Özge Tutar (M.Sc.)

March 2018

Distribution of genetic diversity and gene expression response to environmental forcing in *Posidonia oceanica* (L.) Delile, 1813

Università degli Studi di Milano-Bicocca

Mediterranean Marine Sciences Ph.D. School

March, 2018

Table of Contents

ABSTRACT

Posidonia oceanica and *Cymodocea nodosa* are marine angiosperms widely distributed in the Mediterranean Sea. As habitat builders, they have crucial importance for coastal ecosystems; however, because of human activities and environmental changes populations are in regression.

My thesis focuses on the effect of temperature on the distribution and persistence of *P. oceanica*. I approached the topic from two different perspectives. First, I looked at heat-induced oxidative stress response of the species, in comparison with the other Mediterranean seagrass C*. nodosa*, assessing the variations in gene expression of antioxidant genes. Second, I looked at the putative role of latitudinal and bathymetric thermoclines in shaping distribution and genetic diversity patterns of the species. Among all populations analyzed, two main clusters draw attention on Anatolian coastline: the Aegean metapopulation and the Mediterranean one. Between these, Kas population appears to be a transition area. While results suggest a panmixia in Aegean area we see a more structured population on the Mediterranean Basin. The gene expression study results suggest in both experiment there is a difference between two species and two ecotypes analyzed. *P. oceanica* shows more intense response to heat stress comparing to *C. nodosa*.

CHAPTER 1: INTRODUCTION

1.1 Seagrasses

Seagrasses are clonal marine angiosperms which are distributed all over the world. Seagrasses are present worldwide with 60 species belonging to 4 families which are Posidoniaceae, Cymodoceae, Hydrocharitaceae, and Zosteraceae. Among those families, Posidoniaceae harbor a single genus which is only distributed in the Mediterranean Sea and along the coastline of Australia; Zosteraceae and Cymodoceae have four and five genera respectively, while 3 marine genera are present in the Hydrocharitaceae, that include also freshwater genera (Les, Cleland, & Waycott, 1997). Even though they are represented by really few species comparing to their terrestrial relatives, the convergence of their morphological characters to adapt to marine environment causes ambiguity of their taxonomy (Olsen et al., 2016; Papenbrock, 2012; York et al., 2017)

Seagrasses are part of the Alismatales order of Monocots and secondarily colonized marine environments. The adaptation to the marine environment took place at least in three independent events (Les et al., 1997). The change of environment from terrestrial to submerged in the marine realm required morphological and physiological adaptations. Seagrasses lack stomata and guard cells, and they do internal gas transport (Kuo & Den Hartog, 2007). They have epidermal chloroplast because they require more oxygen to support their nonphotosynthetic rhizomes and roots which are often growing in sediment with high-level sulfide and they do hydrophilous pollination (Les et al., 1997; Olsen et al., 2016).

Seagrass meadows fulfill important ecological services, including high carbon storage and oxygen production (Green & Short, 2003) not only by themselves but also by macro- and microepiphytes that they host. They fulfil an important role for blue carbon reserve (Oreska, Mcglathery, & Porter, 2017); for example, the *Posidonia oceanica* matte has a millennial stability potential (Arnaud-Haond, Duarte, Diaz-

Almela, Marba, et al., 2012). By producing a high level of oxygen, they improve the water quality (Green & Short, 2003). Some of the species trap sediment and prevent coastal erosion by lifting the sea bottom through an organogenic structure called matte, formed by remains of plant tissue, on which the meadow overgrows. They also create banquettes, that are wedge-shaped leaf deposits from a few cm to meters on the seashore (Mateo, Sánchez-Lizaso, & Romero, 2003). They are an important food source for larger marine animals like sea turtles, manatees or several species of fishes, and they are an important nursery area for many species. Intertidal seagrasses create a supralittoral habitat and host insects and crustacean (Defeo et al., 2009).

Despite the importance and great value of seagrass ecosystems (Costanza et al., 1997), an accelerated decline has been observed since 1940 (Waycott, Michelle, Duarte et al., 2009). Seagrass loss is directly or indirectly caused by human activities, and by natural processes. Direct human activities are boat mooring (Hastings, Hesp, & Kendrick, 1995), dredging (Erftemeijer & Lewis, 2006), anchoring, fishing and aquaculture (Marbà, Santiago, Díaz-Almela, Álvarez, & Duarte, 2006), coastal construction (Fyfe & Davis, 2007) and introduced exotic species (Williams, 2007). Indirect human activities are water quality degradation through nutrient addition (Burkholder, Tomasko, & Touchette, 2007) and sediment run-off, overfishing, and climate change (Waycott, Michelle, Duarte et al., 2009). Natural causes are extreme events like flood, pathogen, hurricanes, sea level rise, and heat waves (Duarte, Marbà, & Santos, 2004).

There are four seagrass species in the Mediterranean which are *Posidonia oceanica*, *Cymodocea nodosa*, *Zostera noltii* and *Zostera marina* (Procaccini et al., 2003). Recently, another species, *Halophila stipulacea*, entered the Mediterranean Sea through Suez Canal and is spreading in the basin (Den Hartog, 1972; Gambi, Barbieri, & Bianchi, 2009; G. Procaccini, Acunto, Famà, & Maltagliati, 1999). *P. oceanica* and *C. nodosa* are the most abundant seagrass species in the basin and they are the subject of this study.

1.2 The Mediterranean Sea

The Mediterranean Sea is the largest semi-closed sea, and is located between latitude 30˚ and 46˚ N and longitudes 6˚ W and 36˚ E. There are eleven sub-basins in the Mediterranean, which are Alboran, North-Western, South Western, Tyrrhenian Sea, Ionian Sea, Adriatic Sea, Central, Aegean Sea, North-Levantine and South-Levantine (Laubier, 2005) (Figure 1). It is connected to the Atlantic Ocean on the western side through the Strait of Gibraltar and to the Red Sea on the South-East side through the man-made Suez Canal. The Marmara Sea separates the Mediterranean from the Black Sea.

Figure 1: Map of the Mediterranean Sea and its sub-basins

The Mediterranean sea is a hot spot of species diversity (Lejeusne, Chevaldonné, Pergent-Martini, Boudouresque, & Pérez, 2010) and it continues to receive newly introduced species through Suez Canal and ballast waters of ships (Williams, 2007). Besides species invasion, it is under the effect of climate change which facilitates species introduction from a warmer climate (Raitsos et al., 2010). According to Micheli et. al. (2013), the cumulative impact of 22 anthropogenic drivers (i.e. acidification, SST increase, UV increase, fisheries) has the highest average in the Alboran and Levantine Basins in the context of ecoregions. Among those 22 drivers, climate change (acidification, SST increase, UV increase), demersal fisheries-high bycatch and shipping have the greatest average impact in the Mediterranean and the Black

Sea (F. Micheli et al., 2013). On Figure 2, climatic and land-based drivers effect are given. The Levantine basin is under high impact by climatic drivers, higher in comparison to the western Mediterranean, while land-based drivers have high impacts especially at North-Western, Adriatic, Central, Aegean and Levantine basin.

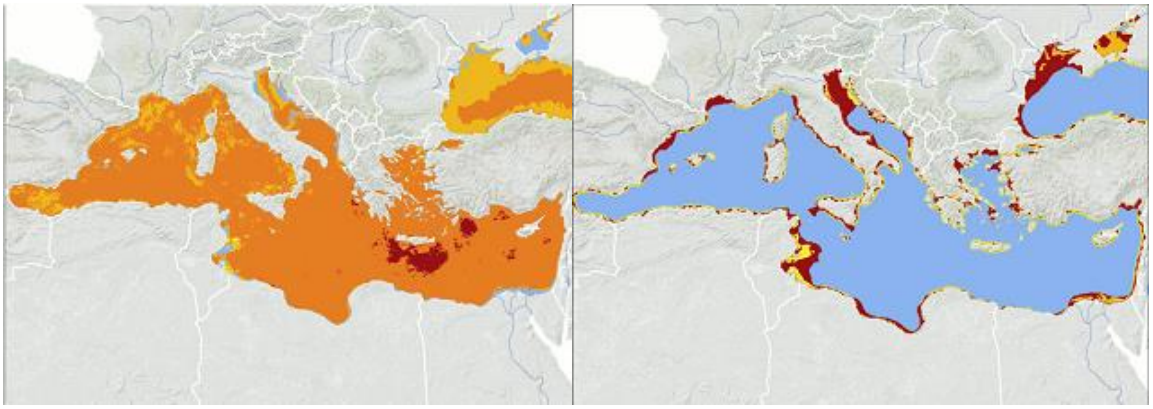


Figure 2: Spatial distribution of cumulative impact of driver categories. Driver categories are: climate (i.e. the combined cumulative impact of temperature and UV increase, and acidification; left) and land-based drivers (nutrient input, organic pollution, urban runoff, risk of hypoxia and coastal population density; right) (figure modified from (F. Micheli et al., 2013) doi:10.1371/journal.pone.0079889.g004)

On Figure 3, sea surface temperature (SST) time series shows an annual positive trend in all sub-basins of the Mediterranean sea (Shaltout & Omstedt, 2014). Temperature increase can have an important impact on species turnover, by increasing beta-diversity and decreasing the compositional stability of communities (Hillebrand, Soininen, & Snoeijs, 2010).

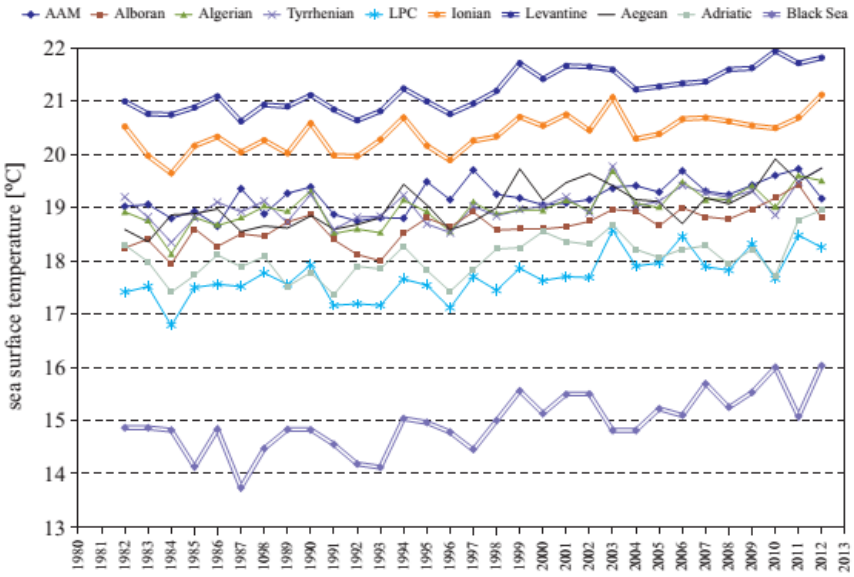


Figure 3: SST time series for the various sub-basins based on daily AVHRR data (AAM= Active Atlantic Mediterranean sub-basin to the west of the Strait of Gibraltar) (taken from Shaltout & Omstedt, 2014)

The Atlantic water is much warmer than the Alboran Sea water and it enters as surface water into the Mediterranean Sea (Figure 4) (Millot & Taupier-Letage, 2005). While this water entrance creates two clockwise circulations following each other on the eastern side of Alboran Sea, it continues to flow eastward by creating the Algerian current. The current sometimes loses its stability and creates eddies but a strong current comes from Egypt to Northern Levant and turns to west on Turkish coastline. In general, there are two anticlockwise currents, one is in the Western basin and the other is in Eastern one.

