

# A CITIZEN SCIENCE APPROACH FOR THE GENETIC ANALYSIS OF A *POSIDONIA OCEANICA* MEADOW IN THE ITALIAN SOUTHERN ADRIATIC SEA

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*POSIDONIA OCEANICA*  
SEAGRASS  
UNDERWATER VISUAL CENSUS  
EPIPHYTE  
MICROSATELLITE  
GENOTYPING  
CITIZEN SCIENCE  
GENETICS

**ABSTRACT.** – A citizens’ observatory, the Osservatorio del Mare a Molfetta (OMM), monitors the health status of the marine/coastal environment of the city. *Posidonia oceanica* (Linnaeus) Delile meadows are considered as bioindicators revealing the health status of the related marine/coastal ecosystem. To gather information necessary for the preservation of this ecosystem, OMM investigated the health status of the local San Vito-Barletta *P. oceanica* meadow extending along the coast of the Apulia region in the Italian Southern Adriatic Sea, which has been lately graded as ‘disturbed’. Volunteers conducted underwater visual census of the epiphytes on *P. oceanica* leaves. Two campaigns of investigations at three different stations, namely Molfetta, Giovinazzo and Mola di Bari, evidenced differences in the distribution of the epiphytic communities. Furthermore, a population genetics study performed together with a scientific research centre using highly variable microsatellite markers showed an excess of heterozygosity and demonstrated the existence of at least two subpopulations within the meadow. Since the sampling areas were situated along the coasts of different urban centers, this situation may reflect the diverse anthropogenic pressures differing from city to city. Therefore, this study highlights the need to promote good conservation practices to preserve the San Vito-Barletta meadow and the whole related coastal/marine ecosystem.

## INTRODUCTION

*Posidonia oceanica* (Linnaeus) Delile meadows are good biological indicators of the health status of marine ecosystem. Anthropogenic and environmental factors are causing the alarming decline of this marine flowering plant (Boudouresque *et al.* 2006, Montefalcone *et al.* 2019). The withdrawal of *P. oceanica* correlates with decay in associated biodiversity and ecosystem services. Yet public awareness of the importance of preserving *P. oceanica* meadows is still very low. The benefits provided by the ecosystem located in the intertidal zones are poorly understood (Nordlund *et al.* 2018). The European Union’s Habitat directive (92/43/CEE) considers *P. oceanica* beds among priority habitats, which are among the major targets for the protection of the Mediterranean Sea (Telesca *et al.* 2015). Unfortunately, seagrass meadows are often not taken into account for the purposes of coastal management (Grech *et al.* 2012).

The Osservatorio del Mare a Molfetta (OMM, sea observatory at Molfetta) is a non-profit organization of volunteers operating in the city of Molfetta in the Apulia region of southern Italy.

Very limited information is publicly available on the widespread San Vito-Barletta meadow, a Site of Community Importance (SCI) located along the coast of the Apulia region. In 1991, a first map assessed this meadow as “not very lush” (Ministero Marina Mercantile 1991).

An update performed in 2004 revealed a severe decline in the spatial distribution of the *P. oceanica* meadow, in particular northwards of Bari (Costantino *et al.* 2010). More recently, a local incidence assessment study related to the extension of the harbor of the city of Molfetta performed in April 2010 and October 2011 showed that *P. oceanica* had survived only within the natural cove named Cala San Giacomo. The meadow was highly fragmented displaying numerous patches of dead matte with a mean density value assessed as ‘low sub-normal density’ (LSD), thereby classified as ‘very disturbed’ (Andreassi 2016). So far, no other studies have been carried out on the San Vito-Barletta meadow.

Herein we have explored the health status of the San Vito-Barletta meadow using leaf-epiphyte biomass as biological quality elements (BQE) that are efficient indicators of the health status of *P. oceanica* (Costa *et al.* 2015). Then, the spatial pattern and level of genetic variability of this meadow was determined using highly variable microsatellite molecular markers allowing exploration of the correlation between genetic/genotypic variability and longevity of the *P. oceanica* population.

