenolic compounds were extracted from 100 mg (fresh weight) of apical (near the meristem), intermediate and basal sections of the rhizome, ground in liquid N_2 using a mortar and pestle. The powdered tissue was first extracted in 4 ml of 0.1 N HCl and kept overnight at 4° C. After centrifugation (15'; 3000*xg*), the pellets were re-suspended in 4 ml of 0.1N HCl and centrifuged. The supernatants from the two centrifugations were collected and brought to 10 ml with 0.1 N HCl. According to the Booker and Miller (1998) protocol, 50 µl of phenolic extracts were placed in a 1 ml cuvette with 475 µl of 0.25 N Folin-Ciocalteau reagent; after 3' 475 µl of Na_2CO_3 (7.5 %) were added. After 60' total phenol content was determined spectrophotometrically (UV/visible Spettrophotometer Beckmann 473) at 724 nm. A calibration curve was made with Chlorogenic acid in five different concentrations (0 - 25 - 50 - 100 - 200 µg/ml).

Zostera noltii

Total phenol determination protocol was modified for *Z. noltii* plants, due to their lower content of phenols. 200 mg of rhizomes or leaves were homogenized homogenized (and not ground as Posidonia tissues) for 3' in 9 ml of 0.1 N HCl by Ultraturrax and kept overnight at 4 °C, under constant agitation. The homogenization was performed by Ultraturrax, as in the protocol used in the past for *P. oceanica*. After centrifugation (20'; 6000*xg*), the pellets were resuspended in 9 ml of 0.1N HCl and centrifuged. The supernatants from the two centrifugations were collected and brought to 20 ml with 0.1 N HCl. 42 µl of phenolic extracts were

placed in a 1.5 ml cuvette with 400 µl of Folin-Ciocalteau reagent 0.25 N; after 3' 400 µl of Na_2CO_3 (7.5 %) were added. A calibration curve was made with Chlorogenic acid in five different concentrations (0 - 2.5 - 5 - 10 - 20 µg/ml).

Two-Dimensional electrophoretic analysis

Total proteins were extracted according to the protocol of Wang et al. (2006), originally developed for recalcitrant plant tissues. This protocol, previously utilized for *P. oceanica* leaves (Bucalossi et al., 2006), was modified for *P. oceanica* rhizomes (Migliore et al., 2007).

250 mg of basal section from each selected rhizome were ground in liquid N_2 using a mortar and pestle (4 rhizomes with the highest phenol content and 4 with the lowest were chosen each year). The powdered tissue was subjected to phenol extraction in the presence of SDS, as described in Wang et al. (2006).

The protein pellet was dried and dissolved in 2-DE rehydration solution [8 M urea, 2 M thiourea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.2% carrier ampholytes], supplemented with protease and phosphatase inhibitors. Protein content was measured by Bradford protein assay using Bovine Serum Albumine as standard.

Protein samples (12 µg) in 155 µl of 2-DE rehydration solution, protease and phosphatase inhibitors were applied to 7 cm Readystrip IPG (pH 3-10 non linear), by incubating overnight. The isoelectrofocusing (IEF) was performed at room temperature using the ZOOM IPG Runner™ Mini-cell (Invitrogen), applying 175 V for 15', 175-2000 V for 45' and 2000 V for 30'. Focused strips were equilibrated using DTT and iodoacetamide solutions and subjected to protein separation by a second dimension electrophoresis.

In the original protocol 12% acrylamide SDS-PAGE minigels 1 mm thick (Laemli, 1970) were used for the second dimension electrophoresis (Migliore et al., 2007). However, the 2008-2009 analyses were performed with Precast Gradient Gels (4%-12% acrylamide), allowing the resolution of a greater number of protein spots in the whole range of molecular weights.

Electrophoresis was performed at 80 V in NuPAGE Running Buffer MOPS-SDS 1X, according to standard procedures.

After electrophoresis, resolved proteins were visualized by acidic silver staining that allows the detection of values as low as 0.5-1 ng of protein per spot (Switzer, 1979).

Each protein sample was subjected to at least 2 parallel runs of isolectrofocusing and second dimension electrophoretic separation to assess proteomic pattern reproducibility. Silver-stained gels were acquired using a camera, generating 6.2 Mb Tiff format images that were then imported into the PD Quest 2-D Analysis Software (Biorad, version 8.0.1) for analysis. Spot selection was performed using default selection parameters. The protein spots were characterized for isoelectric point (p*I*) and relative molecular weight (MW) according to an 'internal standard', *i.e.* a mixture of proteins with known identities as p*I* markers.

Heavy metal/element content

P. oceanica plants, sampled in 2009 from S. Marinella (Rome, Italy) meadow, were analyzed by a certified laboratory (Istituto Superiore di Sanità, Dip. Sanità Pubblica Veterinaria e Sicurezza Alimentare) for the quantification of five trace elements (Cu, Zn, As, Cd and Pb).

Frozen samples (rhizomes and leaves) were thawed at room temperature and sectioned using stone implements, homogenized with a HMHF Turbo Homogenizer (PBI International, Italy) and subjected to acid mineralization (in HNO_3) at high pressure in a microwave (Milestone Ethos Plus, Italy). Certified reference materials were used to ensure the quality of analytic data. Elements were analysed using a quadrupole inductively coupled plasma mass spectrometer (Q-ICP-MS; Elan 6000: Perkin-Elmer SCIEX, Norwalk, USA). The metal concentrations were expressed as µg/g plant dry weight.

All the methods used were validated in compliance with UNI CEI EN ISO/IEC Regulation 17025.

Genetic analysis using random amplified polymorphic DNA (RAPD) markers

Genetic analysis was carried out in duplicate on five plants from four stations. Immediately after sampling, plants were washed in distilled water and the growing portions of younger leaves were stored in liquid N_2. Genomic DNA was extracted from about 2 g of leaves, following a protocol of Micheli et al. (2005).

Quantification of DNA was done by using UV-VIS spectrophotometer (Beckmann 473). The optical density (OD) was measured at 260 nm and the ratio of OD 260/280 was measured to check for the RNA/protein impurities in the sample.

DNA amplification was performed in 30 µl reaction volume containing 30 ng genomic DNA, 200 mM each of dATP, dCTP, dGTP and dTTP, 10 mM of a primer, 0.4 µl of AmpliTaq DNA polymerase, 10 mM $MgCl_2$ and 3 µl incubation buffer containing 100 mM Tris–HCl, 500 mM KCl and 25 mM $MgCl_2$. The sequences of the primers (5'–3'), chosen for their capacity to discriminate and score bands as present/absent (Micheli et al., 2005, 2010; Waycott, 1995), are reported in Table 2.2.

The amplification was performed in a thermocycler (Perkin-Elmer/Cetus, USA). The cycling conditions were: 2' denaturation cycle at 94 °C and, subsequently, 40 cycles of 30 sec. at 37 °C annealing temperature and 2' at 72 °C synthesis temperature.

Table 2.2. Sequences of primers, relative GC contents and melting temperatures (Tm).

Primer	Sequence	% C+G	T_m (°C)
BY11	5'- ATCCACTGCA -3'	50	30
BY12	5'- GGTCGCAGGC -3'	80	36
BY13	5'- CCTTGACGCA -3'	60	32
BY15	5'- CTCACCGTCC -3'	70	34
DN4	5'- GTCGTGCTAT -3'	50	30
DN5	5'- CCGACGGCAA -3'	70	33
DN6	5'- TGGACCGGTG -3'	70	34
UB24	5'- GGGTGAACCG -3'	70	34
UB26	5'- CGCCCCCAGT -3'	80	36
UB28	5'- GCTGGGCCGA -3'	80	36

Amplification products were separated by electrophoresis in 1.4% agarose gel stained with ethidium bromide in TAE buffer. Gels were visualized on UV light and acquired using a camera. DNA fragment sizes were estimated by comparison with standard DNA marker, 1 kb DNA ladder.

All the electrophoresis runs were repeated in the same experimental conditions, to verify the method reproducibility. Reproducibility and repeatability of amplification products were tested for each primer used in the experiments.

Genetic data analysis focused on the number of molecular fragments for each specimen examined, both monomorphic and polymorphic, following Waycott (1995).

All the RAPD data were elaborated using NT-SYS-PC (Numerical Taxonomy and Multivariate Analysis System) computer package. The Nei's coefficients of similarity between each pair of samples were used to construct a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA).

The bands were recorded as present (1) or absent (0) and assembled in a data matrix table. Then similarity coefficients (Dice index) were obtained (Simqual data matrix, NT-SYS-pc) and their average and standard deviation were calculated.

Antioxidant enzyme activity, soluble proteins and photosynthetic pigment content in leaves

Enzyme extraction, activity assays and soluble protein content

Antioxidant enzymatic activity was measured in *P. oceanica* leaves collected and immediately frozen in liquid nitrogen. Both ascorbate peroxidase (APx) and dehydroascorbate reductase (DHAR) were determined in the same leaf extract. Foliar extract was obtained as in Polle and Morawe (1995) and ascorbate was added in the extraction and in the elution buffers to avoid APx inactivation (Nakano and Asada 1987). The protocol for enzymes extraction, previously utilized for *Helianthus annuus* (Barrote, 2005) and *Z. noltii* (Barrote *et al.,* 2009), was modified for *P. oceanica* and optimized for the examined enzymes. Approximately 1 g (fresh weight) of *P. oceanica* leaves ware ground in liquid nitrogen, then 500 mg PVPP

(polyvinylpolypyrrolidone) and 200 mg sea sand were added. The resulting powder was suspended in 4 ml of 100 mM phosphate buffer (pH 7.8) with 2%Triton-x and 10 mM ascorbate. The suspension was centrifuged at 3000*xg* for 30'. 2.5 ml of the resulting supernatant were purified and desalted on a PD-10 column using 3.5 ml of 100 mM potassium phosphate buffer pH 7 with 1 mM ascorbate as eluent. The eluate was used for enzyme assays. PD-10 column was equilibrated with 20 ml of 100 mM potassium phosphate buffer pH 7 with 1 mM ascorbate before sample loading. All steps of the extraction procedure were carried out at 1–4 °C. All extracts were subjected to three repeated assays for both enzymes and the results were expressed as means of the three measurements.

Soluble protein content in the enzyme extracts was determined spectrophotometrically according to the Bradford method using bovine serum Albumine as a standard.

Ascorbate peroxidase (APx) activity was determined based on the method described by Nakano and Asada (1987). APx activity was measured in 1 ml of reaction mixture containing 50 mM potassium phosphate buffer pH 7.0, 800 μM ascorbate and 50 μl of the enzyme extract. The reaction was initiated by the addition of hydrogen peroxide (2 mM final concentration). The hydrogen peroxide dependent oxidation of ascorbate was measured at constant temperature of 25 °C following, the decrease in absorbance at 290 nm, during 3 minutes, on a Schimadzu UV-160 spectrophotometer. APx activity was calculated using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$, expressed as μmole of ascorbate oxidized per milligram of soluble protein and per minute.