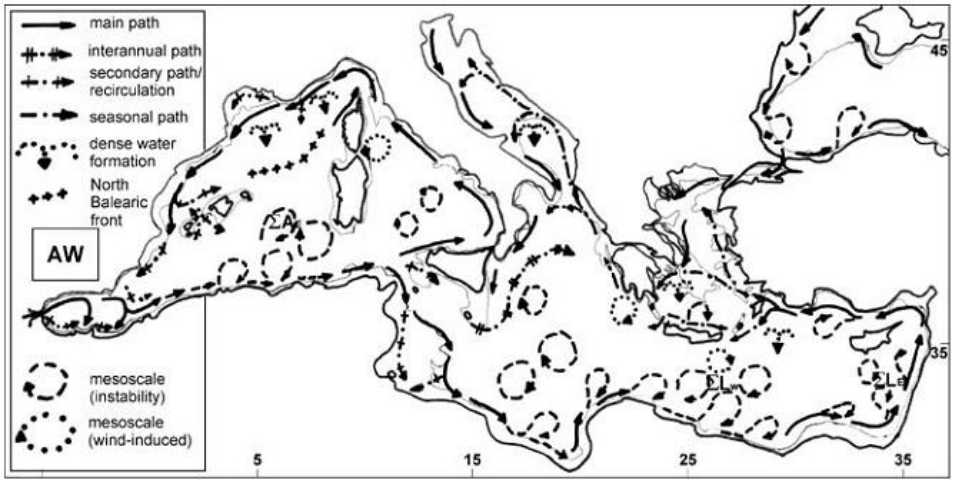


Figure 4: Circulation of Atlantic water in the Mediterranean Sea (taken from (Millot & Taupier-Letage, 2005))

1.2.1 Aegean vs. Levantine Basin

Aegean and Levantine basins are directly connected however they are contrast at some aspects like geography and bathymetry. While Aegean coastline is quite indented and there are approximately 3000 islands, Levantine basin constitutes less geographical barriers. As shown in *Figure 5* there is a temperature gradient in the Aegean Sea and there is a circulation which comes from south to north and two gyres in the middle of the Aegean Sea.

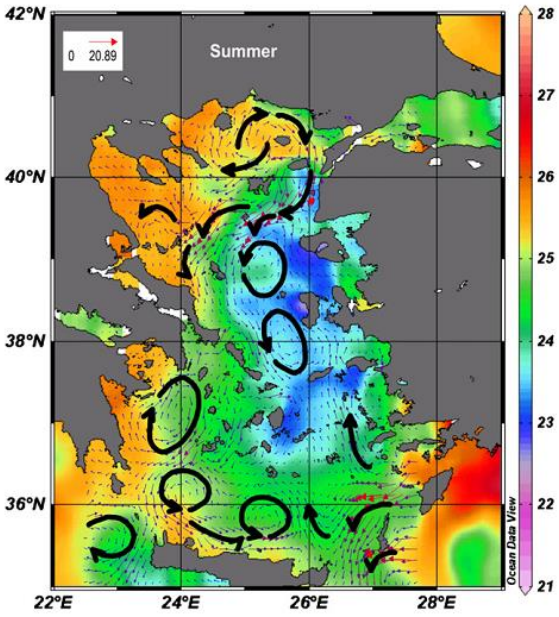


Figure 5: Summer general circulation pattern of Aegean Sea with average SST field of summer 1991 on the background (taken from Sayın, Eronat, Uçkaç, & Beşiktepe, 2011)

1.3 General Characteristics of *P. oceanica* and *C. nodosa*

The most abundant seagrass species in the Mediterranean basin are *P. oceanica* and *C. nodosa*. *C. nodosa* is considered an opportunistic species, due to its relatively fast shoot turnover rate and time to reach sexual maturity, physiological resistance, and response to disturbance (Cancemi, Buia, & Mazzella, 2002); *P. oceanica* is a persistent species with slow shoot turnover (Marbà & Duarte, 1998), long lifetime (Arnaud-Haond, Duarte, Diaz-Almela, Marbà, et al., 2012) longer life cycle, and slower recovery (Kilminster et al., 2015).

P. oceanica is endemic to the Mediterranean Sea where it is distributed almost all over the coast (Chefaoui, Assis, Duarte, & Serrão, 2016) including the Marmara Sea (Meinesz et al., 2009). However, it doesn't exist along most of the Egypt coastline (Por, 1978) and in the Black Sea (Gobert et al., 2006) and it is absent between Mersin and its eastern side on the Turkish coasts (Celebi, Gucu, Ok, Serdar, & Akoglu, 2007) (Figure 6).

It is a slow growing plant, leaf longevity can reach 303 days and leaf length is up to 1.4 m being 75 cm in average with 1 cm wideness (Pérez-Lloréns et al., 2014). Its horizontal rhizome elongation rate is 2 cm/y and vertical rhizome elongation rate per year is 1 cm (Marbà & Duarte, 1998). *P. oceanica* is a monoecious plant with irregular sexual reproduction and has mainly a vegetative growth (Pérez-Lloréns et al., 2014). *P. oceanica* does not form seed banks and seeds germinate in ten days (Pérez-Lloréns et al., 2014) after dispersing on the sea surface according to the hydrodynamics of the area (Ruiz-Montoya, Lowe, Van Niel, & Kendrick, 2012).

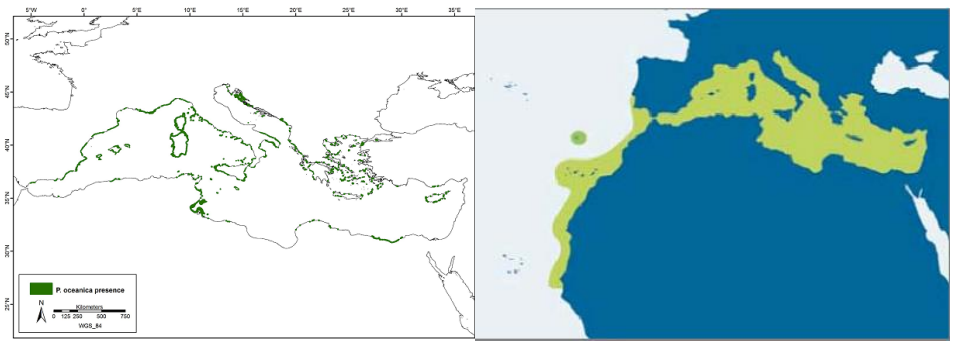


Figure 6: Current distribution of *P. oceanica* meadows, right (taken from (Telesca et al., 2015)); current distribution of *C. nodosa*; green area shows the distribution, left (taken from Espino et al. (2008))

C. nodosa is present throughout the Mediterranean, Portugal and North-Western coast of Africa up to Senegal, including Canary Islands (Chefaoui et al., 2016). It is a pioneer species and can be found up to 30-40 m depth depending on water clarity on sandy or sandy-muddy bottoms. It has a tropical origin and is tolerant to 10-30˚C and to 26-44 salinity (Pérez-Lloréns et al., 2014). *C. nodosa* is a dioecious species, flowering frequency depends on the locality (Terrados, 1993) and occurs in spring-summer on more than one year older shoots. Its horizontal elongation rate is quite high with 40 cm per year (Marbà & Duarte, 1998; Pérez-Lloréns et al., 2014).

1.4 Seagrass dispersal and genetic connectivity

Seagrasses are rhizomatous plants and create meadows which vary from small patches to a field of tens of thousands ha and they can extend up to 50 m depth (Telesca et al., 2015; Unsworth & Cullen-Unsworth, 2017).

Seagrasses can disperse sexually through the passive transport of pollen and fruits by water current and asexually with the dispersal of disassembled shoots. The experiment of Rasheed (2004), shows that in some tropical species the main dispersal is through the vegetative way. The dispersal distance changes species to species. The longest distance known for fruit dispersal belong to *Enhalus acroides* with 400 km and it is 76 km for *Posidonia australis* (Kendrick et al., 2012). Fruit dispersal distance depends from fruit

persistence and duration but also from patterns (direction and speed) of marine currents. Dispersal simulations based on sea currents can allow to infer are used to determine the potential connectivity (Jahnke et al., 2017; Serra et al., 2010). Thus, realized and potential connectivity can be related, and priority areas for genetic hotspots can be identified to be used for management purposes (Jahnke et al., 2017).

1.4.1 Dispersal and genetic connectivity of *P. oceanica*

Genetic diversity of *P. oceanica* is a well-studied subject for Western Mediterranean and it continues to improve with dispersal models (Arnaud-Haond et al., 2014; Arnaud-Haond, Marbà, Diaz-Almela, Serrão, & Duarte, 2009; Jahnke et al., 2017; Serra et al., 2010). After usage of microsatellites, detection of genetic variability has increased (Alberto et al., 2003; G Procaccini, Orsini, Ruggiero, & Scardi, 2001; G Procaccini & Waycott, 1998; Ruggiero, Turk, & Procaccini, 2002). Thus an East-West cleavage has been suggested for *P. oceanica* and Siculo-Tunisian Strait is an important genetic boundary (Arnaud-Haond et al., 2007; C. Micheli et al., 2005).

1.5 Heat Stress on Seagrasses

As mentioned above, one of the effects of climate change is elevated temperature. All over the world heatwaves started to be observed more frequently, intense and longer (Perkins, Alexander, & Nairn, 2012). Seagrass meadows are also potentially impacted by high temperatures. According to Jordà, Marbà, & Duarte (2012), if we don't do anything to stop warming and remove local impacts, meadow functionality will be lost in 30 years due to a rapid decrease of shoot density.

1.5.1 Heat Stress Induced Oxidative stress

Heat stress affects plants in different aspects including protein unfolding, lipid peroxidation, changes in membrane fluidity, cytoskeleton disassembly and enzymatic activities (Ruelland & Zachowski, 2010). We

observe the effect of these changes in molecular, metabolic, and cellular level. One of the organelle which is affected by heat stress is chloroplast.

As aerobic organisms, plants continuously produce reactive oxygen species (ROS) as a result of different metabolic pathways (Mittler et al., 2011). Under normal conditions ROS are scavenged by antioxidants and reduced to less damaging forms by enzymes. Under stress conditions, which can be heat, chilling, light, drought etc., scavenging mechanism cannot sufficiently transform the molecules and so oxidative stress occurs. ROS gives damages to protein, DNA and membranes which drive the cell to homeostatic unbalance and eventually to death (Sharma, Jha, Dubey, & Pessarakli, 2012).

The pioneers of oxidative stress defense are chaperons and ROS scavengers. After oxidative stress occurs, signaling molecules step in and start to regulate gene expression of chaperons, ROS scavenging enzymes. Hydrogen peroxidase (H_2O_2) is one of the signaling molecule as a ROS itself (Foyer, Lopez-Delgado, Dat, & Scott, 1997; Neill, Desikan, Clarke, Hurst, & Hancock, 2002).

ROS are singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$). They are scavenged by enzymatic and non-enzymatic molecules and reduced to less damaging molecules.

ROS are generated by mitochondria, NAD(P)H oxidases, peroxisomes and chloroplast (Selles, 2013).

A few of these studies report on the specific response to oxidative stress and on the activity of antioxidants in seagrasses. The MnSOD gene has been shown to play and important role in the response to heat stress tolerance in *Zostera marina* (Winters et al., 2011; Liu et al., 2016). Massa et al., 2011 suggest that Glutathione-S-transferase (GST) participates to heat stress response in *Zostera noltii*.

Thesis aims

In this study, I aimed to characterize genetic diversity of *P. oceanica*'s Turkish coastline population and to analyze the stress response of the seagrasses *P. oceanica* and *C. nodosa* in controlled conditions to enhanced heat.

I collected samples from edges of the population distribution of Turkey and filled the between considering geographical barriers of the area. By collecting samples from above and below summer thermocline, I wanted to have the opportunity to characterize population by latitudinal and by depth gradient.

I analyzed the changes in the expression of genes involved in different steps of the stress response, with particular focus on antioxidants. We selected genes codifying for proteins involved in the regeneration of glutathione or ascorbate, the ROS scavengers, heat-shock proteins, an apoptosis-related gene, and other generic stress-responsive genes. Through this approach, I also aimed to identify target genes that can be utilized as indicators of plant response to heat stress.

CHAPTER 2

Genetic characterization of *P. oceanica* populations along Turkish coastline

2.1 Introduction

Posidonia oceanica is distributed all around the Mediterranean basin. Meadows cover about 1,225,000 ha, along 12,000 km of coastline (Telesca et al., 2015). Meadow distribution is affected by the presence of suitable substrate and of freshwater inputs, that impede species survival in proximity of river deltas. Human impact is having a profound effect on the distribution and density of *P. oceanica* meadows.

P. oceanica meadows show different levels of genetic diversity all around their distribution. Meadow' genetic diversity result for the interplay of recruitment of new offspring and clonal growth of existing genotypes. Almost uniclonal meadows have been observed in different localities (Arnaud-Haond et al., 2007; Ruggiero et al., 2002) raising concern about their capability to face abrupt environmental changes. A metadata analysis showed that meadows with low genetic diversity are absent in localities with high human-driven cumulative impact, suggesting a sensitivity of the species toward environmental alterations that overcome its resilience threshold (Jahnke, Olsen, & Procaccini, 2015) and stressing the importance of genetic polymorphism in facing adverse conditions. The species suffers in particular from enhanced turbidity, lowering light availability, increased sea water temperature and direct removal (Boudouresque, Bernard, Pergent, Shili, & Verlaque, 2009). Recent studies provided experimental evidence on the mechanisms of response to high temperature and to changes in the light (Dattolo, Marín-Guirao,

Ruiz, & Procaccini, 2017; Marin-Guirao, Entrambasaguas, Dattolo, Ruiz, & Procaccini, 2017; Lazaro Marín-Guirao, Ruiz, Dattolo, Garcia-munoz, & Procaccini, 2016). Shallow and deep portions of the same meadow (i.e. about 5 and 20 m depth) seem to be adapted to their local conditions, and modulate their metabolism accordingly (Gabriele Procaccini et al., 2017), with low plasticity to perform in reciprocal conditions (Dattolo et al., 2017).