## MATERIALS AND METHODS

*Community-based environmental monitoring:* OMM volunteers are of all ages, origins, gender, educational level, religious

or political beliefs. We are determined to preserve the marine and coastal ecosystem of the city of Molfetta. For this purpose, numerous actions such as photographic exhibitions, conferences, seashore and seafloor clean-ups are performed to raise the awareness of the general public. The authors of the present study are among the OMM members (including researchers from other research fields) who wanted to perform this study and publish its scientific data. Each of the authors freely contributed to this study according to their own wishes/ability/free time. MdV led and coordinated the teamwork.

This study has been carried out using volunteers' personal devices including, but not limited to, cars, cell phones, GoPro cameras, snorkeling equipment and personal computers. The expenses of this study have been covered by all the volunteers. However, to complete the genetic part of this study and access the required instrumentation, OMM was helped by a public sector scientific research centre.

**Study sites:** The San Vito-Barletta meadow extends over an area of 12,459 ha corresponding to a covered area of 11,213.1 ha for a 65 km stretch of coastline situated in the Maritime Domain of Bari and Molfetta (Apulia Region, Southern Italy) at a depth between 3 and 16 meters, 17°2'9.996"E; 41°4'8"N (Natura2000, Cartografia-IT9120009). This meadow is a SCI (cod. IT9120009) established according to the Habitat Directive 92/43/CEE of the European Union.

The Geographic Information System (SIG) using the open tool Google Earth (where *Posidonia* shoots are visible) was used to select the sites for investigation. For underwater visual census, three shallow water habitats hosting *P. oceanica* were chosen: Molfetta (MF) at Cala San Giacomo (41°12'51.1"N; 16°34'37.0"E), Giovinazzo (GV) at Cala Spiriticchio (41°10'55.8"N; 16°40'56.9"E), and Mola di Bari (ML) (41°03'10.4"N; 17°06'34.1"E) (Fig. 1).

For DNA extraction and microsatellite genotyping, only two of the above sampling stations were chosen: MF at Cala San Giacomo where the seagrass meadow is confined within a semi-

enclosed natural cove, and ML where *Posidonia* shoots extend along a longitudinal coastline (Fig. 1). MF and ML are 48 km apart from each other.

**Underwater visual census and sampling:** In June 2017 and June 2018, MF, GV, and ML sites were monitored by scuba diving between 4 and 6 m depth (5 m depth medium, one dive of about 40 minutes for each year and site). Photographs and videos were recorded using a GoPro Hero3 Black TM.

For DNA extraction and microsatellite genotyping, sampling was performed by scuba diving by two trained volunteers. Importantly, sampling only concerns old single outer leaves from vertical shoots that were selected at a minimum reciprocal distance of 10 meters.

**DNA extraction and microsatellite genotyping:** Epiphytes were removed with a razorblade from the surface of each leaf. Then, leaves were washed in distilled water, cut in half and stored at -80 °C before DNA extraction. Each half of a single leaf was ground to a fine powder in liquid nitrogen in a pre-cooled sterile mortar. 100 mg of powder were transferred to an Eppendorf tube and processed for genomic DNA extraction with Plant DNAzol (Invitrogen) reagent according to manufacturer's protocol.

DNA quality was assessed through 0.8 % (w/v) agarose/1X Tris-borate-EDTA buffer (TBE) gels containing 0.5 mg/ml ethidium bromide and visualized by UV light. DNA purity and concentration was measured using NanoDrop ND-Spectrophotometer (Thermo Fisher Scientific). Samples were genotyped at 14 loci previously developed in *P. oceanica* (Procaccini & Waycott 1998, Alberto *et al.* 2003). Microsatellites were amplified in multiplex PCR reactions. Samples showing missing data were not considered for all the following analysis. To evaluate evolutionary influences, we tested for Hardy-Weinberg genotype frequencies. Hardy-Weinberg equilibrium (HWE) *P*-value, the probability of a given Multi-Locus Genotype (MLG) occurring *n* times as a consequence of different recombination events was calculated with the Genclone software 1.0. Clonal diversity was