Dehydroascorbate reductase (DHAR) activity was determined based on the protocol described by Polle and Morawe (1995). DHAR activity was measured in 1 ml of reaction mixture containing 60 mM potassium phosphate buffer pH 6,1, 800 μM dehydroascorbate, 2mM reduced glutathione (GSH) and 100 μl of the enzyme extract. The reaction was initiated by the addition of the enzyme extract. The reduction of dehydroascorbate to ascorbate catalyzed by DHAR was measured at a constant temperature of 25 °C following the increase in absorbance at 265 nm, during 3 minutes, on Schimadzu UV-160 spectrophotometer. DHAR activity was

calculated using an extinction coefficient of 14 $mM^{-1}cm^{-1}$ and expressed as µmole of dehydroascorbate reduced to ascorbate per milligram of soluble protein and per minute.

Photosynthetic pigment content: Chlorophyll a, Chlorophyll b and Carotenoids

P. oceanica foliar photosynthetic pigments were extracted in dimethyl-formamide (100 mg fresh weight: 5 ml solvent) inside a glass tube sealed with a cotton plug and kept overnight at 4 °C, in the dark. The absorbances of the extract were read at 480, 646.8, 663.8 and 750 nm in a Schimadzu UV-260 spectrophotometer, using a glass cuvette. Two different extractions were done for each sample and all the extracts were read in duplicate. The foliar concentration of pigments was calculated according to Wellburn (1994). The formulas are reported below.

$$C_a\ (g\ ml^{-1}) = 12(A_{663.8} - A_{750}) - 3.11(A_{646.8} - A_{750})$$

$$C_b\ (g\ ml^{-1}) = 20.78(A_{646.8} - A_{750}) - 4.88(A_{663.8} - A_{750})$$

$$C_{x+c}\ (g\ ml^{-1}) = (1000 * A_{480} - 1.12\ C_a - 34.07 C_b)/245$$

C_a=Chlorophyll *a* C_b= Chlorophyll *b* C_{x+c}=Total Carotenoids

Final results were expressed as milligrams of pigment per gram of dry weight and are means of four measurements.

3. RESULTS

Total phenols

Posidonia oceanica 2005-2009

Total phenol content was measured in rhizomes collected yearly from the S. Marinella meadow between 2005 and 2009; values from 20 sampling stations are presented as box-plots (fig. 3.1). The mean rhizome concentrations increased with time: from 18.7 mg/g in 2005 (n=65; SE 0.5), to 25.2 mg/g in 2006 (n=60; SE 0.7), to 26.2 mg/g (n=60; SE 0.6) in 2008, up to a final 27.7 mg/g (n=60; SE 0.7) in 2009. Differences between subsequent years were always statistically significant (ANOVA, 2005-2006: $F=58.3$, $p<<0.01$; 2006-2008: $F=8.3$, $p<0.01$), except for the 2008-2009 comparison.

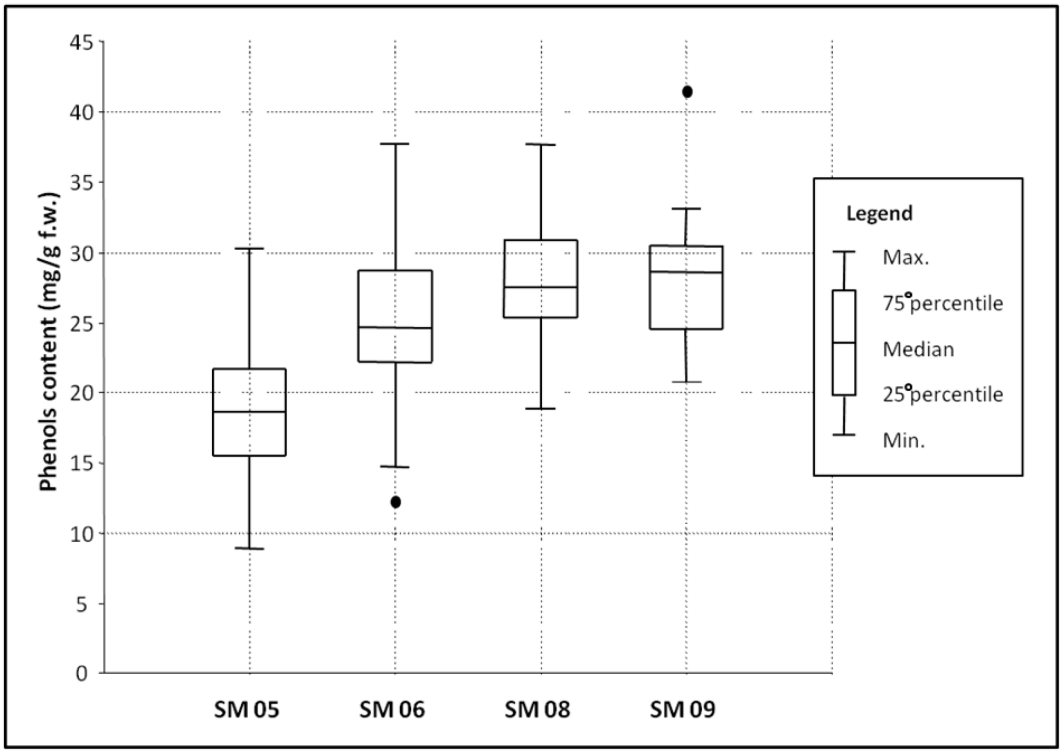

Fig. 3.1. Total phenol content (as mg/g fresh weight, f.w.) in *P. oceanica* rhizomes collected in S. Marinella meadow (Rome, Italy) from 2005 to 2009 (SM05-SM09).

Total phenols were also quantified separately in apical, intermediate and basal sections of rhizomes (fig 3.2). An increase in the total phenol concentration from the basal to the apical sections were always found. The differences in phenol

content among the sections were statistically significant only in 2005 (ANOVA, F=38.1, p<<0.01).

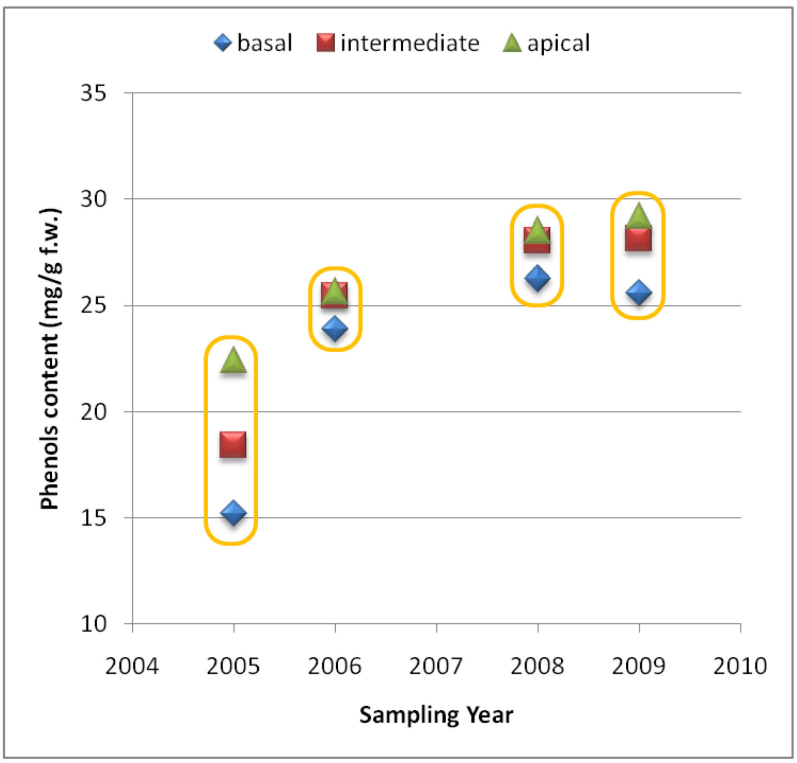

Fig. 3.2. Trend of total phenol content in *P. oceanica* rhizome sections (as mg/g fresh weight, f.w.), during 2005-2009 period, at S. Marinella (Rome, Italy).

Posidonia oceanica 2010

Total phenol content was measured in rhizomes collected in 2010, from S. Marinella and Monterosso a Mare meadows. Data are shown separately because of the modified experimental procedures during sample handling (i.e. freezing and storage). Values from both meadows (20 sampling stations each) are presented as box plots (fig. 3.3).

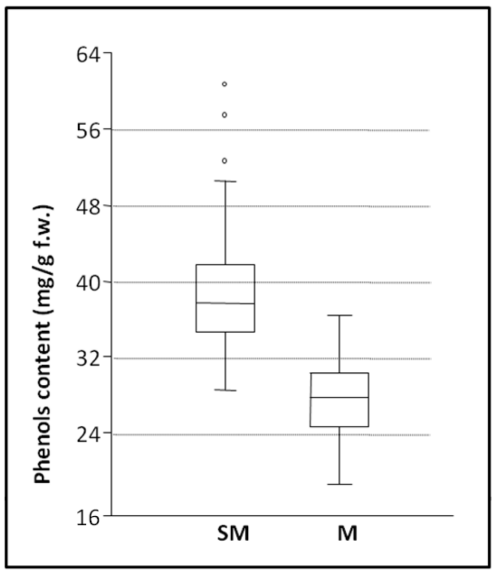

Fig. 3.3. Total phenol content (as mg/g fresh weight, f.w.) in *P. oceanica* rhizomes collected in 2010 at S. Marinella (Rome, Italy; SM) and Monterosso (La Spezia , Italy; M) meadows.

The mean rhizome concentration was 38.9 mg/g (n=62; SE 0.8) in S. Marinella meadow and 27.6 mg/g (n=63; SE 0.5), in Monterosso meadow. Differences between meadows are statistically significant (ANOVA, F=140, p<<0.01).

Zostera noltii

The protocol for the determination of total phenol content was modified *ad hoc* and successfully applied to some *Z. noltii* samples from Ria Formosa lagoon. Total phenol concentrations found in *Z. noltii* tissues, utilized for the set up of the method, are shown in table 3.1.

Table 3.1. Total phenol content (as mg/g fresh weight, f.w.) in *Z. noltii* tissues.

Sample	Phenol content (mg/g f.w.)
Z 1	1.78
Z 2	2.18
Z 3	2.84
Z 4	1.35
Z 5	2.19
Z 6	2.80
Z 7	1.27
Z 8	1.81

2-D electrophoresis analysis

The overall protein expression pattern of *P. oceanica* rhizomes was determined by 2-D electrophoresis, in the 2006, 2008 and 2009 samples.

The polypeptides falling within the experimental window of p*I* and molecular weights and sufficiently abundant to be detected by the silver staining procedure were taken into account. The experimental values of *pI* and molecular weight for each spot were calculated by dedicated computer software.