Adding to that, meadows show a clear genetic structure across the basin, with the presence of clearly identified population groups, connected by low levels of gene flow. Two main population groups are present, the western Mediterranean populations and the Eastern Mediterranean population, connected by a transition zone represented by the Sicily channel (Arnaud-Haond et al., 2007; Serra et al., 2010). Grouping results by the evolutionary history of the species in the basin but also by limited present-day gene flow, constrained by direction of current dispersal and life time of the main dispersal vehicles (i.e. floating fruits).

Little is known about the genetic diversity and structure of *P. oceanica* and the factors limiting its distribution areal at the easternmost boundary of its distribution. The only existing study on Turkish populations points to the isolation of Marmara Sea meadows in respect to Aegean Turkish ones, probably related to the geomorphological history of the enclosed basin and the relatively recent changes in water flows direction through the Dardanelles Strait (Meinesz et al., 2009). No data exist to now on the genetic diversity of *P. oceanica* populations distributed along the Southern coasts of Turkey.

The Mediterranean Sea is going through profound changes in biodiversity, due to the introduction of alien species facilitated by the enhanced ship traffic, opening of new ways of

introduction (best example is the Suez Channel), and increased sea water temperature (Carlo Nike Bianchi, 2007). Distribution of the winter isotherms seem to be one of the main factors driving the distribution of biodiversity and the spreading of new introduced species (Carlo Nike Bianchi, 2007). Almost all Turkish coasts are included in two temperature zones, whose boundaries are defined by the 15°C and the 16°C isotherms. Most of the Aegean coast has winter temperatures comprised between 15°C and 16°C, while the southern coast is comprised between 16°C and 17°C. The 17°C isotherm seems to track quite nicely the easternmost boundary of the species which is known to be the Turgutlar cove in Mersin (Çelebi, 2007).

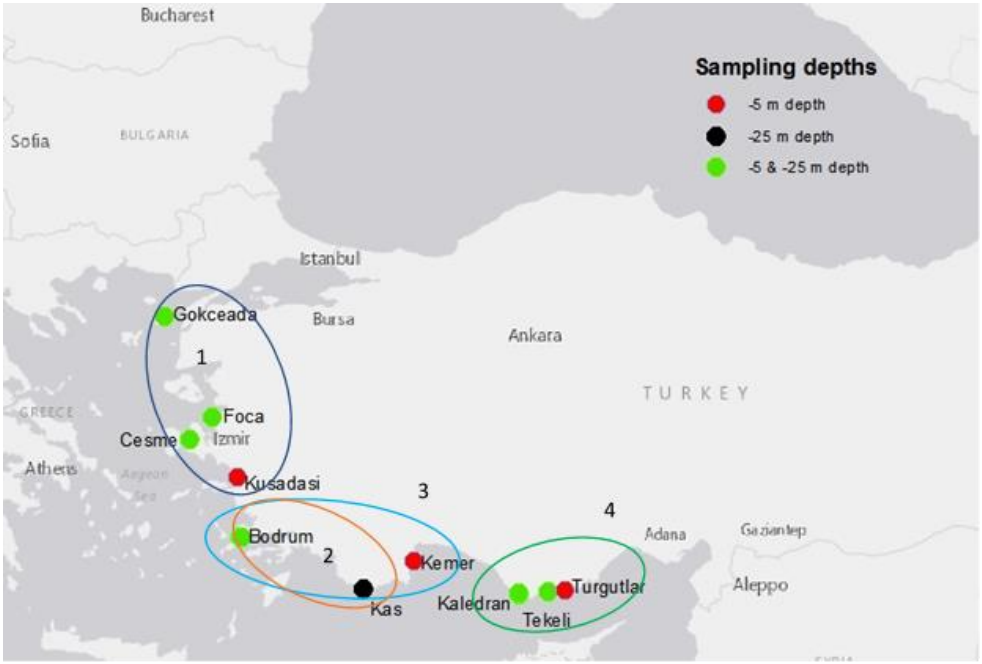
The goal of this study is to assess the genetic diversity and structure of *P. oceanica* along the Turkey coasts. Where possible, populations have been sampled at different depth in order to assess genetic structure along a bathymetric gradient. Resulting patterns of genetic structure have been related with isotherm distribution and with passive current dispersal, in order to disentangle neutral gene flow from adaptive response to temperature, one of the main drivers affecting *P. oceanica* distribution and persistence in the Mediterranean basin.

2.2 Materials and Method

Samples were collected from 10 distinct localities in the regions as indicated in *Figure 7* and *Table 1*, between May 29 and June 14, 2015. Populations along the Southern coasts of Turkey (from Kas to Tu) are from now-on defined as “Mediterranean”, while populations along the Western coasts of Turkey (from Go to Bod) are defined as “Aegean”. During sampling, three leaves were cut from the same shoot. Samples were collected at least five meters apart, to minimize sampling from the same rhizome, following a zigzag route. After diving, samples were cleaned from the epiphytes with a scalpel, without damaging the leaf tissue, in the first suitable place during the

day, wrapped in individual gauze cloths, numbered, and dried by silica gel. In the following days, according to the color-indicating silica gel condition, if necessary silica gel was changed and the samples were kept dry. Although the dive sites were primarily determined by the literature, they have been selected also according to the information and logistical facilities available to the dive centers that have been operating in the area for years. Both shallow and deep-water samples could be collected in 6 localities, while three of the remaining 4 stations could be sampled only

Figure 7: Map of sampling stations. Elipses shows the areas used for different groupings for variance analysis.



at one depth (*Figure 7*). Two of the three sheets collected from each individual were delivered to METU-DBE, Turkey, while one sheet was transferred to the Stazione Zoologica Anton Dohrn in Napoli (Italy) for microsatellite analysis.

2.2.1 DNA extraction and microsatellite analysis

Leaf samples weighting approximately 60 mg were powdered individually in TissueLyser and genomic DNA isolation was carried out using the Macherey-Nagel NucleoSpin 96 Plant II kit,

following a protocol optimized for a Biomek FX robotic station (see Tomasello et al., 2009). After isolation, DNA was visualized by gel electrophoresis, and isolation was repeated if unsuccessful. Of the 639 samples extracted, 480 samples (30 samples from each population or depth) were selected for subsequent analyses.

All samples were genotyped with 20 selected microsatellite regions (Alberto et al., 2003; Arranz et al., 2013; G Procaccini & Waycott, 1998), assembled in two separate multiplexes and amplified by PCR using QIAGEN Multiplex PCR Kit. Selected microsatellite regions and multiplex assembly are reported in Table 2. Genotyping was performed using a ABI Prism 3730 automated DNA sequencer (Applied Biosystems) using the following conditions: 95 °C for 15 min, 35 cycles of 94 °C for 30'', 60 °C for 90'' and 72 °C for 60'', followed by 30' at 60 °C. Peak identification and scoring was performed using the Applied Biosystem Peak Scanner Software 2. Only samples showing <10% missing data were included in the analysis, samples with more missing data were removed from the dataset. One of the marker (Poc-trn) is chloroplastic, because of that it is removed from further analysis. Another marker Pooc-PC003H09 presented at least three similar peaks in more than half of the samples. Because of the genetic mosaicism showed it was also removed from further analysis.

2.2.2 Statistical Analysis

The presence of identical genotypes (MLGs) was assessed by the software RClone (Bailleul, Stoeckel, Arnaud-Haond, & Poisot, 2016), and all the following analysis were performed on MLGs, retaining only one of the identical samples of the same genotype. For each population, genotypic diversity was assessed as the R ratio: R = G-1/N-1, where G is the number of genotypes and N is the number of samples.

In order to identify putative outlier loci, a neutrality test was performed using two different approaches, implemented in the software Lositan (Antao, Lopes, Lopes, Beja-Pereira, & Luikart, 2008) and Bayescan (Foll & Gaggiotti, 2008) . Lositan was used with a confidence interval of 0.95 with 50000 simulations, Neutral mean Fst, and Forced mean Fst options. The Bayescan program was used with default settings.

Genetic diversity indices, genetic distance calculations, PCoA, Mantel's test and AMOVA were performed with the software Genalex 6 (Peakall & Smouse, 2006). Values of number of alleles per locus (Na), effective number of alleles per locus (Ne), observed (Ho) and expected heterozygosity (He), fixation index (F) and percent of polymorphic loci per locus are reported in *Table 1*. The inbreeding coefficient per population was calculated with the FSTAT software (Goudet, 1995). Assignment test was carried out with the GeneClass2 software (Piry et al., 2004) by using Bayesian method (Rannala & Mountain, 1997). For that we used Paetkau, Slade, Burden, & Estoup, (2004) simulation algorithm for probability computation with 1000000 simulation steps and a type 1 error of 0.05. Bayesian cluster analysis was performed with the STRUCTURE software using the three sets of neutral, putative outlier, and all markers together. In all analyses, the options admixture model, run length 100000, 100000 MCMC iterations and related allele frequency, were selected. In order to identify the most suitable grouping, 15 iterations were performed for each grouping level. The STRUCTURE output was processed according to the Evanno method (Evanno, Regnaut, & Goudet, 2005) by the Structure Harvester (Earl & vonHoldt, 2012) program and the correct number of clusters was determined. For visualization of results, STRUCTURE output was used in CLUMPAK software (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015). The program EDENetwork (Kivelä, Arnaud-Haond, & Saramäki, 2015) was used

to measure and visualize genetic distance. In the analysis setup, allele sharing was selected as for distance index.

2.2.3 Dispersal analysis

In order to conduct Lagrangian analyses and infer the physical connectivity along the coasts of Turkey, we released and tracked a set of point-like particles being passively advected by currents. Particles are released on the surface along the coast around each sampling sites in a disk-like area with a radius of Rsite = 4 km. Starting in May and during 90 days, a batch of 25600 particle couples was released from every sampling site and their position was recorded after 28 days, which has been considered the maximum time of fruits floating persistence (see Serra et al., 2010).

From the particles trajectories computed with ROMS, we inferred the fine-grained Lagrangian PDFs which give the probability density function of a particle leaving its initial location and reaching a given location of interest (here any point in the Tyrrhenian Sea), after a given advection time scale. Then, we inferred from the Lagrangian PDFs the physical connectivity matrix which gives the probability that a particle leaving a given sampling site reaches another sampling site. The combined analysis of Lagrangian PDFs and connectivity matrix allows for a complete drawing of the seascape and its related transport dynamic.

2.3 Results

A total of 424 samples, that were under the threshold of missing data, were utilized in the analysis. After genotyping assessment, 365 MLGs were identified. Number of MLGs ranged from 19 in TU-S to 28 in KAL-D, with respective R values of 0.7 and 1 (*Table 1*). The population with the highest number of alleles per locus (Na) was KAL-D, with a value of 3.1, followed by FO-S, where

the highest percent of polymorphic loci was recorded. At the other extreme, there was TEK-S, with Na = 1.9. The population with the lowest expected heterozygosity was CE-S (0.25), while the highest value was recorded in BOD-D (0.36).

Table 1: Genetic diversity indices. Populations are grouped in the two big areas of Aegean Sea and Mediterranean Sea. For each population, the following indices are shown: Depth, the number of samples, with missing values of microsatellite alleles < 10% (N), the number of genotypes (G), the genotypic diversity (R= G-1/N-1), the number of alleles (Na), the effective number of alleles (Ne), the observed (Ho) and expected (He) heterozygosity, the Fixation index (F), the Fis and the percentage of polymorphic loci.