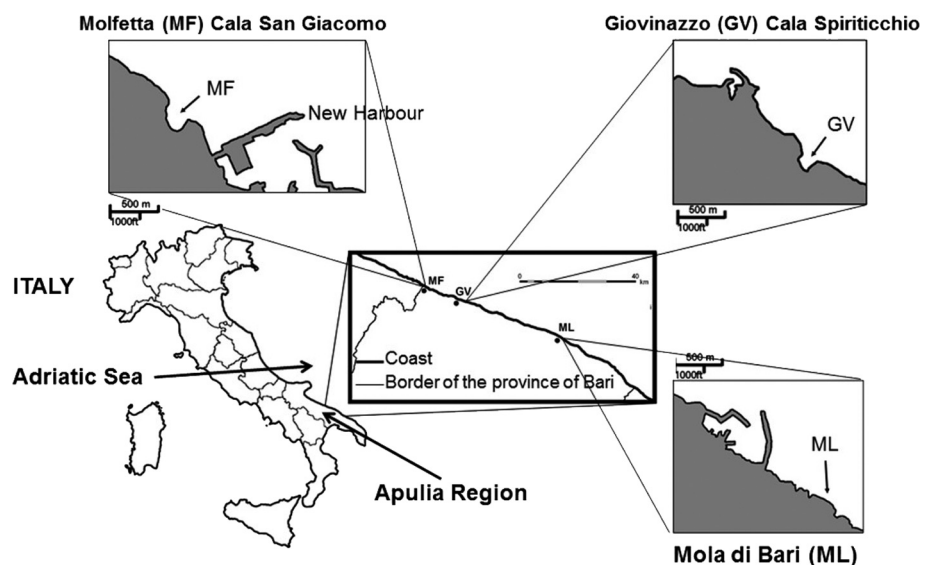


Fig. 1. – Location of the study stations in the San Vito-Barletta meadow. Molfetta (MF), Giovinazzo (GV), Mola di Bari (ML).

Table I. – Population genetic parameters for the two *Posidonia oceanica* populations analyzed. The following parameters are shown: number of samples genotyped (N), percent of polymorphic loci, number of multilocus genotypes (MLG), genotypic diversity ( $R_{MLG}$ ), total number of alleles (Na), number of private alleles (Pa), observed (Ho), expected (He) and unbiased expected (uHe) heterozygosity and fixation index (Fis). All parameters are based on MLG values.

Station	N	Polym. Loci (%)	MLG	$R_{MLG}$	Na	Pa	Ho (SE)	He	uHe (SE)	Fis (SE)
Molfetta	20	71.43	13	0.632	27	5	0.362 (0.106)	0.301 (0.064)	0.314 (0.067)	-0.228 (0.155)
Mola di Bari	21	71.43	14	0.65	25	1	0.347 (0.105)	0.263 (0.062)	0.273 (0.064)	-0.101 (0.184)

estimated as  $R = G - 1/N - 1$ , with G representing the number of MLGs discriminated in the sample ramets after correcting for null alleles. The analysis on the HWE *P*-value as well as all the following analysis was performed on MLGs.

**Genetic and genotypic diversity:** The software GenAIEx 6.502 was used to estimate the number of alleles (Na), the effective number of alleles (Ne), the significance of Hardy-Weinberg (HW) deviations per each locus, the number of alleles per locus (Na/L), private alleles (Pa), observed (Ho), expected (He) and unbiased expected heterozygosity (uHe), Fixation index (Fis) per population (Table I).

**Genetic differentiation:** The inbreeding coefficient value (Fst) between the two sub-populations was calculated with GenAIEx 6.502. To visualize genetic relatedness, a Bayesian clustering analysis implemented in STRUCTURE 2.3.4 was utilized on the MLGs matrix. The number of clusters (K) was estimated using the online software STRUCTURE HARVESTER. To assess the component of genetic variance associated with different levels of analysis present in our dataset, an analysis of molecular variance (AMOVA) was performed using GenAIEx ver. 6.502. Components of genetic variance were computed at these hierarchical levels: between MF and ML, among and within sub-populations.