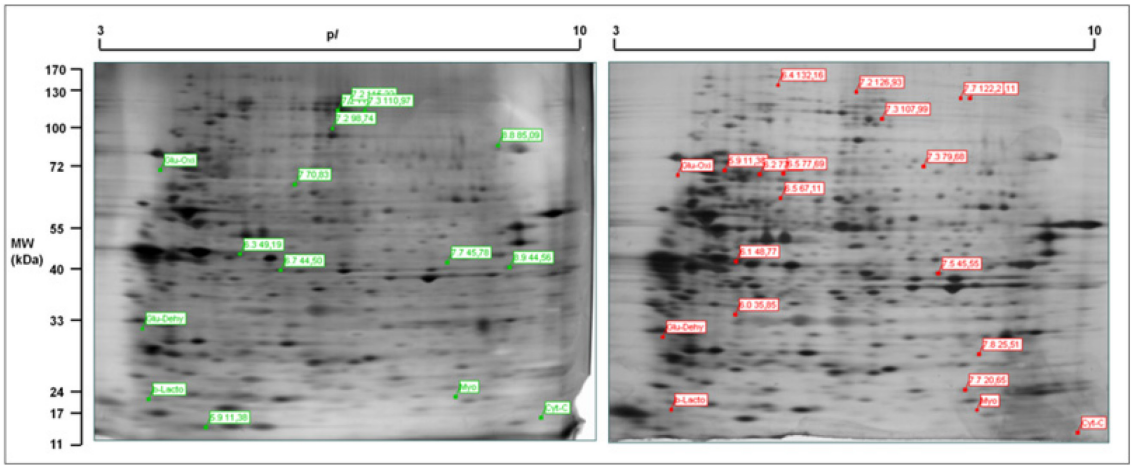

Fig. 3.4. Representative 2-DE maps from 2008 plants characterized by high (right) or low (left) total phenol content. High phenols-specific protein spots are highlighted and labeled in red, low phenols–specific protein spots in green. Protein markers (in alphabetic order) are: Cyt-C (Cytochrome C); Glucose-1-Dehydronase (Glu-Dehy); Glucose oxidase (Glu-Oxi); β-Lactoglobuline (b-Lacto); Myoglobine (Myo).

Analyses were performed on rhizomes characterized by the lowest or the highest phenol content. Maps from both low and high phenols showed a comparable number and arrangement of spots (see fig. 3.4, tab. 3.2).

Table 3.2. Comparisons among protein expression pattern analyses performed on 2006, 2008 and 2009 *P. oceanica* rhizome.

	2006	2008	2009
Total spots expressed	437	480	472
High phenols-specific spots (N)	27	32	9
Low phenols-specific spots (N)	22	13	6
High phenol values (mg/g f.w.)	28÷35	34÷37	32÷33
Low phenol values (mg/g f.w.)	7÷14	18÷22	16÷18

Distinctive features in protein patterns from low and high phenol samples were highlighted. Differences in specific spot presence/absence, between the two phenol conditions, were always found: some spots were exclusively expressed in low phenol rhizomes and others are exclusively expressed in high phenol ones.

Heavy metal/element content

The protocol to quantify five elements (Cu, Zn, As, Cd and Pb) has been developed for *P. oceanica* rhizomes and leaves, seawater and sediment samples in 2009 from S. Marinella meadow.

Metal/element content in the sediments and in the water column is reported in table 3.3.

Table 3.3. Element concentrations in sediment (µg/g) and water column (µg/l) collected in proximity of the S. Marinella meadow. The mean values of duplicates with standard deviations less than 5 ppb are reported.

	As	Cd	Pb	Cu	Zn
Sea water	2.69	0.00	0.00	0.75	2.2
Sediment	3.57	0.00	0.01	0.87	1.87

Mean metal/element content in leaves and rhizomes are reported in fig. 3.5, showing separately nonessential and essential elements. Values were homogeneous among the 20 different samples in the meadow.

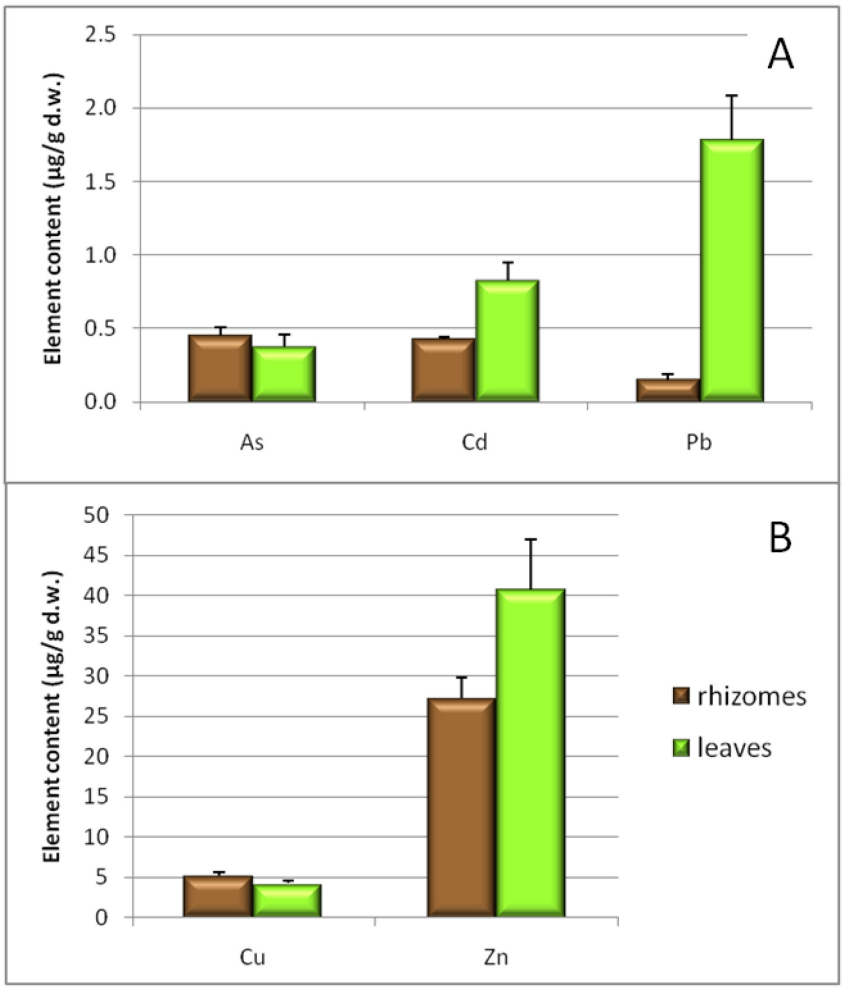

Fig. 3.5. Content of nonessential (A) and essential (B) elements in *P. oceanica* leaves and rhizomes. Values are mean based on 20 independent determinations and bars indicate standard deviations.

As and Cu were found at comparable concentrations in leaves and rhizomes, while Cd, Pb and Zn were found at higher concentrations in leaves. In particular, the highest difference was found for Pb, with a ratio leaves:rhizomes 10:1, while Cd and Zn showed a 2:1 (Cd) and 3:2 (Zn) ratio, respectively.

The ratio between essential (Cu, Zn) and nonessential (Cd) mean metal concentrations in rhizomes were calculated in each sample: the ratio Zn/Cd was 63.6 (n=20; E.S.=6.3) and the ratio Cu/Cd was 12.2 (n=20; E.S.=0.8).

RAPD genetic analysis

The overall percentage of polymorphism in the S. Marinella meadow, obtained on 2005 samples through the calculation of amplified polymorphic and monomorphic fragments, was 63.41%.

All the 10 primers gave high reproducibility of their electrophoresis pattern both in the signal intensity and in the number of bands for each different analysis performed.

Cluster analysis (fig. 3.6) confirmed that similarity between samples is very high (the highest genetic distance being 0.82). The average of all similarity coefficients among the samples was 0.87±0.03. The Mantel test comparing Nei's distance and cophenetic matrices had a strong and statistically significant correlation with a value of r = 0.9 as a measure goodness of fit for cluster analysis.

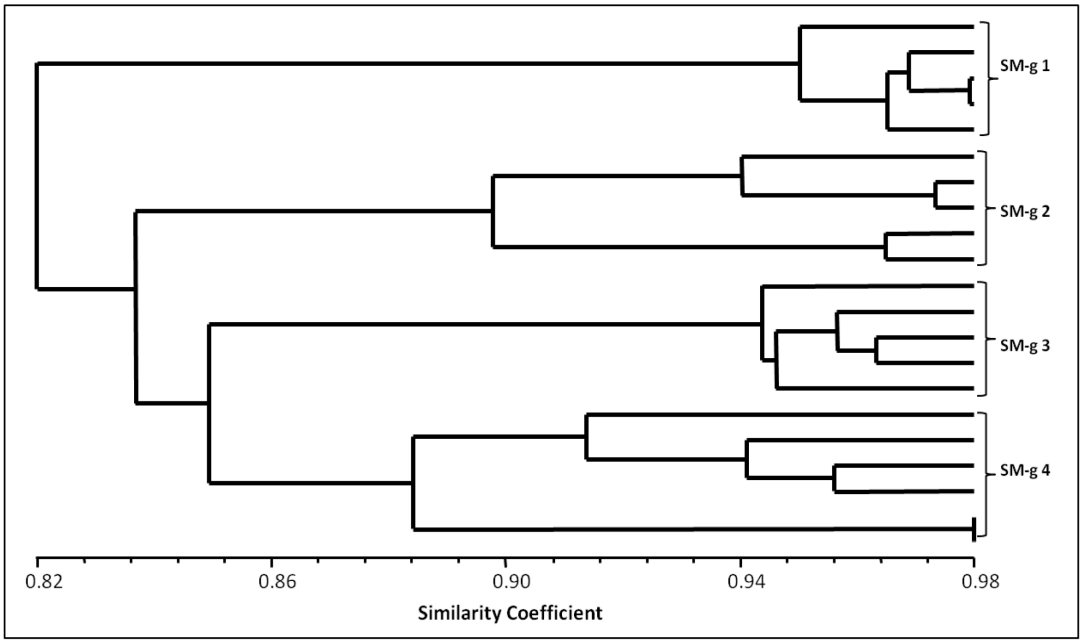

Fig. 3.6. UPGMA phenogram constructed from the matrix of RAPD-based genetic distances among *P. oceanica* samples at S. Marinella.

Antioxidant enzyme activity and photosynthetic pigments

A protocol for the activity determination of two enzymes involved in antioxidant defence (DHAR, dehydroascorbate reductese, and APx, ascorbate peroxidase) was developed for the first time on *P. oceanica* leaves. Preliminary data on S. Marinella and Monterosso 2010 samples are reported in table 3.4 and fig. 3.7.

Table 3.4. Dehydroascorbate reductase (DHAR) and ascorbate peroxidase (APx) activities in *P. oceanica* leaves, sampled in 2010 from S. Marinella (SM) and Monterosso (M) meadow. Mean concentration ± SE (standard error) from three replicates.