Sea	Populations	Depth	N	G	R	Na	Ne	Ho	He	F	Fis	% of Polymorphic Loci
Aegean Sea	**GO-S**	5 m	30	22	0.7	2.4	1.5	0.24	0.26	0.15	0.26	77.78%
	GO-D	25 m	23	22	1.0	2.4	1.7	0.28	0.29	0.00	0.23	61.11%
	FO-S	5-9 m	24	23	1.0	2.7	1.7	0.29	0.32	0.33	0.28	88.89%
	FO-D	25-27 m	25	19	0.8	2.2	1.6	0.31	0.27	0.06	0.08	66.67%
	CE-S	5-7 m	26	24	0.9	2.4	1.6	0.29	0.25	-0.07	0.10	61.11%
	CE-D	25-27 m	26	23	0.9	2.4	1.7	0.29	0.31	0.20	0.27	72.22%
	KUS-S	5 m	28	25	0.9	2.6	1.8	0.33	0.33	0.10	0.24	66.67%
	BOD-S	7-9 m	28	20	0.7	2.6	1.8	0.33	0.32	0.10	0.24	77.78%
	BOD-D	25 m	22	19	0.9	2.6	1.9	0.32	0.36	0.26	0.21	77.78%
	KAS-D	22 m	24	24	1.0	2.8	1.9	0.39	0.34	0.01	0.09	72.22%
Mediterranean Sea	**KEM-S**	7-12 m	27	24	0.9	2.2	1.7	0.32	0.29	0.04	0.03	72.22%
	KAL-S	6 m	29	27	0.9	2.9	1.9	0.30	0.32	0.13	0.17	77.78%
	KAL-D	20-22 m	29	28	1.0	3.1	1.7	0.31	0.32	0.02	0.20	83.33%
	TEK-S	6 m	29	24	0.8	1.9	1.6	0.33	0.29	-0.07	-0.07	72.22%
	TEK-D	20 m	27	22	0.8	2.1	1.7	0.41	0.33	-0.02	-0.22	83.33%
	TU-S	5 m	27	19	0.7	2.0	1.5	0.28	0.28	0.13	-0.07	66.67%

Ho and Fis showed regional differences. While Aegean populations has higher Fis with less variation, Mediterranean has opposite features ($p<0.01$). Observed heterozygosity is higher in the Mediterranean populations and variation is higher than Aegean ones ($p<0.05$) (Figure 2).

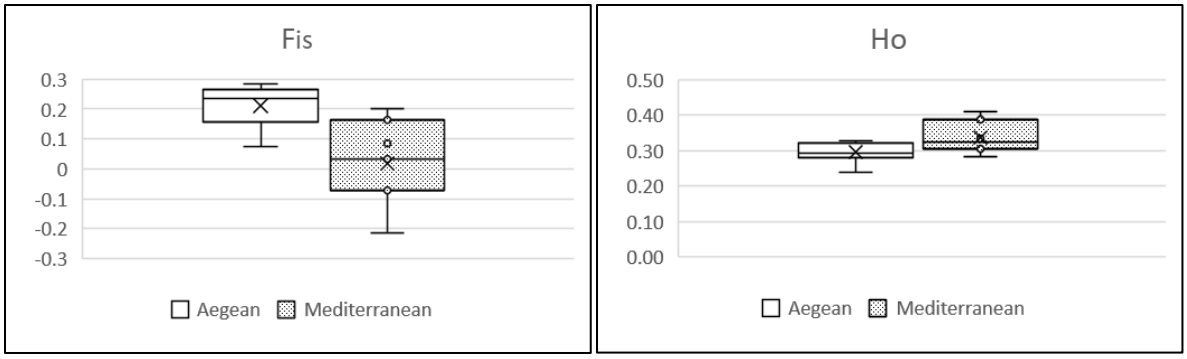


Figure 8: Regional differences of Fis and Ho

The outlier analysis performed with Lositan identified Poc-5 and Pooc-229 as loci under positive selection (Figure 3), while Pooc-PC047G07, Po-5-39 and Pooc-330 were in balancing selection. Bayescan gave the same result except for Pooc-330 that was not identified as a putative outlier.

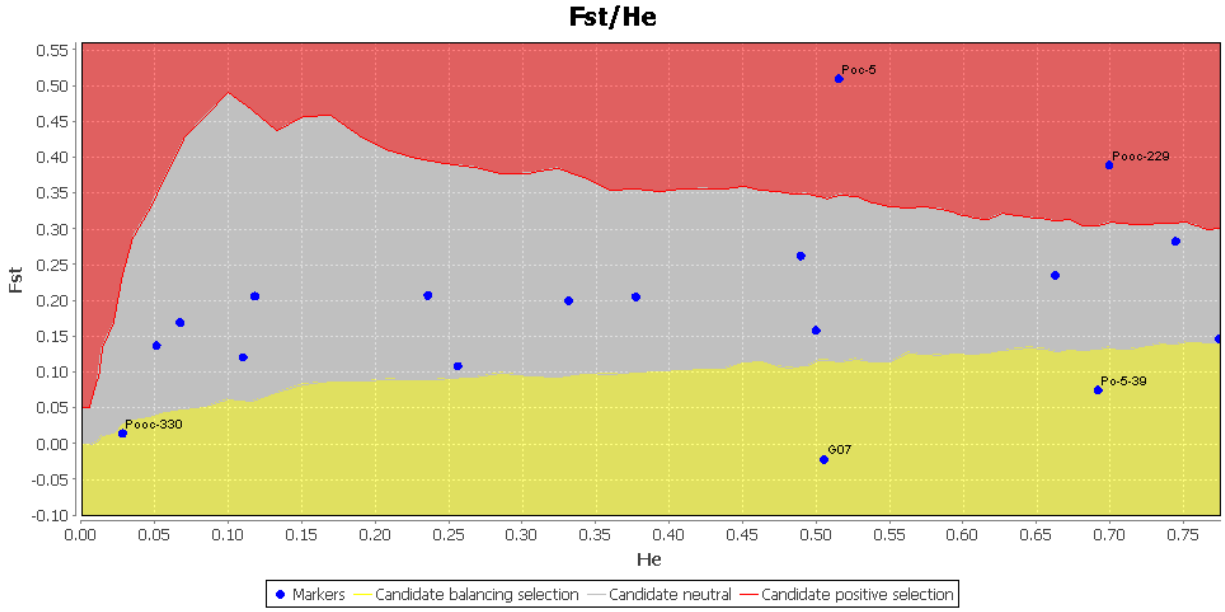


Figure 9: Results from the outlier analysis performed in LOSITAN

A total of 245 genotypes (67%) were correctly assigned to the populations in which they were sampled. The population with the lowest number of correctly assigned individuals is GO-S (27%) and the percent of correct assignment increases going from Northern to Southern populations along the Aegean coasts. The best correctly assigned population is KAS-D (88%). Among

Mediterranean populations, all but KAL-D show values above 70%. We can almost see a boundary between area 1 and 3-4 (*Figure 7*) in terms of percentage of receiving migrants (see 1). Considering together shallow and deep portions of the same locality, Figure 4 shows that KAS has the highest retention level and GO has the lowest one. KAL is an important source for genotypes dispersal by giving genotypes to five different and far distance locations. GO receives genotypes from five different sources. The highest genotypes dispersal traffic is among Aegean populations, while the two populations TEK and TU are the only ones that do not give any genotype to other populations.

Among Region	All	No-outlier	only-outlier
Sea	24%	11%	51%
Depth	0%	0%	0%
Within Populations	All	No-outlier	only-outlier
Sea	57%	68%	39%
Depth	63%	71%	34%
Among Populations	All	No-outlier	only-outlier
Sea	19%	21%	10%
Depth	37%	29%	66%

Table 2: Results of AMOVAs performed with two groupings; Sea includes Aegean and Mediterranean, and Depth includes shallow and deep habitats. Values shows percentage of explained variance.

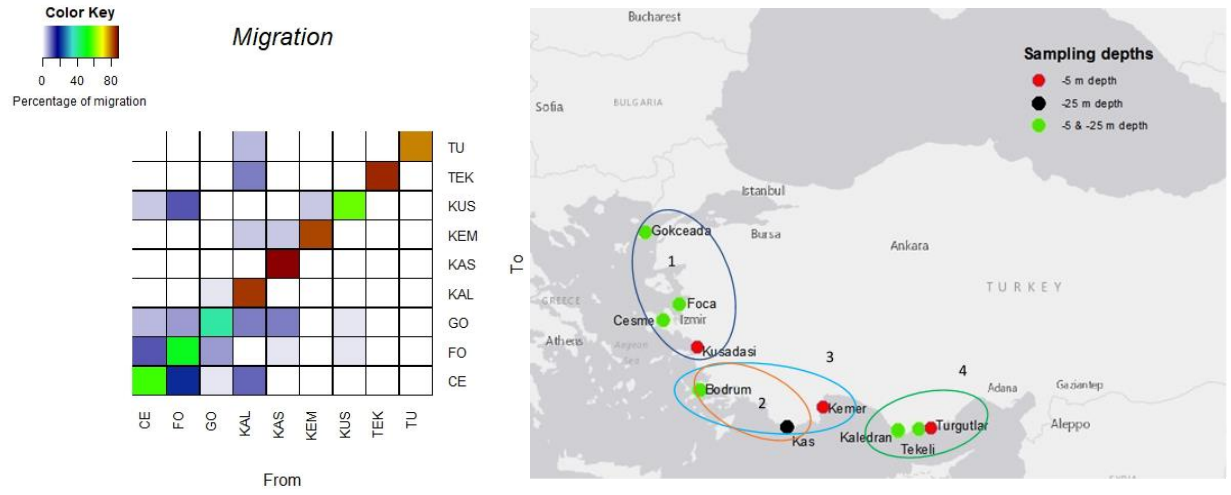


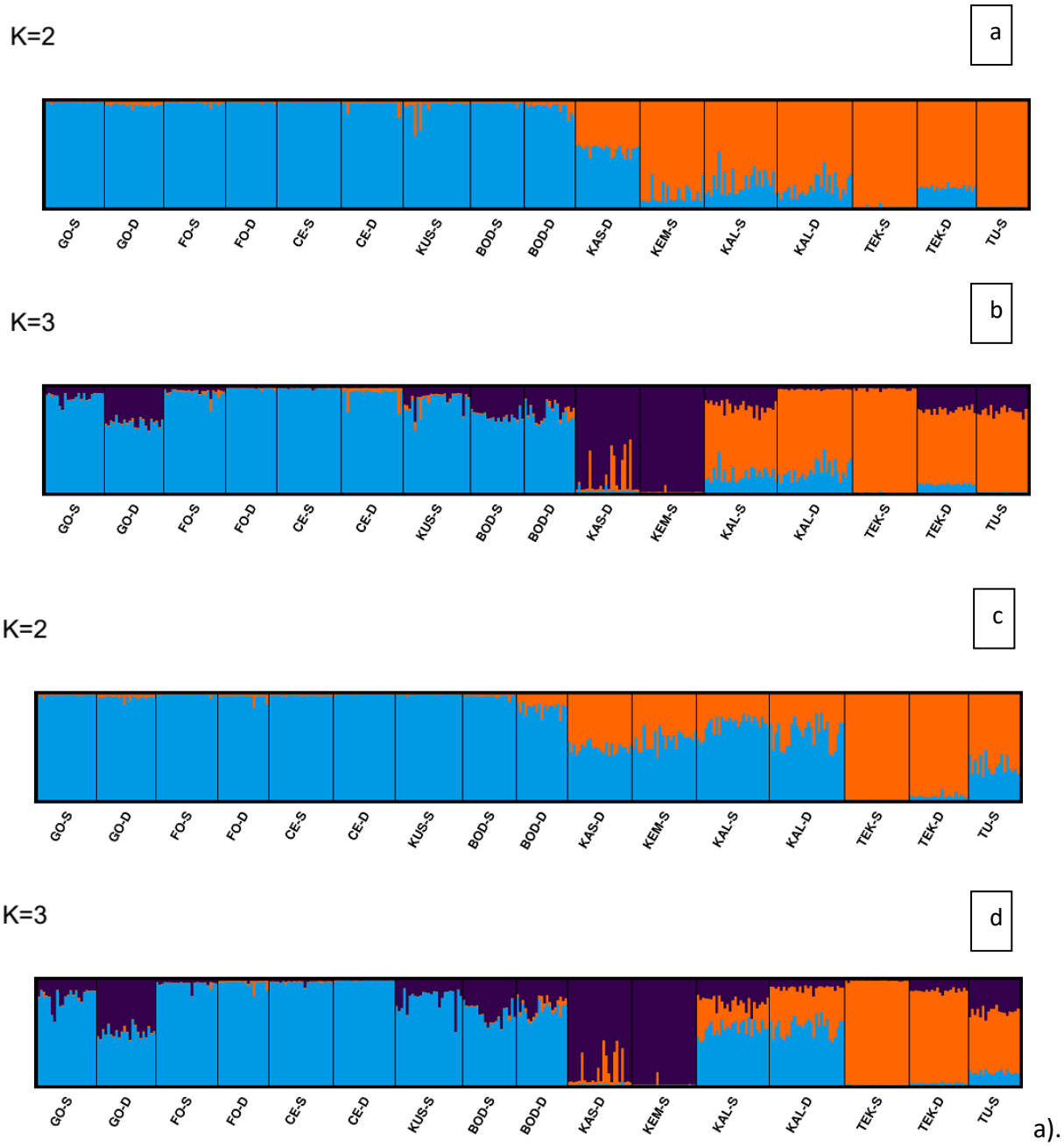
Figure 10: Migration plot. The plot shows the percentage of immigrants that are given to- or received from- any population considered in the analysis.