## RESULTS

### *Underwater visual census of leaf epiphytes*

At all stations, the epiphytic organisms on *Posidonia* leaves showed a clear apical-basal zonation mainly on the apical portion of the leaves. Differences were observed in both abundance and composition of leaf epiphytic communities (Fig. 2). In particular, biomass of filamentous epiphytes was more abundant on the leaves of station MF compared to GV and ML. These data imply that the health status of the San Vito-Barletta meadow is heterogeneous.

### *Genetic diversity*

Genetic analysis was performed using highly variable microsatellites markers. The number of individuals sampled for each population was  $N = 20$  at MF and  $N = 21$  at ML. The proportion of polymorphic loci (71.43 %) was identical. The number of private alleles was significantly higher at MF (Pa = 5) than at ML (Pa = 1). 14 MLGs were found in ML and 13 in MF. Genotypic richness was lower than the average reported value in the literature (Jahnke *et al.* 2015) and slightly but not significantly higher at ML ( $R = 0,650$ ) compared to MF ( $R = 0,632$ ). Both MF and

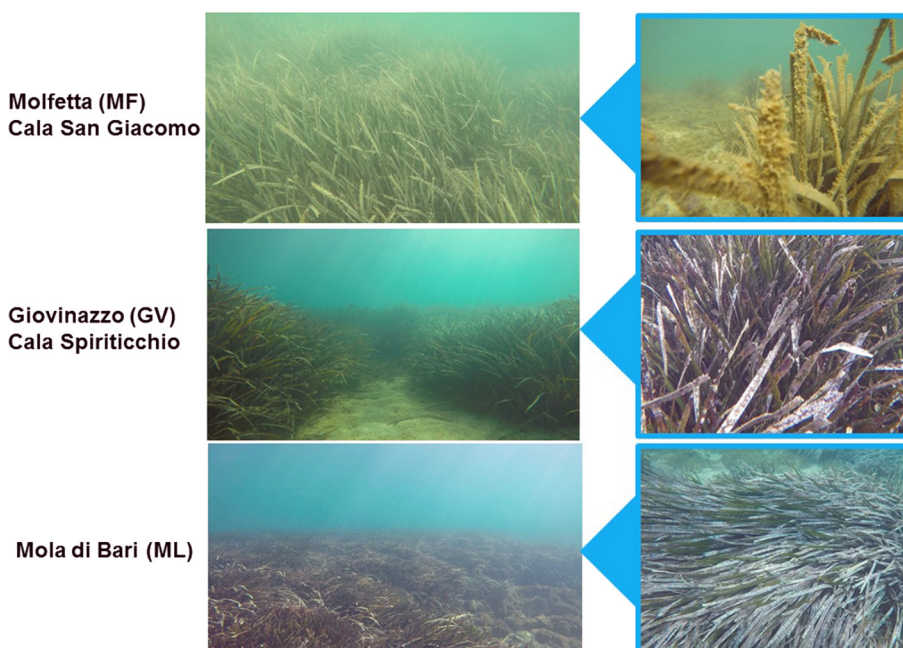


Fig. 2. – Distribution of *P. oceanica* leaf epiphytes. Molfetta (MF), Giovinazzo (GV) and Mola di Bari (ML).

ML populations exhibited an excess of heterozygosity values ( $H_o$ ), which were higher than expected as shown by the  $uHe$  values. The fixation index ( $F_{is}$ ) was less than zero in both populations indicating an excess of outbreeding (Table I).