	DHAR	APx
Sample	(µmole of ascorbate formed/mg protein/min)	(µmole of ascorbate oxidized/mg protein/min)
SM 1	0.176 ± 0.003	0.547 ± 0.015
SM 2	0.283 ± 0.001	0.495 ± 0.037
SM 3	0.437 ± 0.009	0.434 ± 0.170
SM 4	0.220 ± 0.004	0.828 ± 0.037
SM 5	0.101 ± 0.000	0.504 ± 0.001
SM 6	0.498 ± 0.016	1.289 ± 0.431
SM 7	0.440 ± 0.007	1.085 ± 0.005
SM 8	0.250 ± 0.002	0.860 ± 0.008
SM 9	0.331 ± 0.002	0.875 ± 0.016
SM 10	0.324 ± 0.002	0.731 ± 0.014
M 1	0.164 ± 0.003	0.353 ± 0.017
M 2	0.157 ± 0.002	0.523 ± 0.006
M 3	0.143 ± 0.002	0.356 ± 0.017
M 4	0.177 ± 0.011	0.670 ± 0.004
M 5	0.167 ± 0.002	0.483 ± 0.004
M 6	0.144 ± 0.004	0.517 ± 0.008
M 7	0.188 ± 0.012	0.622 ± 0.008
M 8	0.206 ± 0.008	0.942 ± 0.016
M 9	0.178 ± 0.003	0.566 ± 0.009
M 10	0.270 ± 0.021	0.479 ± 0.005
M 11	0.143 ± 0.005	0.386 ± 0.002
M 12	0.256 ± 0.008	0.477 ± 0.010

A good correlation ($r=-0.8$; $p<0.01$) between the two enzymes' activities and significant differences between the two sampling sites were found (DHAR: ANOVA, $F=8.016$, $p<0.02$; APx: ANOVA, $F=8.23$, $p<0.01$).

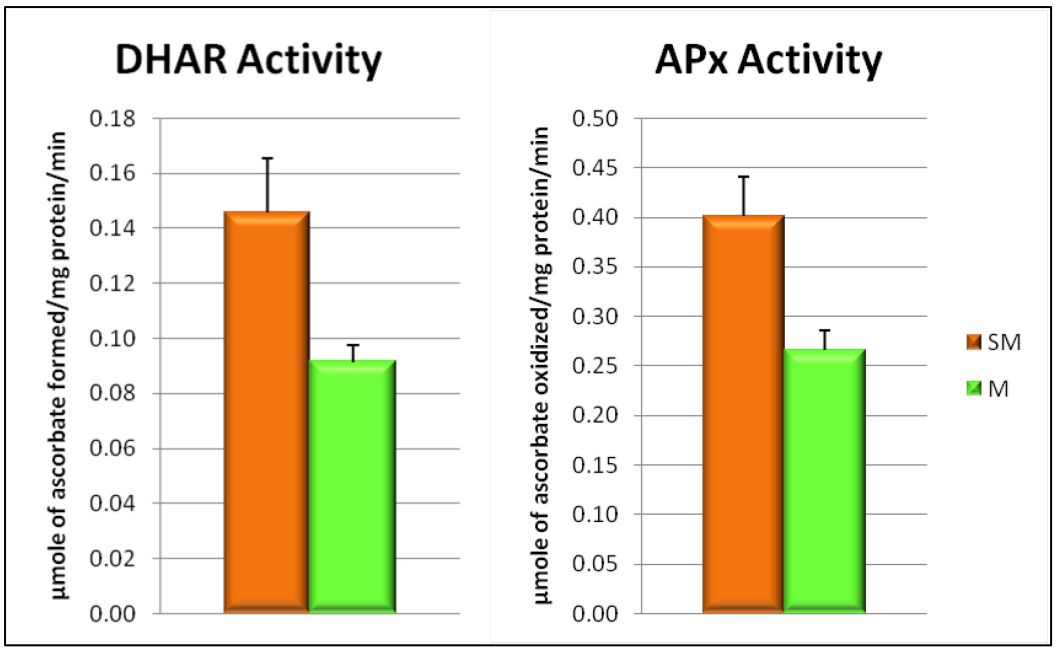

Fig. 3.7. Enzymatic activity levels of dehydroascorbate reductase (DHAR) and ascorbate peroxidase (APx) in *P. oceanica* leaves collected in 2010, at S. Marinella (SM) and Monterosso (M) meadows. Mean values are based on at least 10 independent determinations and bars indicate standard deviations.

A protocol for the spectrophotometric determination of Chlorophylls a and b and total Carotenoids was also applied for the first time on *P. oceanica* leaves. Preliminary data collected on S. Marinella and Monterosso 2010 samples are reported in table 3.5.

Table 3.5. Chlorophyll a (C_a) and b (C_b) and total Carotenoid (C_{x+c}) concentrations (as mg/g dry weight, d.w.,) in *P. oceanica* leaves sampled in 2010 from S. Marinella (SM) and Monterosso (M) meadow. Mean concentration ± SE (standard error) from four replicates are reported.

	Ca		Cb		Cx+c	
Sample	(mg/g d.w.)					
SM 1	1.41	± 0.17	0.45	± 0.15	0.65	± 0.17
SM 2	1.36	± 0.13	0.45	± 0.11	0.58	± 0.13
SM 3	1.15	± 0.06	0.37	± 0.06	0.53	± 0.06
SM 4	2.11	± 0.07	0.73	± 0.07	0.80	± 0.07
SM 5	1.59	± 0.06	0.53	± 0.07	0.80	± 0.06
SM 6	2.51	± 0.16	0.79	± 0.06	1.03	± 0.16
SM 7	0.75	± 0.02	0.24	± 0.04	0.31	± 0.02
M 1	1.14	± 0.14	0.42	± 0.07	0.56	± 0.14
M 2	2.72	± 0.00	1.10	± 0.05	1.19	± 0.00
M 3	3.16	± 0.04	1.26	± 0.14	1.30	± 0.04
M 4	1.96	± 0.04	0.75	± 0.05	0.82	± 0.04
M 5	1.51	± 0.21	0.58	± 0.18	0.69	± 0.21
M 6	2.28	± 0.01	0.97	± 0.02	1.05	± 0.01
M 7	1.11	± 0.13	0.43	± 0.10	0.54	± 0.13
M 8	0.87	± 0.02	0.33	± 0.03	0.40	± 0.02
M 9	2.61	± 0.20	1.02	± 0.20	1.20	± 0.20

4. DISCUSSION

Total phenols

Phenolic compounds are a class of secondary metabolites widely distributed in all organs of both terrestrial (Bate-Smith, 1968; Karolewski and Giertych, 2000) and aquatic plants (Mc Clure, 1970, Pip, 1992; Zapata and McMillan, 1979). They have several structural and physiological roles, including plant defence. In *P. oceanica* phenolic compounds have been quantified as a biochemical marker of environmental stress (Cuny et al., 1995; Dumay et al., 2004; Fresi et al., 2004; Cannac et al., 2006; Migliore et al., 2007), as in terrestrial plants (Dixon and Paiva, 1995; Loponen et al., 1998; Robles et al., 2003).

Total phenol concentration in *P. oceanica* rhizomes was determined, using comparable experimental procedures, during a five year period (2005-2009) in S. Marinella meadow. It was chosen to utilize only rhizomes tissues. In fact, the use of phenolic compound as a stress marker is hampered in *P. oceanica* leaves because they are temporary structures, in which total phenol concentration varies physiologically with depth and season (Dumay et al., 2004; Fresi et al., 2004). On the contrary, in rhizomes the levels of synthesis and accumulation of phenolic compounds were found to be more stable (Fresi et al., 2004). Furthermore, the long lifespan allows the maintaining memory of previous environmental pressures (Fresi et al., 2004; Migliore et al., 2007).

In this survey total phenol concentration in rhizomes showed a significant increase with decreasing conservation status of the meadow, confirming the reliability of this tool. In fact, if phenol concentrations increase, an inverse correlation with classical descriptors has to be found. With this aim total phenol concentration was challenged with meadow density values (fig. 4.1 A). The comparison during the 2005-2009 period showed an inverse relation between the two descriptors: to the increase of mean phenol concentrations corresponded a lowering of the meadow density. In particular, the maximum density values (the highest among all sampling stations per year) clearly decrease with the increase of the mean of total phenol concentrations (linear correlation -0.99, $p<0.01$). But also, at the highest phenol concentration the minimum density values were also lower,

ranging from very sparse beds to semi beds, according to the Giraud classification (1977).

In 2010, samples from S. Marinella and Monterosso a Mare meadows were frozen in liquid N_2 immediately after sampling. This sample handling procedure allows all the analyses to be performed on the same specimen (*i.e.* total phenol determination, protein pattern analysis, genetic analysis, oxidative stress enzyme assay). This modified procedure determined a burst of the extracted and measured phenols: values were 13% higher than those obtained with the old procedure.

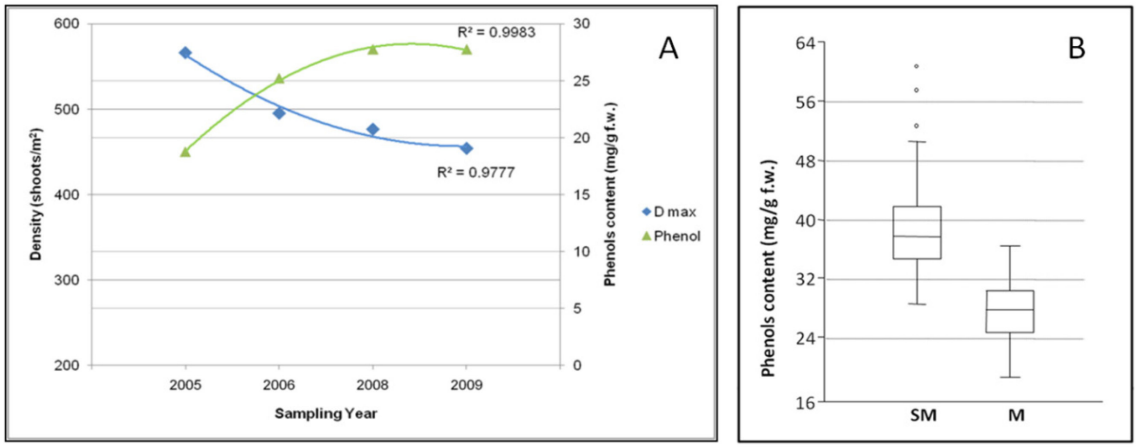

Fig. 4.1. A: variation of total phenol content (as mg/g fresh weight, f.w.) and plant density (as number of shoots/m2) in the S. Marinella meadow, during the 2005-2009 period. Plant density was evaluated in situ by counting the number of shoots using 40 x 40 cm standard frames, five measurements at each site (by the courtesy of Dr. Luigi Valiante). B: total phenol content (as mg/g fresh weight, f.w.) in *P. oceanica* rhizomes collected in 2010 froma S. Marinella (SM) and Monterosso (M) meadow.

Comparison between S. Marinella and Monterosso 2010 samples showed significant differences in mean content (fig. 4.1 B), confirming once again the capability of this easy and cheap analysis to give a clear picture of the plant health status. Indeed, S. Marinella is a disturbed site with turbid waters, near the mouth of the Tiber River; the meadow is highly fragmented (Diviacco et al., 2001; Dante, 2010; Rotini and Valiante, personal observation). While Monterosso has higher values of transparency and better environmental conditions (part of the bed is located inside the 'Cinque Terre' National Park), although there are pressures, mainly of anthropogenic nature, related to the proximity to residential areas, destination of intense nautical tourism (Cavazza et al., 2000; Peirano et al., 2005).