We tested isolation by distance (IBD) via Mantel test by utilizing different sets of data, according to the grouping of *Figure 7*. Results showed that there is a significant IBD when we utilize all samples (r= 0.470, p=0.01). When we utilize only Aegean samples we obtained lower correlation (r=0.171, p=0.01), while Mediterranean samples have a similar correlation to all samples' result (r=0.424, p=0.01).

The AMOVA was also performed with different sets of loci and with different grouping, in order to identify the highest variance in the set of data (**Error! Reference source not found.**). The highest variance value was observed in the analysis separating Aegean and Mediterranean populations, using only outlier loci (variance = 51%). The same value dropped down to 11%, using only neutral loci. Variance within regions, instead, was higher for neutral loci (68%) compared to outliers (39%).

The Bayesian grouping performed with STRUCTURE indicates that the most significant K value is 2, with both all markers and only outlier loci. Using all loci, KAS appears as a transition zone

between the clearly identified Aegean and Mediterranean groups (



a).

Although less significant, the analysis obtained with K = 3 shows that both KAS and KEM represent a transition between the two main population groups (Figure 5b). In the analysis performed with only neutral loci, instead, the KAS, KEM, and KAL (S and D) populations present a mixed

composition, with almost all individuals shared between the two groups (Figure 5c). Also, in this analysis, KAS and KEM separate from the rest when K=3 was considered (Figure 5d).

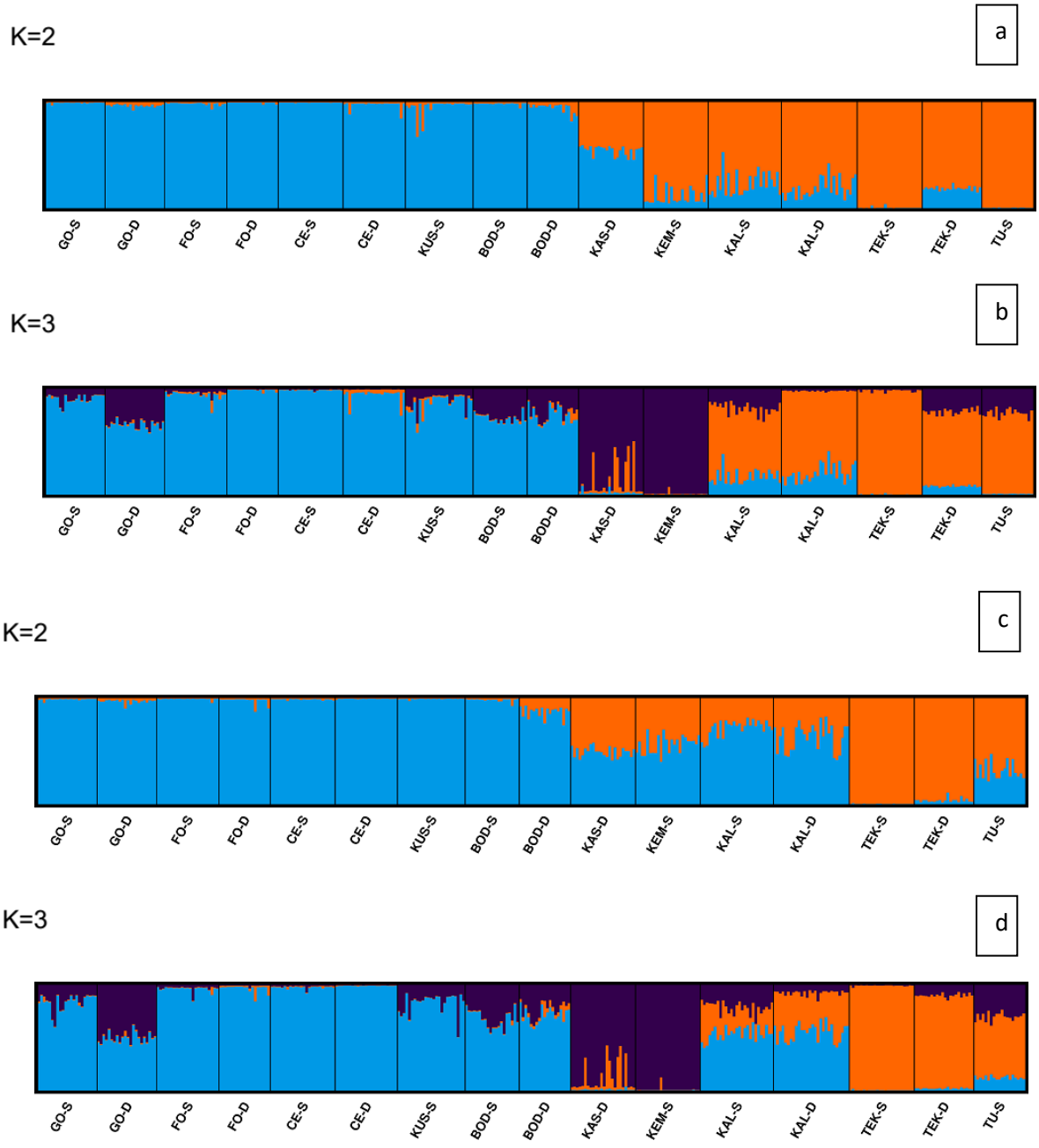


Figure 11: STRUCTURE analysis results in neutral loci and all loci used, respectively. Figures a and b: all loci; figures c and d: outlier loci.

A similar pattern was obtained by the PCoA analysis performed on different sets of data. The analysis conducted using all loci identified a pattern similar to STRUCTURE, with KAS located as a

middle point along the first axis (61%) between the Aegean and Mediterranean groups. In most of the cases, shallow and deep populations of the same locality fall close to each-other in the PCoA space. The shallow population of TEK represents one of the exceptions, being located on the far negative side of the first axis. (Figure 6).

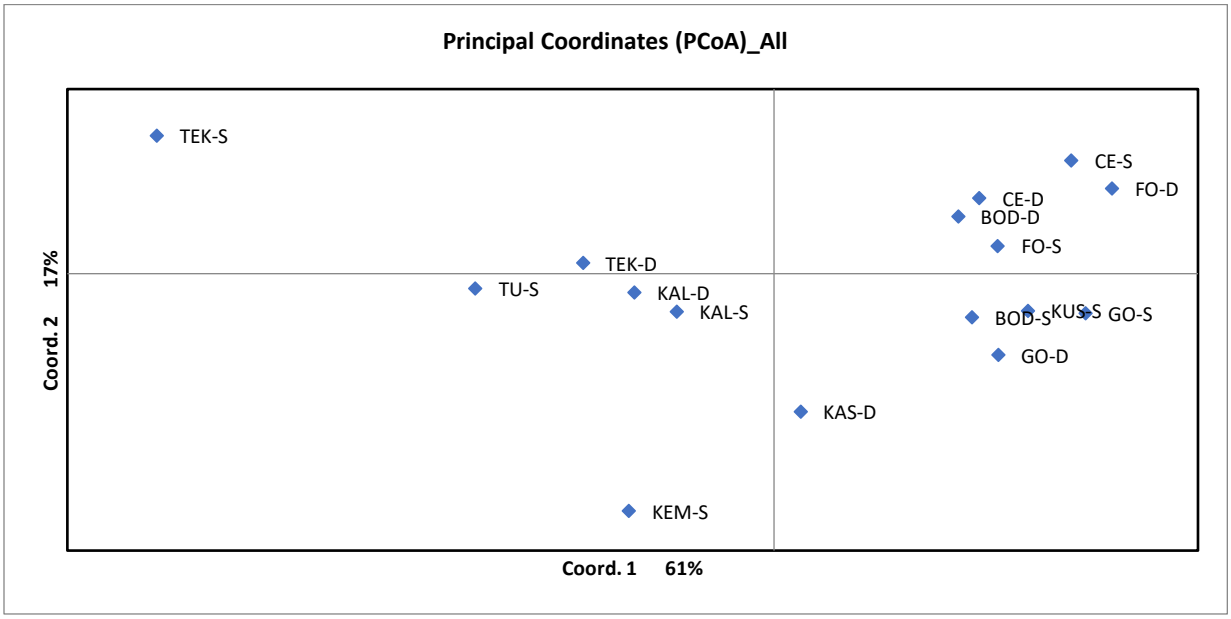


Figure 12: PCoA plot showing that populations are mainly distributed along the first axis, explaining the 61% of the total variance

The separation between Aegean and Mediterranean populations appears evident also from the EDENetwork analysis shown in Figure 7. The Aegean group appears more homogeneous, with individuals from the peripheral southern populations of Bodrum distributed at boundaries of the main group. Mediterranean populations are more scattered. Most of the connections between Aegean and Mediterranean samples occur through the individuals of the shallow populations of KAL and BOD.

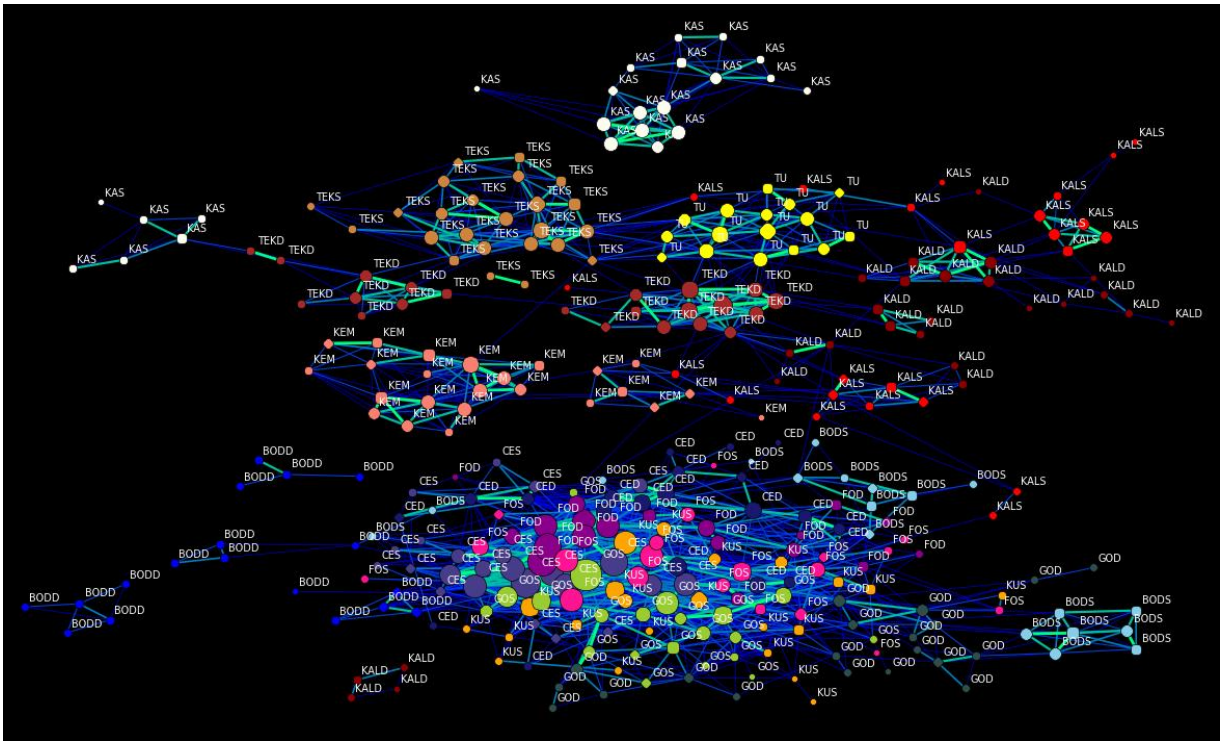


Figure 13: EDENetwork genetic distance analysis result

2.1. Lagrangian dispersal simulation

The dispersal simulation analysis was performed on sea current data from the years 2010-2013. The panels in Figure 8, collect all the simulations where the particles were released from the distinct sampling stations along 90 days, and trajectory was calculated for 10 and 28 days of passive dispersal. The main patterns of current circulation agree in identifying two main areas of dispersal, which correspond to the Aegean and the Mediterranean coasts. CES, TU, KAL, TEK, KEM and FO are the populations with the highest source strength. KUS has the highest retention strength and after it FO and GO have the highest. Particle released from the Aegean populations never reach Southern localities remaining trapped with the system of islands surrounding the Aegean Turkish coast. Particles originated from GO are drifted by current only to Northern part. FO and CES originated particles trapped in the island system and due to their distance, the

dispersal areas overlap. Unfortunately, dispersal simulations from Bodrum were made impossible due to the coastal morphology and the model resolution. Particle released from the Mediterranean populations move toward the west, but never reaching the Aegean populations in the due time. TU and TEK sourced particles drifted to both west and east side and they have a possibility to reach Cyprus coasts. On the other hand, KAL originated particles drifted to Antalya Bay, which connects KEM population to KAL. It must be said that particles released in the last days of the releasing period are less probable to represent a real fruits migration, since fruits release is easier to happen during the first two months (i.e. March and April).