### Genetic differentiation

The genetic divergence between MF and ML populations was assessed using the average differentiation value,  $F_{st}$ . Its value,  $F_{st} = 0.065$  ( $p = 0.021$ ) shows a slight but significant divergence among ML and MF populations to a level comparable to sub-population structure detected in other *Posidonia* meadows of the Mediterranean Sea (Micheli *et al.* 2015, Table II). Furthermore, the level of gene flow is sufficiently high to restrict the effect of genetic drift ( $Nm = 3.614$ ) between ML and MF stations. Based on Evanno's delta K statistics, the Bayesian analysis of population structure led to the identification of 5 genetic groups (Fig. 3; mean  $\ln P(K) = -365.26$ ). Lastly, the percentage of variance among sub-populations (14 %) shows the existence of a sub-population structure within the San Vito-Barletta meadow (Table II).

## DISCUSSION

Using a citizen's science approach and non-destructive methodologies, we have assessed the health status of the *P. oceanica* San Vito-Barletta meadow. Firstly, underwater visual census showed that the meadow is heterogeneous exhibiting obvious differences at the three sampling sites. Then, genetic investigations further illustrated this heterogeneity indicating the existence of at least two sub-populations with significant genetic differentiation.

Leaf-epiphyte biomass is one out of nine descriptors of *P. oceanica* judged as appropriate for the application of the European Water Framework Directive (Costa *et al.* 2015). We have observed substantial differences. Epiphyte biomass on the leaves of *P. oceanica* at

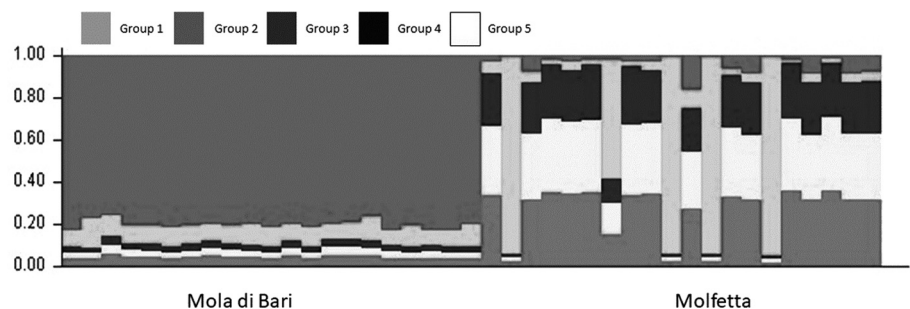
station MF is more abundant than those at ML and GV. The difference between stations MF and ML may be due to the fact that these two stations are located 48 km apart (GV is in between stations MF and ML, 10 km away from MF). However the key features causing the above differences can be one or several environmental factors, either natural or anthropic, or concurrent. One of the main factors influencing leaf-epiphyte biomass is the enrichment in nutrients frequently resulting from human activities. Alteration in the flux of nutrients in waters alongside the coasts is crucial and can be initiated by physical disturbances such as piers, harbor extension and/or coastal erosion (Howarth & Marino 2006). Thus, it is possible that the enlargement of the harbor at Molfetta (which began in 2008) has modified the seawater currents, altering the flux of nutrients and explaining the increase in leaf-epiphyte biomass observed at station MF. In addition, suspended particles in the water column reduced water transparency at station MF. This may be another consequence of the construction of the new harbor at Molfetta, which through changes in coastal currents and swells may have impacted the dynamics of sedimentation. Light reduction mostly due to sediment resuspension is known to reduce growth rate, shoot biomass, storage of starch in the rhizomes, positive C-balance and diminution of the meadow (Ruiz & Romero 2001).

Genetic diversity improves resistance and resilience of seagrass populations to environmental changes (Hughes & Stachowicz 2004). The slow-growing and long-lived *P. oceanica* is a marine plant capable of enduring environmental change through its genetic reservoir (Procaccini *et al.* 2007). Genetic variability of the MF and ML populations was assessed using microsatellite markers. Stations MF and ML are located 48 km apart from each

Table II. – Hierarchical analysis of molecular variance (AMOVA). AMOVA is based on the 14 microsatellite loci utilized.  $P$ -values were based on 1000 permutation tests. The following parameters are shown: degrees of freedom (df), Mean Square (MS), Variance Components, Percentage Variation.