A similar protocol for total phenol determination was successfully applied to another seagrass (*Z. noltii*), opening interesting perspectives on a possible future application of this tool to a large number of marine phanerogams. To this end the most significant variation in the protocol was made at the extraction step: homogenization in HCl was used (and not grinding in liquid N_2). The entire plant (leaves, roots and rhizome) was used, due to the morphological characteristics of the plant, which do not allow an easy separation. These solutions were preferred because they allow the better extraction and detection of total phenols. Preliminary results showed that total phenol concentration in *Z. noltii* tissues is about 10 times lower than in *P. oceanica*; this is probably due to both the different structural features of the plants and to the good environmental conditions of the sampling site (Ria Formosa; Andrade, 1990; Santos at al., 2004).

Hence, phenolic compounds seem to have all the potentialities to constitute reliable biomarkers of environmental quality; the technique proposed here is cheap, easy to do and standardizable, and it can be utilized to screen the health status of different seagrass meadows.

2-D electrophoresis analysis

The proteomic approach, based on the simultaneous separation of hundreds of proteins in the same two dimensional electrophoretic (2-DE) gel, is a powerful tool to monitor the health status of living organisms. In fact, the technique allows the comparison of protein expression patterns in organisms under different physiological and environmental conditions (Hu et al., 2003; Lopez, 2007; Nunn and Timpermann, 2007; Rossignol et al., 2006). The application of proteomics in monitoring of marine ecosystems is a relatively new tool and only a few studies have introduced the proteomic approach to study seagrasses (Bucalossi et al., 2006; Migliore et al., 2007; Mazzuca et al., 2009; Rotini et al., 2011).

The analysis of protein expression patterns of *P. oceanica* rhizomes from S. Marinella meadow, led to the construction of the first 2-DE map of *P. oceanica* rhizome, composed of 437 protein spots, each characterized by p*I* and molecular weight (2006 samples). The analysis was replicated on 2008 and 2009 samples, by a modified 2nd dimension separation. This modification allowed the detection of a

slightly higher number of spots with a better spatial resolution, particularly those with higher molecular weights (see fig. 4.2). All the maps showed a comparable number of spots (about 460), arranged in comparable 'constellations' in the experimental window of p*I* and MW.

The protein expression patterns of *P. oceanica* rhizomes containing the lowest or the highest phenol amount were compared. Differences in the presence/absence of specific spot have been consistently found between the two phenol conditions, although the number of proteins exclusively expressed in each condition varied among years. Probably there is a threshold level reflecting plant condition (i.e. poor preservation of the meadows) that induces high phenol production and influences the expression of specific proteins/variants (irreversible changes?). This may correspond to over-expression or absence of some protein spots and/or *de novo* expression of proteins/variants normally not present in the so called "healthy meadow".

Adaptation to stress represents an early phenomenon in *P. oceanica* which results in the increase of phenol content and the rapidly modified expression of certain proteins. The number of spots in the "lowest phenol" samples (i.e. the "low phenol-specific spots) is dramatically reduced from 2006 to 2009, although the total spot number is not. The same trend is found in the high phenol-specific spots from 2008 to 2009. Consistently, low phenol values increase from 2006 to 2008 and remain constant in 2009. The correspondence between phenol content and specific protein patterns confirms preliminary observations (Migliore et al., 2007) and opens interesting perspectives on the validation of the combined tool phenol content/2-DE protein pattern combined in a single tool to evaluate the 'health' of *P. oceanica* meadows.

The next step of this work will be the quantitative analysis with the identification of peculiarly expressed proteins (by mass spectroscopy or western blotting) that may lead to the development of specific biomarkers, which are able to identify and quantify early alterations in the plant's adaptive response.

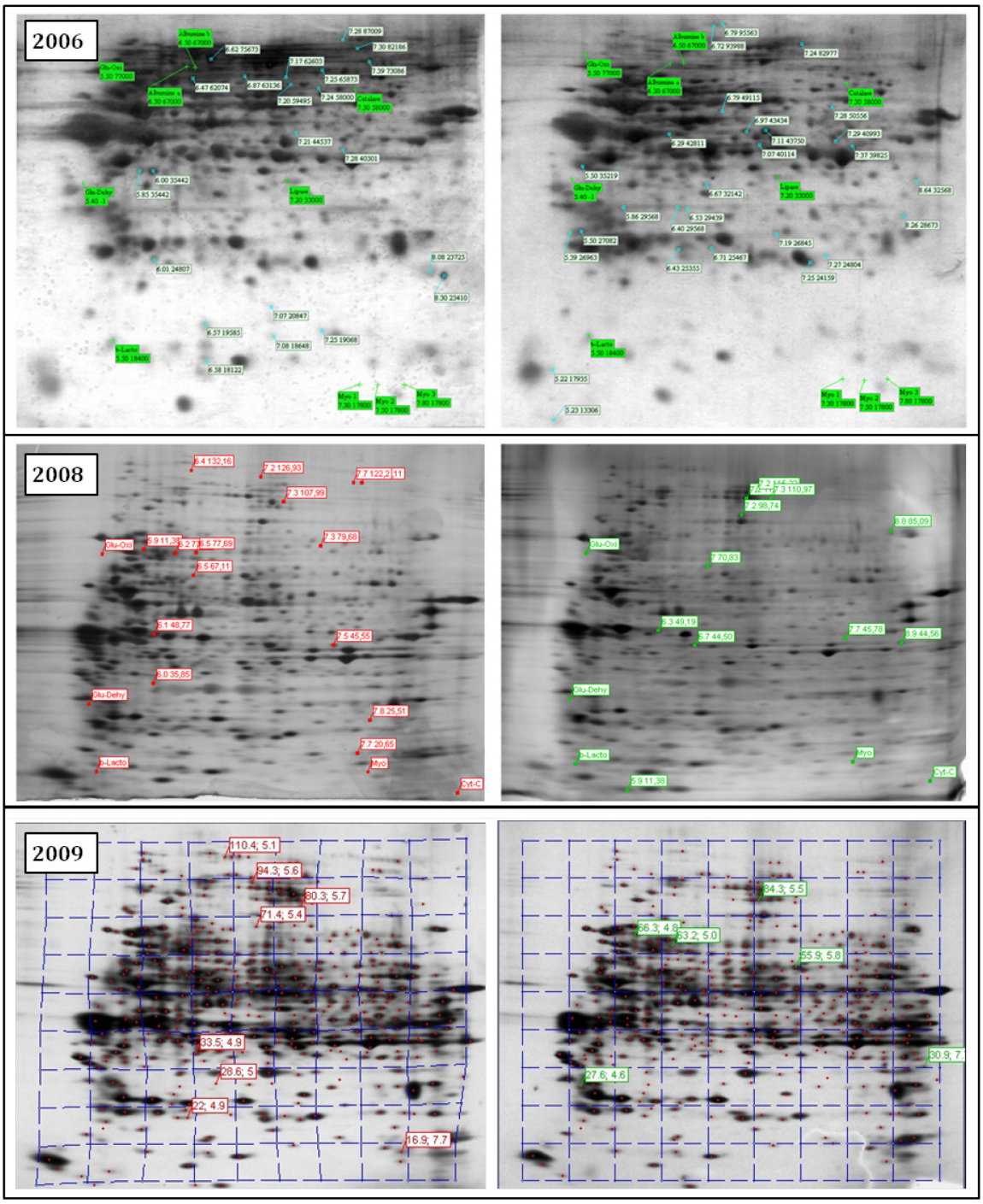

Fig. 4.2. Representative 2-DE maps obtained in 2006, 2008 and 2009 from plants showing high (left) or low (right) total phenol content. Specific protein spots expressed in the two phenol conditions samples are highlighted and labelled.

Heavy metal/element content

The analysis of element/metal content in environmental matrices such as seawater or sediments provides a picture of the total contaminant load, while the use of organisms allows the evaluation of their biological availability and effects on the biota. *P. oceanica* has been utilised by several authors as a trace metal biomonitor as reported by Conti et al. (2010).

Our results on metal/element concentrations in seawater and sediment and in *P. oceanica* tissues are generally in the order of magnitude of those found in the literature; the absolute metal contents recorded in this study fall in the range of the lowest reported values (Campanella et al., 2001; Gosselin et al., 2006; Conti et al., 2010; Tovar-Sànchez et al., 2010; Ruiz Chacho et al., 2010; Llorente-Mirandes et al., 2010).

In S. Marinella meadow Cd, Pb and Zn concentrations in *P. oceanica* tissues were higher in leaves than in rhizomes. The preferential accumulation of Cd and Zn in leaves rather than in rhizomes is a well-established pattern; our results match those of many previous studies (Warnau et al., 1995; Schlacher-Hoenlinger and Schlacher, 1998, Campanella, 2001). This model of accumulation seems based upon surface uptake; *i.e.* in leaves the wider surfaces promote absorption kinetics. An active carry over in leaves is strongly suggested by the dramatically lower metal content in the abiotic compartment.

Cu levels do not differ between leaves and rhizomes. An almost constant Cu content in plant tissues has already been observed; it can be supposed to arise from a certain regulation capability of the phanerogams. (Warnau et al., 1995; Campanella et al., 2001)

For As levels, no significant differences were found between leaves and rhizomes but concentrations in the abiotic compartment were higher than those registered in *P. oceanica* tissues. As concentrations in leaves are in the same order of magnitude reported in the literature (Ruiz Chacho et al., 2010; Llorente-Mirandes et al., 2010). Concentrations in rhizomes are original data and cannot be compared with others. A particularly meaningful result is the difference between plant tissues and abiotic compartment contents. The inverted accumulation ratio suggests a different uptake mechanism and/or a specific defence capability towards this element.

In plant tissue concentrations decrease according to the order: Zn>Cu>As>Cd>Pb in rhizomes and Zn>Cu>Pb>Cd>As in leaves. This is the sequence commonly observed, although sometimes with the occasional inversion of Cd and Pb (Costantini et al., 1991; Warnau et al., 1995; Schlacher-Hoenlinger and Schlacher, 1998a; Conti et al., 2010).

The Zn/Cd and Cu/Cd ratios were also estimated. These ratios could be utilized as indicators of deficiencies and imbalances in contaminated areas, as Zn and Cu are involved in many metabolic pathways (Hänsch and Mendel, 2009).

This study confirms that *P. oceanica* can be considered a sentinel organism for the evaluation of metal and element contamination in marine environments. *P. oceanica* leaves (which have a lifespan of months) can give indications of the metal/element occurrence in the environment on a short time, while rhizomes give indications over a wider time period because of their longer lifespan (years), maintaining memory of historical contamination.

RAPD genetic analysis

Genetic analysis must be included within *P. oceanica* descriptors, because the lowering of genetic diversity (often caused by a dominance of clonal growth in *P. oceanica*) may result in a low resistance, low resilience and a limited adaptability to environmental changes (Procaccini et al., 2007; Arnaud-Haond et al., 2010).