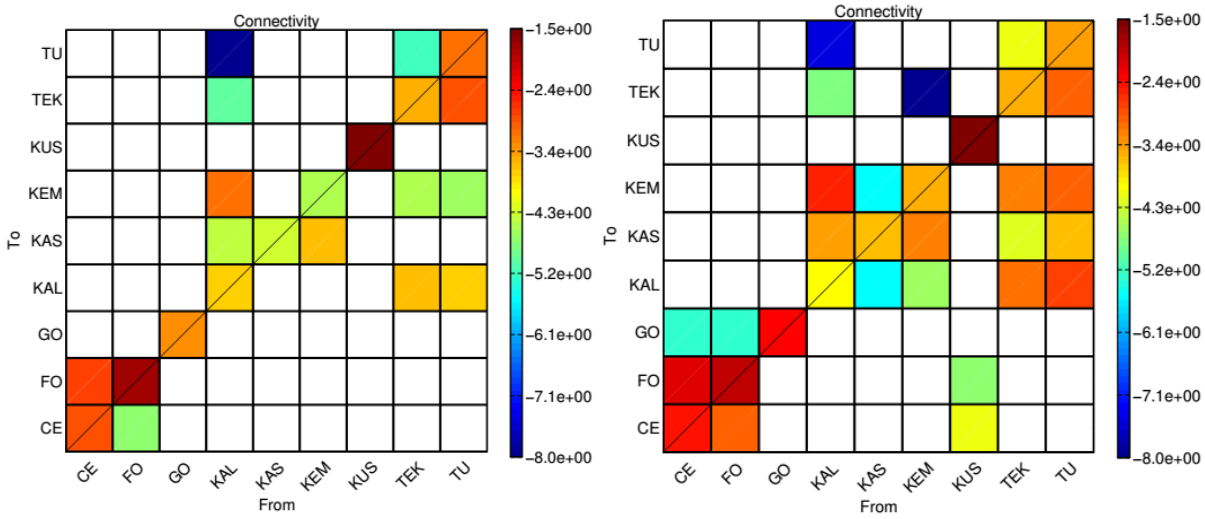


Figure 14: Connectivity between populations, 10 days (left), and 28 days (right) simulation results

2.4 DISCUSSION

In this study, we combined genetic and physical connectivity approaches to investigate the genetic structure of Anatolian *P. oceanica* populations and their possible connectivity through surface currents. Microsatellite analysis identified two main genetic clusters, corresponding to the Aegean populations collected along the Western Turkey coasts and the herein called Mediterranean populations, collected along the southern Turkey coasts. Mediterranean populations appear more heterogeneous, while Aegean populations are more homogeneous and are connected by higher levels of gene flow, which is indicative of panmixia which has been observed also for other species (Giantsis, Mucci, Randi, Abatzopoulos, & Apostolidis, 2014).

The indented Aegean coastline reflects in a complex surface circulation system (Olson, Kourafalou, Johns, Samuels, & Veneziani, 2007) while the Mediterranean coast of Anatolia is mostly under the effect of a dominant westward current (C. N. Bianchi et al., 2012). The complex coastal profile and the presence of small islands along the coasts act as a barrier to long distance dispersal, isolating the Aegean populations form the Southern coast of Turkey and resulting in the lack of a directional gene flow and IBD.

The separation of the two population groups is confirmed both by the genetic connectivity data (realized connectivity, sensu Jahnke et al., 2016) and by the current dispersal modelling (potential connectivity). Nevertheless, realized and potential connectivity do not always correspond. Potential connectivity data indicate the Aegean locality of KUS as the one with the highest retention strength after 10 days of simulated dispersal, although according to the realized connectivity assessment, the Eastermost populations of TEK and TU are the ones with the highest

retention level. The model obtained after 28 days of dispersal indicates that propagules from KUS can reach the nearby populations of CE and FO, suggesting a correspondence between potential and realized connectivity for longer dispersal times. The considerable maritime traffic, mainly due to touristic activities, among Aegean localities can also mask natural dispersal patterns and act as a vehicle for moving around vegetative propagules. This could be at the basis of the high realized connectivity (higher genetic homogeneity) among Aegean populations, in comparison to Mediterranean ones.

2.5 Appendix

			not assigned to			
		G	its own pop	any pop	total	%
Aegean	GO-S	22	13	3	16	27
	GO-D	22	14	1	15	32
	FO-S	23	10	4	14	39
	FO-D	19	8	1	9	53
	CE-S	24	11	1	12	50
	CE-D	23	9	2	11	52
	KUS-S	25	7	3	10	60
	BOD-S	20	2	2	4	80
	BOD-D	19	2	1	3	84
Mediterranean	KAS-D	24	0	3	3	88
	KEM-S	24	2	2	4	83
	KAL-S	27	5	2	7	74
	KAL-D	28	9	3	12	57
	TEK-S	24	4	1	5	79
	TEK-D	22	1	2	3	86
	TU-S	19	1	3	4	79

Appendix 1: GeneClass2 assignment results

GO-S	GO-D	FO-S	FO-D	CE-S	CE-D	KUS-S	BOD-S	BOD-D	KAS-D	KEM-S	KAL-S	KAL-D	TEK-S	TEK-D	TU-S	
0.000																GO-S
0.046	0.000															GO-D
0.036	0.053	0.000														FO-S
0.051	0.068	0.028	0.000													FO-D
0.052	0.083	0.037	0.042	0.000												CE-S
0.070	0.087	0.032	0.038	0.062	0.000											CE-D
0.033	0.041	0.025	0.049	0.064	0.058	0.000										KUS-S
0.056	0.038	0.046	0.056	0.073	0.078	0.036	0.000									BOD-S
0.073	0.084	0.055	0.062	0.062	0.074	0.057	0.045	0.000								BOD-D
0.134	0.083	0.106	0.129	0.153	0.113	0.099	0.084	0.110	0.000							KAS-D
0.134	0.114	0.147	0.196	0.187	0.165	0.127	0.119	0.155	0.087	0.000						KEM-S
0.124	0.104	0.101	0.124	0.140	0.111	0.104	0.080	0.098	0.096	0.084	0.000					KAL-S
0.128	0.119	0.103	0.137	0.150	0.116	0.118	0.092	0.112	0.117	0.095	0.025	0.000				KAL-D
0.254	0.225	0.210	0.251	0.239	0.206	0.226	0.207	0.196	0.196	0.188	0.114	0.105	0.000			TEK-S
0.153	0.122	0.121	0.154	0.138	0.129	0.124	0.117	0.105	0.091	0.106	0.074	0.073	0.066	0.000		TEK-D
0.168	0.164	0.143	0.181	0.176	0.155	0.165	0.158	0.169	0.147	0.101	0.089	0.067	0.109	0.075	0.000	TU-S

Appendix 2: Pairwise Fst results of all loci used data set

CHAPTER 3

Assessment of oxidative stress response to heat stress of *P. oceanica* and *C. nodosa*

3.1 Material & Methodology

3.1.1 Study area, sample collection and experimental design

Samples were collected along Spanish Mediterranean coast from two distant regions (ca. 700 km) and with contrasting thermal regimens were selected in this study. In these regions, dense and healthy shallow *P. oceanica* (5-7 m) and *C. nodosa* (1-2 m) meadows were chosen to sample plants from contrasting thermal environments (Fig. 1). *P. oceanica* plants were collected in extensive meadows in Cala Montgò (Catalonia: 42° 6.38' N, 3° 10.27' E) and in Isla Grosa (Murcia: 37° 43.7' N, 0° 42.75 W) for cold-adapted (C-plants) and warm-adapted (W-plants) plants respectively (Beca-Carretero et al., in press). Mean summer temperature (±SD), from July to September (i.e. the period in which the experiment was conducted), naturally experienced by *P. oceanica* C-plants and W-plants is 22.78 ± 0.89°C and 25.17 ± 0.88°C respectively, according to the temperature time series available from the network of oceanographic buoys of the Spanish Ministry of Development (http://www.puertos.es/es-es/oceanografia/Paginas/portus.aspx).

C. nodosa plants were collected also in Isla Grosa and in a locality at the Ebro Delta (Catalonia: 40° 35.23' N, 0° 37.38' E). Summer water temperatures are higher in the Delta due to the shallow and semi-enclosed geomorphological nature of the system, where they can rise levels above 30°C, and consequently, plants from the Delta are considered W-plants (warm-adapted) while those from Isla Grosa are considered C-plants, as evidenced in Beca-Carretero et al. (In press).

Plant collection was conducted from May 28 to June 3, 2015. Large fragments of rooted *P. oceanica* and *C. nodosa* rhizomes bearing apical growth meristems and a large number of connected shoots were carefully collected by scuba divers to maintain clonal plant integrity. Plants were transported in large coolers under controlled conditions (i.e. temperature and oxygen concentration) to be transplanted in the mesocosms systems of the Oceanographic Centre of Murcia within 36 hours after uprooting. The experimental system for *P. oceanica* is composed of twelve 500-liter tanks described in detail in Marín-Guirao et al (Lázaro Marín-Guirao, Sandoval-Gil, Bernardeau-Esteller, Ruíz, & Sánchez-Lizaso, 2013), and that for *C. nodosa* has similar characteristics but is composed of lower volume tanks (100-liter).

P. oceanica plant fragments of similar size and a similar average number of interconnected vertical shoots (i.e. 42 ±8) were individually transplanted in plastic pots filled with coarse carbonate sediments. Four randomly selected pots (transplantation units, TUs) were allocated inside each experimental tank. *C. nodosa* apical plant fragments with no less than 10 interconnected vertical shoots were selected and fixed with plastic clamps to plastic grids (3-4 fragments per grid, 41 ± 10 shoots grid-1). Four randomly selected grids (i.e. TUs) were allocated in each experimental tank and superficially buried in sediments from the collection site. Sediments employed in the experiment were thoroughly washed and sun-dried before transplantation to remove organic matter and reduce the potential increase in phytotoxic gases promoted by high temperatures (e.g. H_2S; Knoblauch and Jorgensen, 1999). The sediment redox potential measured in TUs at the end of the heatwave showed positive and similar levels in control and heated treatments (i.e. from 130 to 156 mV in *P. oceanica* TUs and from 110 to 163 mV in *C. nodosa* TUs) evidencing the absence of enhanced anoxic conditions due to warming.

After transplantation, experimental plants were allowed to acclimate under constant salinity (37.5 psu) and a light regime of 300 ± 10 and 340 ± mmol photons m-2 s-1, for P. oceancia and *C. nodosa* respectively, and a 14/10 h light/dark photoperiod. During plant acclimation, water temperature in experimental tanks was progressively increased (0.2 ° C day-1) from the temperature levels recorded at the time of sampling up to mean summer temperatures: *P. oceanica* C-plants from 19.5 °C to 23° C, *P. oceanica* W-plants from 23 °C to 25 °C, and *C. nodosa* C-plants and W-plants from 23 °C and 24 °C, respectively, to 25 °C. After an acclimation period of three weeks, temperature in half of the tanks containing plants of each species and thermal environments was progressively increased of 4 °C (rate of 0.5 °C day-1) to simulate a heatwave. The exposure lasted six weeks after which temperature was returned to control levels to allow plants to recover for another six weeks.

Leaf tissues were sampled in two-time points during the heat exposure to follow the molecular mechanisms involved in warming-induced flowering: two weeks after the beginning of temperature increase (T1) and four weeks later (T2), at the end of the heat exposure. A 7 cm leaf segment from the middle part of the youngest fully developed leaf were collected (14:00 h) from one randomly selected shoot per pot and immediately cleaned from epiphytes and stored in RNA later. Leaf pieces of similar biomass (25 mg W.W.) of the four pots of each tank were pooled (n=3 per treatment).