Source of Variation	df	MS	Variance components	Percentage variation	$F_{st}$
Among Subpopulations	1	11.170	0.573	14	0.065 ( $p = 0.021$ )
Within Subpopulations	25	3.439	3.439	86	
Total	73		4.013	100	

Fig. 3. – Bar plot resulting from the Bayesian analysis. The analysis was performed with the software STRUCTURE. For the two study stations, each multilocus genotype is represented as a vertical bar of different grey intensity in proportion to estimated membership coefficient ( $Q$ ) to the K clusters.



other exposed to diverse urban environments. Our experimental data mirror this physical separation indicating the differentiation of stations MF and ML into two populations that are still closely related to each other. As shown by HWE *P*-values, not all of the loci were in HW equilibrium suggesting that these loci were under evolutionary influences generated by still unknown factors. Thus, stations MF and ML are most likely affected by diverse disturbance factors. However, both populations displayed an excess of heterozygosity that often occurs in *P. oceanica* (Micheli *et al.* 2015). Heterozygosity is related to fitness in various ways. The Fixation index suggests an excess of outbreeding in both populations, however *P. oceanica* is also a clonal species. Therefore, better-fitted heterozygous genotypes can become dominant via asexual propagation in a very confined environment with limited gene flow (Tomasello *et al.* 2009). This situation may pertain to the MF population, which is confined within a natural cove. Another possibility is that the excess of heterozygosity is due to the exposure to heterogeneous environmental conditions such as suspended particles in the water column observed at station MF altering flowering events. The existence of two populations at stations MF and ML that are genetically different is reinforced by the *F<sub>st</sub>* value of 0.065 ( $p = 0.021$ ). Moreover, the AMOVA analysis indicates genetic variations of 14 % between stations MF and ML and further supports the presence of two subpopulations.

In conclusion, our population genetic analysis was performed at two different sites of the San Vito-Barletta meadow using a minimum replication sample to decrease the impact of sampling on the meadow. Our data show: (i) the presence of two subpopulations, which have evolved under the pressure applied by diverse environmental and/or anthropogenic factors selecting more resistant clones; (ii) the moderate genetic and allelic richness displayed by both subpopulations is in line with the previously published means for *P. oceanica* meadows.

This study is the very first step in a wider ecosystem-based approach. It constitutes the first insight into the current health status of the ecosystem along the coasts of the Apulia region. *P. oceanica* meadows are biological indicators of the health status of the whole ecosystem because the ecosystem is dependent on *P. oceanica*, which provides oxygen, shelter, and stabilizes the coast, preserving it from erosion. However, to achieve a global view, and in phase with our ecosystem-based approach, we are also monitoring other parameters such as phytoplankton or *Ostreopsis ovata* Fukuyo, 1981 blooming (to be published elsewhere). Furthermore, our present data show that the two subpopulations of *P. oceanica* exhibit different health status; MF being more altered than ML. Therefore, it is more likely that the preservation of the whole ecosystem will require specific actions from site to site.

The previous genetic diversity of the San Vito-Barletta meadow is unknown. Thus, this study will be useful for

a broader meta-analysis on *P. oceanica* populations with the goal of resolving the long-standing debate on the correlation between genotypic diversity and resistance/resilience. Our idea is to foster the use of genetic diversity as an essential tool in conservation management.