The genetic analysis conducted in S. Marinella meadow demonstrates a low variability in the specimens. RAPD analyses showed a high similarity value within the population (0.87), much higher than that found at the Monterosso al Mare meadow (0.66) or at the Mediterranean basin (0.81) scale (Micheli et al., 2005).

Samples from the same station cluster in a well characterized groups and differences among stations can be found.

These results confirm that this repeatable, cheap and easy technique is suitable for the genetic screening of *P. oceanica* populations.

Antioxidant enzyme activity and photosynthetic pigments

Posidonia is a recalcitrant material (Wang et al, 2006), from which high-quality proteins, maintaining their enzymatic activity, are difficult to be isolate. Notwithstanding, enzymatic activities are widely utilized to evaluate the physiological status of many organisms; in particular, antioxidant enzymes are considered suitable stress biomarkers. In fact, several environmental stressors are known to induce activation of antioxidant mechanisms in different species (Ferrat et al., 2003; Monserrat et al., 2007).

The method to quantify the activity of two enzymes involved in antioxidant defence (DHAR and APx) has been developed for the first time on *P. oceanica* leaves to be included as a putative descriptor of health status of *P. oceanica* plants. An increment of ROS formation and the consequent increase in ROS scavenging enzyme activity are a common response to several environmental stresses. Enzymatic activities were analyzed exclusively in leaves, because leaf tissues represent an easy to handle source of enzymes compared to rhizomes, which harbour a negligible antioxidant activity as demonstrated by preliminary assays. These methods will be further implemented to check their reproducibility and the enzymatic activities may be included as putative descriptors of the health status of *P. oceanica* plants.

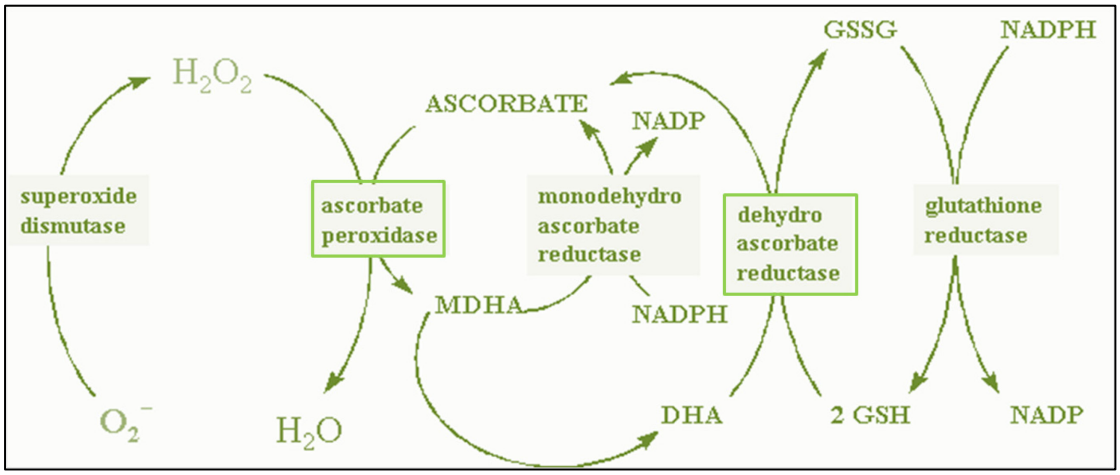

Fig. 4.3. Superoxide dismutase-ascorbate-glutathione pathway for H_2O_2 removal in plants, which involves ascorbate peroxidase and dehydroascorbate reductase, highlighted by green box (modified from Pennsylvania State University site: http://cropsoil.psu.edu/Courses/AGRO51Oxygen.htm#biological last access 2005).

Both APx and DHAR enzymes are involved in the ascorbate-glutathione pathway (*i.e.* the removal of H_2O_2 and regeneration of ascorbate, see fig. 4.3; Noctor and Foyer,1998; Mittler and Puolus, 2005). Reliability of our results is supported by the high correlation found between their activity levels ($r=-0.8$; $p<0.01$, see fig. 4.4).

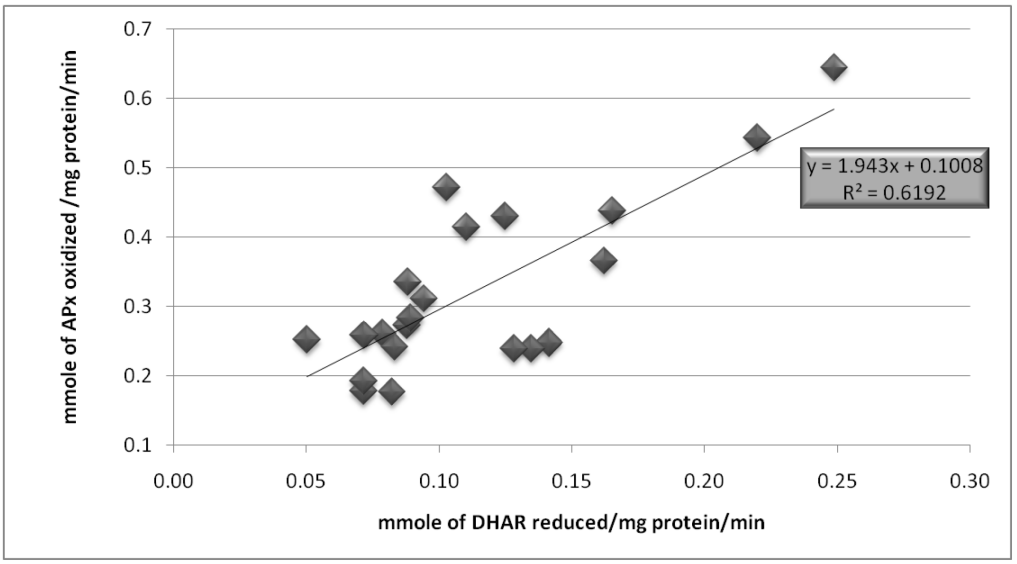

Fig. 4.4. Correlation between the activity levels of ascorbate peroxidase (APx) and dehydro ascorbate reductase (DHAR) measured in each sample.

Comparison between S. Marinella and Monterosso 2010 samples showed significant differences in mean enzymatic activity. This superimpose to the environmental conditions of the two sites: S. Marinella is a disturbed meadow, while Monterosso is exposed to better environmental conditions. These data are also in agreement with the total phenol content in plants.

Photosynthetic pigments content was also quantified in samples collected in 2010 from S. Marinella and Monterosso meadows. Chlorophyll a and b and Carotenoid concentrations represent additional information which may give an indication of oxidative stress due to the light regimes. In fact, stressed plants increase their carotenoid content to provide protection against free radicals. A decrease in the ratio chlorophylls/carotenoids is often found under stressed conditions (Lichtenthaler and Badani, 2004).

In the 2010 samples from S. Marinella and Monterosso meadow photosynthetic pigments (Chlorophylls a and b and total Carotenoids) content was quantified. Significant differences were not found between the two sampling sites

5. CONCLUSIONS

Deleterious effects on *P. oceanica* meadows are often difficult to detect in time: the health status of the interconnected shoots - generally expressed in terms of biomass and production - does not directly reflect the environmental degradation. By the time that *P. oceanica* meadow shows signs of regression, the rest of the ecosystem has been already largely affected. Furthermore, when *Posidonia* meadow is endangered, it is often too late to plan a management procedure that recovers the meadow; to this end, early-warning indicators of seagrass health are necessary. The choice and combination of measurable, sensitive and integrative variables that adequately reflect the environmental alterations is a challenge for the whole scientific community.

All methodologies developed and/or tested in this research project were aimed to fulfil this goal: results provide evidences of their convenient application as markers of the health status of *P. oceanica* and/or other seagrass meadows; some of them are early-warning indicators of plant stress responses. Furthermore, the proposed tools are standardizable, provide detailed information about physiological status of the plants and can be integrated to the traditional methods of investigation. According to the variability of the disturbances (e.g. nutrient inputs, decrease of water transparency, modified hydrodinamism, kind of pollution), a combination of the proper descriptors can be used. Due to the low amount of biological material necessary for each determination, it is also possible to evaluate several descriptors on a single shoot with a double result: to get a picture of the health status of each individual, improving our understanding of stress-response processes in seagrasses, and to reduce the impact of these 'destructive' indicators.

The increasing pace of human-induced environmental change worldwide has created a demand for effective bioindicators; particularly important are those able to monitor marine systems. The approach proposed by this research project makes available several tools to establish the linkage between stressor and seagrass response and to better understand the effects of complex disturbances. Biomonitoring is an expensive and time-consuming activity, therefore, the appropriate choice of indicators can provide the most unequivocal information about the quality of the environment at the relevant spatial scale is crucial.

6. REFERENCES

Andrade C. (1990) - O ambiente barreira da Ria Formosa, Algarve, Portugal. Ph.D. Dissertation. Faculty of Sciences, University of Lisbon, Algarve, Portugal.

Apostolaki E.T., Marbà N., Holmer M., Karakassis I. (2009) - Fish farming enhances biomass and nutrient loss in *Posidonia oceanica* (L.) Delile. *Estuar Coast Shelf Sci*, 81: 390–400

Arnaud-Haond S., Marbà N., Diaz-Almela E., Serrão E.A., Duarte C. M. (2010) - Comparative analysis of stability-genetic diversity in seagrass (*Posidonia oceanica*) meadows yields unexpected results. *Estuar Coast*, 33: 878–889

Balestri E., Benedetti-Cecchi L., Lardicci C. (2004) - Variability in patterns of growth and morphology of *Posidonia oceanica* exposed to urban and industrial wastes: contrasts with two reference locations. *J Exp Mar Biol Ecol*, 308:1-21

Barrote I. (2005) - Efeitos a posteriori dos défices hídricos sobre a actividade fotossintética. Mecanismos de fotoprotecção e sistema enzimático antioxidante. PhD Thesis, Algarve University, Faro, Portugal .

Booker F.L., Miller J.E. (1998) - Phenylpropanoid metabolism and phenolic composition of soybean (*Glycine max* L.) leaves following exposure to ozone. *J Exp Bot*, 49 (324): 1191-1202.

Boudouresque C.F., Bernard G., Bonhomme P., Charbonnel E., Diviacco G., Meinesz A., Pergent G., Pergent-Martini C., Ruitton S., Tunesi L. (2006) - Préservation et conservation des herbiers à *Posidonia oceanica*. RaMoGe publication, Monaco.

Bucalossi D., Leonzio C., Casini S., Fossi M.C., Marsili L., Ancora S., Wang W., Scali M. (2006) - Application of a suite of biomarkers in *Posidonia oceanica* (L.) Delile to assess the ecotoxicological impact on the coastal environment. Short communication. *Mar Environ Res,* 62: 327-331.

Buia M.C., Gambi M.C., Dappiano M. (2004) - The seagrass ecosystems. In: Gambi M.C., Dappiano M. (eds), Mediterranean Marine Benthos: a manual for its sampling and study. *Biol Mar Medit*, 11(1): 133-183.