3.1.2 RNA extraction and cDNA synthesis

RNA was obtained from RNA later® fixed samples using the BIORAD Aurum™ Total RNA mini kit. Total RNA was analyzed at Nanodrop for quality and quantity, and tested with gel electrophoresis

to confirm purity from DNA contamination. Retrotranscription has been done with BIORAD iScript™ cDNA synthesis kit according to the manufacturer.

3.1.3 Target gene selection and primer design

Target genes were selected considering the most affected cell compartments, key stress enzymes and proteins (Table 1). More in detail, Glutathione reductase (GLURED), Monodehydroascorbate reductase (MDHAR), Glutathione synthetase (GLUSYN), Glutathione-S-transferase (GST) and two isoforms of Ascorbate peroxidase (APX3 and cytosolic APX) are ascorbate-glutathione cycle enzymes involved in hydrogen peroxide (H_2O_2) detoxification. Glutathione peroxidase (GPX) is an important enzyme that also contributes to H_2O_2 removal in plants (Rouhier et al., 2008). Superoxide dismutase (SOD), Catalase (CAT) and Peroxiredoxin Q (PrxQ) are ROS scavengers. The former directly converts the superoxide radical (O_2^-) into O_2 and H_2O_2 and the latter two catalyze the decomposition of H_2O_2 into water and oxygen. Two isoforms of SOD, Fe-SOD (FSD) and Mn-SOD (MSD), located at chloroplast and mitochondria, respectively were selected. Alternative oxidase (AOX) is a respiratory protein of the mitochondrial electron transport chain that increases its activity under stress conditions to protect cells from oxidative damage by reducing respiratory ROS formation (Vanlerberghe, 2013). Lipoxygenase (LPX) is an indicator of lipid peroxidation and its activity responds to oxidative damage in plant tissues under temperature stress (Ali et al., 2005). Heat shock proteins (sHSP and HSP90) are stress messengers and chaperones, which repair and protect unfolded proteins. The luminal binding protein (LBP) is also a molecular chaperon that participates in the constitutive function of the endoplasmic reticulum (ER) and protects the cell against stresses (Liu and Howell, 2010). The death-specific protein 5 (dsp5) has

a suggested role in the signal transduction of the stress to the cell death machinery (Chung et al., 2008).

Some of the primers were already available (Table 1). New *C. nodosa* primers were designed on conserved regions of aligned sequences of the target genes, obtained in a recently published transcriptome for the species (Ruocco et al., 2017), selecting amplicons between 100 and 250bp long. Alignments were performed by using BioEdit Software version 7.2.5 (Hall, 1999), primers were designed using Unipro UGENE v1.16.2 (Okonechnikov et al., 2012). Amplification efficiency of the primer pairs was calculated from the slopes of standard curves of the threshold cycle (CT) of five cDNA dilutions using the equation $E = 10^{-1/slope}$.

3.1.4 RT-qPCR

After the efficiency test of the primers, RT-qPCR reactions were performed by using Fast SYBR® Green Real-Time PCR Master Mix in the ViiA7 384 wells format RT-qPCR machine (Applied Biosystems). For each RT-qPCR reaction three replicates were conducted per sample and for each primer pair three no-template negative controls were included. The RT-qPCR data were analyzed at ViiA7 Software v1.0 (Applied Biosystems) to obtain threshold cycles (CT values) for each gene and sample. The CT values were normalized with primer efficiency value for each sample. Subsequently, -ΔCT values were calculated as the negative difference in CT values between reference genes and each target gene (-ΔCT = CT (ref genes) – CT (target genes)). Fold expression change values were calculated for graphical concerns according to the equation

$$Fold\ expression\ change = \pm 2^{(|(-\Delta CTtreatment)-(-\Delta CTcontrol)|)}$$

3.1.5 Statistical analysis

Permutational multivariate analysis of variance (PERMANOVA) with all analyzed target genes (−ΔCT values) was performed to identify significant differences in the overall gene expression patterns among control and heated plants at the different time points. A two-way PERMANOVA (permutations: 9999) was conducted on the PRIMER 6 & PERMANOVA+ software package (Anderson et al., 2008). I decided to conduct the analysis with each species independently since not all the genes were analyzed in both species (*P. oceanica* = 12 genes, *C. nodosa* = 10 genes). Therefore, I used two factors: thermal origin (O: cold vs warm) and treatment (T: control vs heated) and the analyses are conducted independently for each of the two species. Pair-wise comparisons were subsequently run to search for significant differences using Monte Carlo p-values.

Two-way ANOVA analysis with the same factors was conducted to search for differences in the level of expression of each selected target gene in each sampling time with STATISTICA 7 software (StatSoft, v. 7.0). Data normality and homocedasticity was previously checked through graphic visualization and Cochran's test, respectively. To explore subsequent pair wise comparisons Newman-Keuls test *post hoc* analyses were performed. Heatmap analysis was performed on RStudio Version 0.99.483 (RStudio Team, 2015) by using package 'ggplot2' version 1.0.

3.2 RESULTS

According to PERMANOVA results *P. oceanica* showed an overall response that were significantly different depending on the thermal origin of plants (Factor Origin: Cold vs Warm) and the treatment they were exposed to (Factor treatment: Control vs Heated), but the interaction of the

two factors was only significant at the end of the heat exposure (T2) and recovery (T3) periods. In T2, heated and control plants from the warm thermal origin were almost significantly different (p=0.055) while plants from the cold environment did not differ (p=0.101). In T3, at the end of the recovery, heated and controls from both thermal origins significantly differed. In general, the overall gene expression response of *C. nodosa* were significantly different depending on the thermal origin of plants but not as a consequence of the experimental heat exposure. The interaction of both factors was only significant in T2 (i.e. after 6 weeks of warming exposure), when only plants from the warm environment showed a significant altered response (p=0.014) with respect to their controls (*Table 3*).

Table 3:Results of the two-way PERMANOVA test for long-term experiment

PERMANOVA		**Posidonia oceanica**			**Cymodocea nodosa**			
T1								
Source	df	MS	Pseudo-F	P(perm)	MS	Pseudo-F	P(perm)	
Origin (Or)	1	13.52	2.42	**0.024**	13.00	5.72	**0.009**	
Treatment (Tr)	1	30.65	5.48	**0.005**	3.46	1.52	0.226	
OrxTr	1	6.71	1.20	0.314	3.28	1.44	0.272	
Res	8	5.59			2.27			
T2								
Source	df	MS	Pseudo-F	P(perm)	MS	Pseudo-F	P(perm)	
Origin (Or)	1	22.99	1.94	0.095	35.28	0.62	0.641	
Treatment (Tr)	1	44.35	3.74	**0.008**	123.68	2.18	0.121	
OrxTr	1	41.62	3.51	**0.012**	200.84	3.54	**0.025**	
Res	8	11.85			56.72			
Pair wise (OrxTr)					**Pair wise (OrxTr)**			
Or (Cold vs Warm)	P (MC)		Tr (Heated vs Control)	P (MC)	Or (Cold vs Warm)	P (MC)	Tr (Heated vs Control)	P (MC)
Control	0.0552		Cold	0.101	Control	0.198	Cold	0.413
Heated	0.1345		Warm	0.055	Heated	0.147	Warm	**0.014**
T3								
Source	df	MS	Pseudo-F	P(perm)	MS	Pseudo-F	P(perm)	
Origin (Or)	1	27.66	16.56	**0.003**	109.32	6.15	**0.030**	
Treatment (Tr)	1	8.19	4.90	**0.003**	6.95	0.39	0.669	
OrxTr	1	8.37	5.01	**0.015**	4.94	0.28	0.751	
Res	8	1.67			17.77			
Pair wise (OrxTr)								
Or (Cold vs Warm)	P (MC)		Tr (Heated vs Control)	P (MC)				
Control	**0.004**		Cold	**0.049**				
Heated	**0.010**		Warm	**0.02**				

The fold expression change values (FC) for all the analyzed genes for both species were given in Appendix 3 and synthesized in the heatmaps (Figure 16). Except the response of *C. nodosa* at T2, *P. oceanica* plants showed relatively stronger response comparing to *C. nodosa* plants, as evident from the intensity of colors.

The ratio between the number of upregulated and downregulated genes was plotted to assess the general pattern of selected genes' activation, regardless of their significance level (Figure 15). In the cold origin *P. oceanica* plants (PC), eight genes were upregulated while four genes (cAPX, GST, B1 and LPX) were down regulated at T1. sHSP showed the highest expression with FC (±SE) of 4 ± 1.4 ($p<0.01$) and cAPX showed the lowest expression with FC of -5.1 ± 1.7. In T2, the up/down ratio was reversed comparing to T1 and only LBP did not change its overexpression. (up/down ratio=0.2) and with LBP, LPX is the other overexpressed gene. At the end of recovery (T3), AOX is the gene which showed the highest expression with FC 4.8 ± 1.4. At T3 the ratio of up/down regulated genes almost returned to the T1 level with up/down ratio=3. sHSP is the only gene which did not change its expression trend (**Error! Reference source not found.**).

In the warm adapted *P. oceanica* plants (PW), the ratio of up- and down-regulated genes decreased gradually during experimental steps (Figure 15). Seven out of twelve genes overexpressed during T1 and T2 and downregulated at T3 while LPX and cAPX gradually increased their downregulated level throughout the experiment, and GST, MSD and CSD1 upregulated at T1 while downregulated at T2 and T3. In all experimental stages among all genes sHSP showed the highest expression level in T2 with FC of 11.8.

In the cold adapted *C. nodosa* plants (CC), the up/down ratio is same in T1 and T2 (up/down ratio=0.7) and increased in T3 (up/down ratio=2.3). Only four out of ten genes were upregulated during heat treatment (GPX, GST, sHSP and dsp5). The highest expressed gene in overall experiment is cAPX with FC of 8.1 in T2. The most downregulated genes are in T2 AOX, B1 and sHSP with FC of -15.6 ± 6.8, -12.5 ± 4.3, and -10.5 ± 4.9.

In the warm adapted *C. nodosa* plants (CW), the up/down ratio is same at T1 and T2 (up/down ratio=0.4) all genes kept the regulation trend in T2. Comparing to other species and thermal origin of the same species, CW showed some extreme expression levels in T2 for four genes cAPX, GPX, dsp5 and CSD1 with FC of -56.9 ± 1.7, -69.3 ± 3.3, -31.1 ± 4 and -183.1 ± 8.8 respectively. Since they are shading other information on the graph, I excluded them on bar graphs (**Error! Reference source not found.**), but they are included to heatmaps (*Figure 16*).