## REFERENCES

- Alberto F, Correia L, Arnaud-Haond S, Billot C, Duarte CM, Serrao E 2003. New microsatellite markers for the endemic Mediterranean seagrass *Posidonia oceanica*. *Mol Ecol Notes* 3: 253-255.
- Andreassi G 2016. Procedura di valutazione ambientale strategica. Studio incidenza ambientale. Piano Comunale delle Coste Comune di Molfetta-Livello I Matrice di screening. [in Italian]
- Boudouresque CF, Mayot N, Pergent G 2006. The outstanding traits of the functioning of the *Posidonia oceanica* seagrass ecosystem. *Biol Mar Medit* 13: 109-113.
- Costa M, Barrote I, Silva J, Olivé I, Alexandre A, Albano S, Santos R 2015. Epiphytes modulate *Posidonia oceanica* photosynthetic production, energetic balance, antioxidant mechanisms, and oxidative damage. *Front Mar Sci* 2: 1-10.
- Costantino G, Mastrototaro F, Tursi A, Torchia G, Pititto F, Salerno G, Lembo G, Sion L, D'Onghia G, Carlucci R, Maiorano O 2010. Distribution and bio-ecological features of *Posidonia oceanica* meadows along the coasts of the southern Adriatic and northern Ionian Seas. *Chem Ecol* 26: 91-104.
- Grech A, Chartrand-Miller K, Erfteimeijer P, Fonseca M, McKenzie L, Rasheed M, Taylor H, Coles R 2012. A comparison of threats, vulnerabilities and management approaches in global seagrass bioregions. *Environ Res Lett* 7, e024006.
- Howarth RW, Marino R 2006. Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: Evolving views over three decades. *Limnol Oceanogr* 51(1, part 2): 364-376.
- Hughes AR, Stachowicz JJ 2004. Genetic diversity enhances the resistance of a seagrass ecosystem to disturbance. *PNAS* 101: 8998-9002.
- Jahnke M, Olsen JL, Procaccini G 2015. A meta-analysis reveals a positive correlation between genetic diversity metrics and environmental status in the long-lived seagrass *Posidonia oceanica*. *Mol Ecol* 24: 2336-2348.
- Micheli C, D'Esposito D, Belmonte A, Peirano A, Valiante LM, Procaccini G 2015. Genetic diversity and structure in two protected *Posidonia oceanica* meadows. *Mar Environ Res* 109: 124-131.
- Ministero Marina Mercantile, Ispettorato Centrale Difesa Mare, 1991. Mappatura delle praterie di *Posidonia oceanica* lungo le coste delle regioni: Liguria, Toscana, Lazio, Basilicata e Puglia, SnamProgetti S.p.a. STECOL/04/91/GR/rtf, Comm. 556. 400, Vol. 1-5. [in Italian]
- Montefalcone M, Vacchi M, Archetti R, Ardizzone G, Astruch P, Bianchi CN, Calvo S, Criscoli A, Fernández-Torquemada Y, Luzzu F, Misson G, Morri C, Pergent G, Tomasello A, Ferrari M 2019. Geospatial modelling and map analysis allowed measuring regression of the upper limit of *Posidonia oceanica* seagrass meadows under human pressure. *Estuar Coast Shelf Sci* 217: 148-157.
- Nordlund LM, Jackson EL, Nakaoka M, Samper-Villarreal J, Beca-Carretero P, Creed JC 2018. Seagrass ecosystem services – What's next? *Mar Pollut Bull* 134: 145-151.

- Procaccini G, Waycott M 1998. Brief communication. Microsatellite loci identified in the seagrass *Posidonia oceanica* (L.) Delile. *J Her* 89: 562-568.
- Procaccini G, Olsen J, Reusch TBH 2007. Contribution of genetics and genomics to seagrass biology and conservation. *J Exp Mar Biol Ecol* 350(1-2), 234-259.
- Ruiz JM, Romero J 2001. Effects of *in situ* experimental shading on the Mediterranean seagrass *Posidonia oceanica*. *Mar Ecol Progr Ser* 215: 107-120.
- Telesca L, Belluscio A, Criscoli A, Ardizzone G, Apostolaki ET, Fraschetti S, Gristina M, Knittweis L, Martin CS, Pergent G, Alagna A, Badalamenti F, Garofalo G, Gerakaris V, Pace ML, Pergent-Martini C, Salomidi M 2015. Seagrass meadows (*Posidonia oceanica*) distribution and trajectories of change. *Sci Rep* 5: 12505.
- Tomasello A, Di Maida G, Calvo S, Pirrotta M, Borra M, Procaccini G 2009. Seagrass meadows at the extreme of environmental tolerance: the case of *Posidonia oceanica* in a semi-enclosed coastal lagoon. *Mar Ecol* 130: 288-300.