Buia M.C., Silvestre F., Iacono G., Tiberti L. (2005) - Identificazione delle biocenosi di maggior pregio ambientale al fine della classificazione della qualità delle acque costiere. Metodologie per il rilevamento e la classificazione dello stato di qualita ecologico e chimico delle acque, con particolare riferimento all'applicazione del decreto legislativo 152/99. APAT, Rome. pp. 269–303.

Cambridge M.L. and Kuo J. (1979) - Two new species of seagrass from Australia, *Posidonia sinuosa* and *Posidonia angustifolia* (Posidoniaceae). *Aquat Bot*, 6: 307-328.

Campanella L., Conti M.E., Cubadda F., Sucapane C. (2001) - Trace metals in seagrass, algae and molluscs from an uncontaminated area in the Mediterranean. *Environ Pollut* 111: 117–126.

Cannac M., Ferrat L., Pergent-Martini C., Pergent G., Pasqualini V. (2006) - Effects of fish farming on flavonoids in *Posidonia oceanica*. *Sci Tot Environ*, 370: 91-98.

Cavazza W., Immordino F., Moretti L., Peirano A., Pironi A., Ruggiero F. (2000) - Sedimentological parameters and seagrasses distribution as indicators of anthropogenic coastal degradation at Monterosso Bay (Ligurian Sea, NW Italy). *J Coast Res* 16 (29): 295–305.

Conti M.E., Bocca B., Iacobucci M., Finoia M.G., Mecozzi M., Pino A., Alimonti A. (2010) - Baseline trace metals in seagrass, algae, and mollusks in a Southern Tyrrhenian ecosystem (Linosa Island, Sicily). *Arch Environ Contam Toxicol*, 58: 79–95.

Costantini S., Giordano R., Ciaralli L., Beccaloni E., (1991). Mercury, cadmium and lead evaluation in *Posidonia oceanica* and *Codium tomentosum*. *Mar Pollut Bull* 22: 362-363.

Cuny P., Serve L., Jupin H., Boudouresque C.F. (1995) - Water soluble phenolic compounds of the marine phanerogam *Posidonia oceanica* in a Mediterranean area colonised by the introduced chlorophyte *Caulerpa taxifolia*. *Aquat. Bot.* 52(3): 237-242.

Dale V.H., Beyeler S.C. (2001) - Challenges in the development and use of ecological indicators. *Ecol Indicators*, 1: 3–10.

Dante G. (2010) - Development of sustainable strategies for conservation and management of *Posidonia oceanica*, (Linneo) Delile 1813, meadow: a case study within a Site of Community Importance. PhD Thesis, University of Tuscia, Viterbo, Italy.

Dayton P.K., Thrush S.F., Agardy M.T. and Hofman R.J. (1995) - Environmental effects of marine fishing. *Aquatic Conservation: Marine and Freshwater Ecosystems,* 5: 205-232.

Dekker A., Brando A., Anstee S., Fyfe S., Malthus T., Karpouzly E. (2006) - Remote sensing of seagrasses, in: Larkum, A.W.D., Orth R.J., and. Duarte C.M (Eds) Seagrasses: Biology, Ecology and Conservation. Springer. pp. 387-408.

Den Hartog C. (1970) - Seagrasses of the World. *Verh. Kon. Ned. Akad. Wetens Afd Naturk Ser,* 2: 59-139

Diviacco G., Spada E., Virno Lambert, C. (2001). Le Fanerogame marine del Lazio. Descrizione e Cartografia delle prateria di *Posidonia oceanica* e dei prati di *Cymodocea nodosa*. ICRAM, Roma, pp. 41.

Dixon R.A., Paiva N.L. (1995) - Stress-induced phenylpropanoid metabolism. *Plant Cell*, 7: 1085-1097.

Duarte C.M. (2002) - The future of seagrass meadows. *Environmental Conservation*, 29: 192–206.

Duarte C.M., Borum J., Short F.T., Walker D.I. (2008) - Seagrass ecosystems: their global status and prospects. In: Polunin NVC (ed), Aquatic Ecosystems, Cambridge University Press, Cambridge. pp. 281–294.

Dumay O., Costa J., Desjobert J.M., Pergen, G. (2004) - Variations in the concentration of phenolic compounds in the seagrass *Posidonia oceanica* under conditions of competition. *Phytochemistry*, 65: 3211-3220.

Fernandez-Torquemada Y., Diaz-Valdes M., Colilla F., Luna B., Sanchez-Lizaso J.L., Ramos-Esplà A.A. (2008) - Descriptors from *Posidonia oceanica* (L.) Delile meadows in

coastal waters of Valencia, Spain, in the context of the EU Water Framework Directive. *ICES J Mar Sci*, 65: 1492–1497

Ferrat L., Pergent-Martini C., Roméo M. (2003) - Assessment of the use of biomarkers in aquatic plants for the evaluation of environmental quality: application to seagrasses. *Aquat Toxicol*, 65: 187–204.

Franconi R., Barcaccia G., Paglialonga A., Micheli C. (1995) - Investigation of genomic polymorphism in *Posidonia oceanica* plants collected in different areas of Mediterranean Sea using RAPD markers. Rapp. Commun. Int. Mer. Me ´dit. 34, 29.

Francour P., Ganteaume A., Poulain M. (1999) - Effects of boat anchoring in *Posidonia oceanica* seagrass beds in the Port-Cros National Park (north-western Mediterranean Sea). *Aquat Cons*, 9: 391-400.

Fresi E., Dolce T., Forni C., Lorenzi C., Migliore L., Rizzelli D., Scardi M. (2004) - La prateria di *Posidonia oceanica* (L.) Delile di Talamone (Grosseto, Italia): struttura e stato di salute. In Proceedings of the Conference "Le scienze naturali, economiche e giuridiche nello studio e per la gestione degli ambienti acquatici". CONISMA-AIOL Terrasini (Palermo, Italy).

Giraud G. (1977) - Essai de classement des herbiers de *Posidonia oceanica* (L.) Delile. *Bot Mar*, 20: 487-491.

Gobert S., Cambridge M.L., Velimirov B., Pergent G., Lepoint G. and others. (2006) - Biology of Posidonia, in: Larkum, A.W.D., Orth R.J., and. Duarte C.M (Eds) Seagrasses: Biology, Ecology and Conservation. Springer. pp. 387-408.

Gobert S., Sartoretto S., Rico-Raimondino V., Andral B., Chery A., Lejeune P., Boissery Pi. (2009) - Assessment of the ecological status of Mediterranean French coastal waters as required by the Water Framework Directive using the *Posidonia oceanica* Rapid Easy Index: PREI. *Mar Pollut Bull*, 58: 1727–1733.

Gosselin M., Bouquegneau J.M., Lefèbvre F., Lepoint G., Pergent G., Pergent-Martini C., Gobert S. (2006) - Trace metal concentrations in *Posidonia oceanica* of North Corsica (northwestern Mediterranean Sea): use as a biological monitor? *BMC Ecology,* 6:12.

Guidetti P., Fabiano M. (2000) - The use of lepidochronology to asses the impact of terrigenous discharges on the primary leaf production of the Mediterranean seagrass *Posidonia oceanica*. *Mar Poll Bull*, 40(5): 449-453.

Hänsch R., Mendel R.R. (2009) - Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). *Curr Opin Plant Biol*, 12: 259–266.

Hemminga M.A., Duarte C.M. (2000) - Seagrass Ecology. Cambridge University Press, UK.

Hu Y., Wang G., Chen G.Y.J., Fu X., Yao S.Q. (2003) - Proteome analysis of *Saccharomyces cerevisiae* under metal stress by two-dimensional differential gel electrophoresis. *Electrophoresis*, 24: 1458–70.

Kuo J., den Hartog C. (2006) Seagrass Morphology, Anatomy and Ultrastructure, in: Larkum, A.W.D., Orth R.J., and. Duarte C.M (Eds) Seagrasses: Biology, Ecology and Conservation. Springer. pp. 51-87.

Kuo J., Cambridge M.L. (1984) - A taxonomic study of the *Posidonia ostenfeldii* complex (Posidoniaceae) with descriptions of four new Australian seagrasses. *Aquat Bot*, 20: 267-95.

Laemmli U.K. (1970) - Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.

Lichtenthaler H. K., Badani F. (2004) - Adaptation and Senescence of the Photosyntetic Apparatus. In: George C. Papageorgiou and Govindjee (eds), Chlorophyll a Fluorescence: A Signature of Photosyntesis. Springer. pp. 716-721.

Llorente-Mirandes T., Ruiz-Chancho M.J., Barbero M., Rubio R., López-Sánchez J.F. (2010) - Measurement of arsenic compounds in littoral zone algae from the Western Mediterranean Sea. Occurrence of arsenobetaine. *Chemosphere*, 81: 867-875.

Lopez J.L. (2007) - Applications of proteomics in marine ecology. *Mar Ecol Prog Ser*, 332: 275-279.

Lopez y Royo, C. (2008) - Utilisation de *Posidonia oceanica* (L.) Delile comme outil de gestion de la qualité écologique du milieu marin. PhD Thesis. Université de Corse, pp. 1–242.

Loponen J., Ossipov V., Lempa K., Haukioja E., Pihlaja K. (1998) - Concentrations and among-compound correlations of individual phenolics in white birch leaves under air pollution stress. *Chemosphere*, 37(8): 1445-1456.

Marbà N., Duarte C.M. (2010) - Mediterranean warming triggers seagrass (*Posidonia oceanica*) shoot mortality. *Global Change Biol,* 16: 2366–2375

Mazzuca S., Spadafora A., Filadoro D., Vannini C., Marsoni M., Cozza R., Bracale M., Pangaro T., Innocenti A.M. (2009) - Seagrass light acclimation: 2-DE protein analysis in *Posidonia* leaves grown in chronic low light conditions. *J Exp Mar Biol Ecol*, 374: 113-122.

Mc Carthy J.F., Shugart L.R. (1990) - Biomarkers of environmental contamination. Lewis, Boca Raton, FL.

Mc Clure J.W. (1970) - Secondary constituents of aquatic angiosperms. in: Harborne, J.B. (Ed.), Phytochemical Phylogeny. Academic Press, London, pp. 233-268.

Micheli C., Paganin P., Peirano A., Caye G., Meinesz A., Bianchi C.N. (2005) - Genetic variability of *Posidonia oceanica* (L.) Delile in relation to local factors and biogeographic patterns. *Aquat Bot*, 82: 210-221.

Micheli C., Spinosa F., Aliani S., Gasparini G.P., Molcard A., Peiranod A. (2010) - Genetic input by *Posidonia oceanica* (L.) Delile fruits dispersed by currents. *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology*, 144(2): 333-339

Migliore L., Rotini A., Randazzo D., Albanese N., Giallongo A. (2007) - Phenols content and 2-D electrophoresis protein pattern: a promising tool to monitor *Posidonia* meadows health state. *BMC Ecology*, 7: 6.

Milazzo M., Badalamenti F., Seccherelli G., Chemello R. (2004) - Boat anchoring on *Posidonia oceanica* beds in a marine protected area (Italy, western Mediterranean): effect of anchor types in different anchoring stages. *J Exp Mar Biol Ecol*, 299: 51-62.

Mittler R., Puolus T. L. (2005) - Ascorbate peroxidase. In: N. Smirnoff (ed.), Antioxidants and reactive oxygen species in plants. Blackwell Publishing Edition, pp. 87-100.

Molinier R., Picard J. (1952) - Recherches sur les herbiers de Phanérogams marines du littoral méditerraneen francais. *Ann Inst Oceanogr*, 27(3): 157-234.

Monserrat J.M., Martinez P.E., Geracitano L.A., Amado L.L., Martins C.M., Pinho G.L., Chaves I.S., Ferreira-Cravo M., Ventura-Lima J., Bianchini A. (2007) - Pollution biomarkers in estuarine animals: critical review and new perspectives. *Comp Biochem Physiol (C)*, 146: 221–234.

Montefalcone M. (2009) - Ecosystem health assessment using the seagrass *Posidonia oceanica*: a Review. *Ecol Indicators*, 9: 595-604

Montefalcone M., Albertelli G., Bianchi C.N., Mariani M., Morri C. (2006) - A new synthetic index and a protocol for monitoring the status of Posidonia oceanica meadows: a case study at Sanremo (Ligurian Sea, NW Mediterranean). *Aquat Conserv: Mar Freshwat Ecosyst* , 16(1): 29–42

Montefalcone M., Morri C., Peirano A., Albertelli G., Bianchi C.N (2007) - Substitution and phase-shift in *Posidonia oceanica* meadows of NW Mediterranean Sea. *Estuar Coast Shelf Sci,* 75: 63–71.

Montefalcone M., Chiantore M., Lanzone A., Morri C., Bianchi C.N., Albertelli G. (2008) - BACI design reveals the decline of the seagrass *Posidonia oceanica* induced by anchoring. *Mar Pollut Bull* 56: 1637–1645.

Moreno D., Aguilera P.A., Castro H. (2001) - Assessment of the conservation status of seagrass (*Posidonia oceanica*) meadows: implications for monitoring strategy and the decision-making process. *Biol Cons*, 102: 325–332.

Morri C. (1991) Présentation d'un indice synthétique pour l'évaluation de l'épiphytisme foliaire chez *Posidonia oceanica* (L.) Delile. *Posidonia Newsletter*, 4 (1): 33–37.

Nakano Y., Asada K. (1987) - Purification of ascorbate peroxidase in spinach chloroplasts; Its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. *Plant Cell Physiol*, 28: 131-140.

Niemi G., Wardrop D., Brooks R., Anderson S. and others (2004) - Rationale for a new generation of indicators for coastal waters. *Environ Health Perspect* 112: 979–986

Noctor G., Foyer C.H. (1998) - Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol*, 49: 246–279

Nunn B.L., Timperman A.T. (2007) - Marine proteomics. *Mar. Ecol. Prog. Ser.*, 332: 281-289

Orth R., Carruthers T., Dennison W., Duarte C., Fourqurean J., Heck K. et al. (2006) - A global crisis for seagrass ecosystems. *BioScience*, 56: 987–996.

Pasqualini V., Pergent-Martini C., Clabaut P., Pergent G. (1998) - Mapping of *Posidonia oceanica* using aerial photographs and side scan sonar: Application off the island of Corsica (France). *Estuar Coast Shelf Sci*, 47: 359–367

Peirano A., Damasso V., Montefalcone M., Morri C., Bianchi C.N. (2005) - Effects of climate, invasive species and anthropogenic impacts on the growth of the seagrass *Posidonia oceanica* (L.) Delile in Liguria (NW Mediterranean Sea). *Mar Pollut Bull*, 50: 817-822

Pergent G., Pergent-Martini C., Boudouresque C. F. (1995) - Utilisation de l'herbier à *Posidonia oceanica* comme indicateur biologique de la qualité du milieu littoral en Méditerranée: Etat des connaissances. *Mesogée*, 54: 3–29.

Pergent-Martini C., Leoni V., Pasqualini V., Ardizzone G.D., Balestri E., Bedini R. and others (2005) - Descriptors of *Posidonia oceanica* meadows: use and application. *Ecol Indicators,* 5: 213–230.

Pergent-Martini C., Boudouresque C.-F., Pasqualini V., Pergent G. (2006) - Impact of fish farming facilities on *Posidonia oceanica* meadows: a review. *Mar Ecol*, 27: 310–319

Pip E. (1992) - Phenolic compounds in macrophytes from the Lower Nelson River system Canada. *Aquat Bot,* 42: 273-279.

Polle A., Morawe B. (1995) - Seasonal changes of the antioxidative systems in foliar buds and leaves of field-grown beech trees (*Fagus sylvatica*, L.) in a stressful climate. *Bot Acta,* 108: 314-320.

Procaccini G., Mazzella L. (1998) - Population genetic structure and gene flow in the seagrass*Posidonia oceanica* assessed using microsatellite analysis. *Mar Ecol Prog Ser*, 69: 133-141

Procaccini G., Buia M.C., Gambi M.C., Perez M., Pergent G., Pergent-Martini C., Romero J. (2003) - Seagrass status and extent along the Mediterranean coasts of Italy, France and Spain. In: Green, E.P., Short, F.T., Spalding, M.D. (Eds.), World Atlas of Seagrass: Present Status and Future Conservation. University of California Press Publ., pp. 56–66.

Procaccini G., Olsen J.L., Reusch T.B.H. (2007) - Contribution of genetics and genomics to seagrass biology and conservation. *J Exper Mar Biol Ecol*, 350: 234–259.

Robles C., Greff S., Pasqualini V., Garzino S., Bousquet-Mélou A., Fernandez C. and others (2003) - Phenols and flavonoids in Aleppo pine needles as bioindicators of air pollution. *J Environ Qual*, 32: 2265-2271

Romero J., Martinez-Crego B., Alcoverro T., Perez M. (2007) - A multivariate index based on the seagrass *Podidonia oceanica* (POMI) to assess ecological status of coastal waters under the Water Framework Directive (WFD). *Mar Pollut Bull*, 55: 196–204.

Rossignol M., Peltier J.B., Mock H.P., Matros A., Maldonado A.M., Jorrin J.V. (2006) - Plant proteome analysis: A 2004–2006 update. *Proteomics*, 6: 5529–5548.

Rotini A., Anello L., Di Bernardo M., Giallongo A., Migliore L. (2011). A Five-year monitoring study of A *Posidonia oceanica* meadow coupling total phenols content and 2-D electrophoresis pattern of proteins. *Aquat Bot*, *submitted*.

Ruiz J.M., Romero J. (2003) - Effects of disturbances caused by coastal constructions on spatial structure, growth dynamics and Photosynthesis of the seagrass *Posidonia oceanica*. *Mar Pollut Bull*, 46:1523-1533

Ruiz-Chancho M.J., López Sánchez J.F., Rubio R. (2010) - Occurrence of arsenic species in the seagrass *Posidonia oceanica* and in the marine algae *Lessonia nigrescens* and *Durvillaea Antarctica*. *J Appl Phycol* 22: 465–472.

Sanchez-Jerez P., Ramos-Esplà A.A. (1996) - Detection of environmental impacts by bottom trawling on *Posidonia oceanica* (L.) Delile meadows: sensitivity of fish and macrobenthic communities. *J Aquat Ecosyst Health*, 5: 239–253.

SANTOS R., SILVA J., ALEXANDRE A., NAVARRO N., BARRO' C., DUARTE C.M. (2004) - Ecosystem Metabolism and Carbon Fluxes of a Tidally-dominated Coastal Lagoon. *Estuaries* 27(6): 977–985.

Savini D., Peirano A., Bianchi C.N. (1999) - Effects of anthropic disturbance on shoot structure of plagiotropic and orthotropic rhizomes of *Posidonia oceanica* (L.) Delile in Monterosso Bay (NW Mediterranean). *Biol Mar Medit*, 6(1): 441–444.

Schlacher-Hoenlinger M.A., Schlacher T.A. (1998). Differential accumulation patterns of heavy metals among the dominant macrophytes of a Mediterranean seagrass meadow. *Chemosphere*, 37: 1511-1519.

Short F.T., Neckles H.A. (1998) - The effects of global climate change on seagrasses. *Aquatic Botany*, 63: 169–196.

Spalding M., Taylor M., Ravilious C., Short F., Green E. (2003) - The distribution and status of seagrasses. In: Green EP, Short FT (eds) World atlas of seagrasses. University of California Press, Berkeley, CA, pp. 5–26

Sureda A., Box A., Terrados J., Deudero S., Pons A. (2008) - Antioxidant response of the seagrass *Posidonia oceanica* when epiphytized by the invasive macroalgae *Lophocladia lallemandii. Mar Environ Res*, 66: 359-363

Switzer R.C., Merrril C.R., Shifrin S. (1979) - A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal Biochem*, 98(1): 231-237.

Tovar-Sánchez A., Serón J., Marbà N., Arrieta J.M., Duarte C.M. (2010) - Long-term records of trace metal content of Western mediterranenan seagrass (*Posidonia oceanica*) meadows: natural and anthropogenic contributions. *J Geophys Res*, Vol 115, G02006.

Wang W., Vignani R., Cresti M., Scali M. (2006) - A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. Short communication. *Electrophoresis*, 27: 2782-2786.

Warnau M., Ledent G., Temara A., Bouquegneau J.M., Jangoux M., Dubois P. (1995) - Heavy metals in *Posidonia oceanica* and *Paracentrotus lividus* from seagrass beds of the north-western Mediterranean. *Sci Total Environ*, 171: 95-99

Waycott M. (1995) - Assessment of genetic variation and clonality in the seagrass *Posidonia australis* using RAPD and allozyme analysis. *Mar Ecol Prog Ser*, 116: 289–295

Wellburn A.R. (1994) - The spectral determination of chlorophylls A and B, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *J Plant Phys*, 144: 307-313

Zapata O., Mc Millan C. (1979) - Phenolic acids in seagrasses. *Aquat Bot*, 7: 307-317.

7. ANNEX A - Reagent list

3-10 Bio-Lyte Ampholyte	Bio-Rad Laboratories
Ascorbate	Sigma
Bovine Serum Albumine	Sigma
Bradford reagent	Bio-Rad Laboratories
Chlorogenic acid	Sigma
Dehydroascorbic Acid	Sigma
Dimethyl-formamide	Sigma
DNA ladder 1kb	Applied Biosystems
dNTPs	Applied Biosystems
Folin-Ciocalteau reagent	Sigma
Glutathione reduced	Sigma
H_2O_2 30%	Merck
HCl	Merck
HNO_3	Merck
KCl	Merck
$MgCl_2$	Merck
Na_2CO_3	Merck
pI markers	SERVA Electrophoresis
Precast Gradient Gels Nupage 4%-12% acrylamide	Invitrogen
proteases and phosphatases inhibitors	Sigma
PVPP (insoluble polyvinylpolypyrrolidone)	Merck
Readystrip Immobilized pH Gradient, pH 3-10 NL	Bio-Rad Laboratories
Sea sand	Merck
Sephadex G-25 PD-10 column	Pharmacia
Taq DNA Polimerase	Applied Biosystems
Triton-X	Sigma