Table 4: Significance level of the two-way ANOVA for common genes of both species. Bold text indicates to the significant results. On post-hoc test results, I accepted as significant only the genes with homoscedasticity and the ones are not homogeneous, but significance level is higher than 0.01.

p	T1	cAPX	GPX	MSD	LBP	GST	sHSP	dsp5	CSD	AOX	B1	LPX	MDHAR
Univariate	Treatment	**0.005**	n.s.	**0**	n.s.	n.s.	**0.0002**	**0.0045**	**0.048**	n.s.	n.s.	n.s.	**0.01**
Tests of	Species	**0**	**0**	n.s.	**0**	**0**	**0**	**0**	**0**	**0**	**0**	n.s.	n.s.
Significance	TrxSp	n.s.	n.s.	**0**	n.s.	n.s.	n.s.	**0.0011**	**0.041**	n.s.	n.s.	n.s.	n.s.
Cochran's test		**>0.05**	n.s.	**>0.05**	**>0.05**	n.s.	**>0.05**	**>0.05**	n.s.	**>0.05**	**>0.05**	n.s.	**>0.05**
Post-hoc test (Newman-Keuls)		**PC 0.0052**	PC 0.024				**PC 0.004**	**PC 0.0005**	PC 0.03	**PW 0.01**			**PC 0.04**
							PW 0.0059	**PW 0.0026**	PW 0.042				

p	T2	cAPX	GPX	MSD	LBP	GST	sHSP	dsp5	CSD	AOX	B1	LPX	MDHAR
Univariate	Treatment	**0.047**	**0.047**	n.s.	n.s.	n.s.	n.s.	n.s.	**0.017**	n.s.	n.s.	n.s.	n.s.
Tests of	Species	**0.019**	n.s.	n.s.	**0**	**0**	**0**	n.s.	n.s.	**0**	n.s.	n.s.	n.s.
Significance	TrxSp	**0.003**	**0.008**	n.s.	n.s.	n.s.	**0.006**	n.s.	**0.038**	n.s.	n.s.	n.s.	n.s.
Cochran's test		n.s.	n.s.	n.s.	**>0.05**	n.s.	n.s.	**>0.05**	n.s.	**>0.05**	n.s.	n.s.	**>0.05**
Post-hoc test (Newman-Keuls)		**CW 0.0066**	**CW 0.007**				PW 0.035		CW 0.02				
		CC 0.042					**CC 0.01**						

p	T3	cAPX	GPX	MSD	LBP	GST	sHSP	dsp5	CSD	AOX	B1	LPX	MDHAR
Univariate	Treatment	**0**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**0.02**	n.s.	n.s.	n.s.
Tests of	Species	n.s.	**0**	**0.025**	**0**	**0**	**0**	**0**	**0**	**0**	**0.02**	**0**	n.s.
Significance	TrxSp	**0**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Cochran's test		n.s.	n.s.	n.s.	**>0.05**	**>0.05**	**>0.05**	n.s.	n.s.	**>0.05**	n.s.	**>0.05**	**>0.05**
Post-hoc test (Newman-Keuls)			PW 0.042										

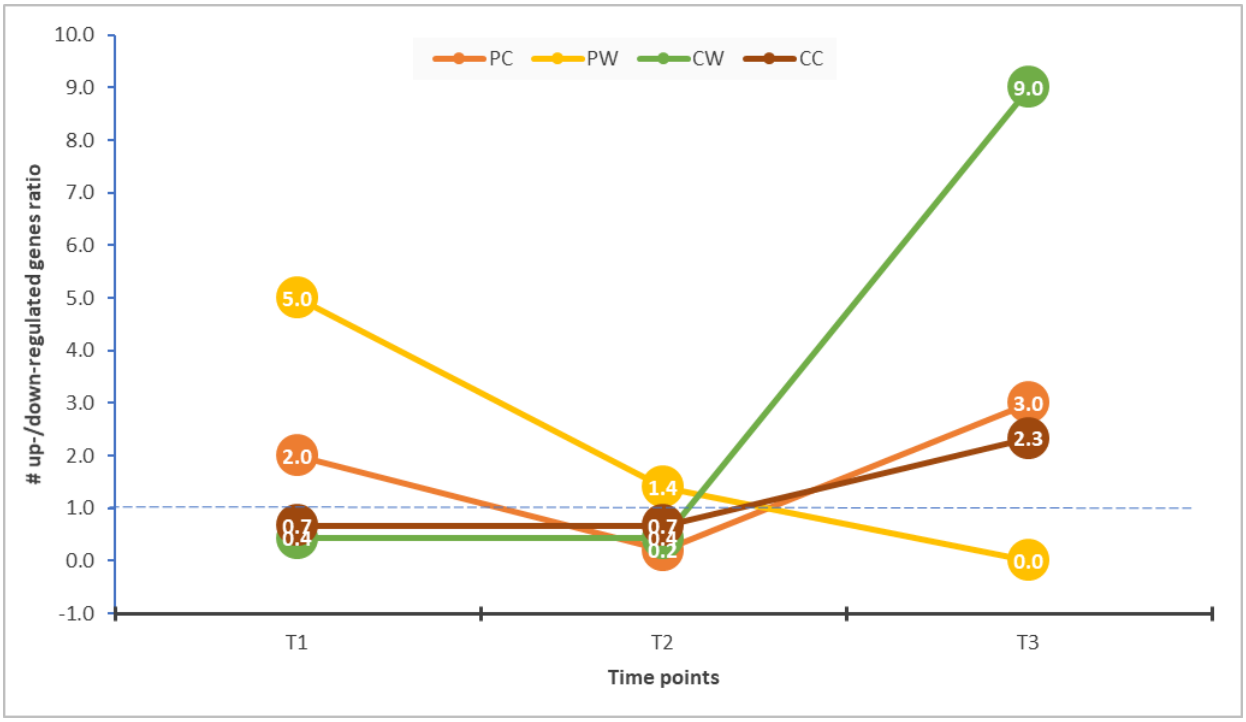


Figure 15: Ratio between the number of upregulated and downregulated genes in cold and warm thermal origins of *P. oceanica* and *C. nodosa* after

Heatmaps shows that *P. oceanica* plants were more responsive than *C. nodosa* plants in general as indicated by colors (*Figure 16*). AOX, B1 and sHSP is in close relationship in both thermal origin of both species. Considering the treatment relationship, *C. nodosa* plants gave similar response in each treatment and grouped treatment by treatment. *P. oceanica* plants grouped in a more mixed way. For example, PW-T2 is different from the rest and PW-T1 showed closer responses with PC-T3 and PC-T2 with PW-T3. Even though there are some obvious regulation changes there are few significant regulations. sHSP is the only significantly downregulated gene in CC ($p<0.01$). GPX and cAPX significantly downregulated by CW plants at the end of heat exposure (both $p<0.01$). *P. oceanica* plants from both thermal origin showed significant regulations only on the early stages of heat exposure (T1). dsp5 significantly upregulated during T1 by PC and PW with $p<0.001$ and $p<0.01$ respectively. sHSP significantly upregulated by PC and PW (both $p<0.01$).

AOX upregulated by PW, cAPX downregulated and MDHAR upregulated by PC in T1 ($p<0.01$, $p<0.01$ and $p<0.0$ respectively).

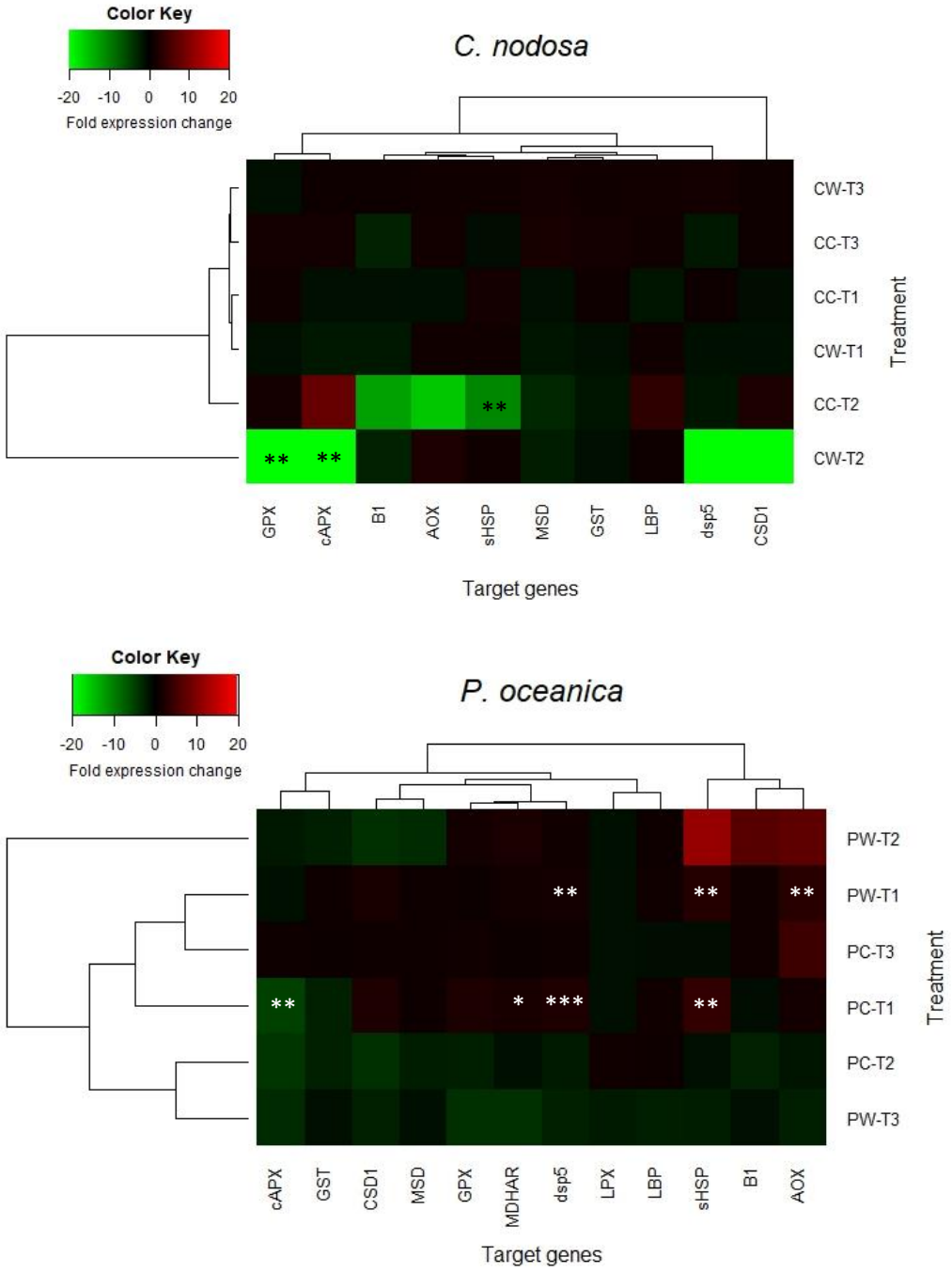


Figure 16: Haetmaps representing fold expression change levels of target genes in *P. oceanica* warm and cold thermal origin and *C. nodosa* warm and cold thermal origin. Asterisks indicate significant differences between control and heated plants: *p<0.05; **p<0.01; ***p<0.001

The constitutive expression of selected genes was also compared between two thermal origin for both species. All genes downregulated in CW controls with respect to CC controls.

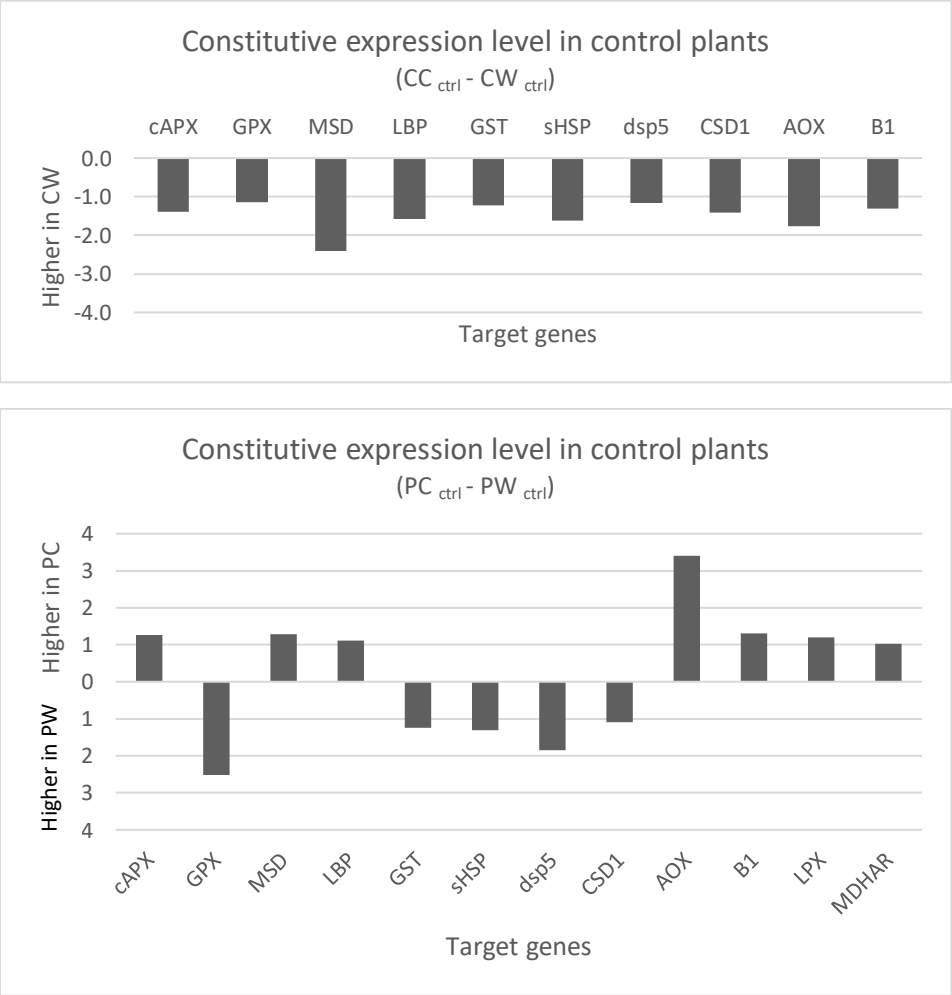


Figure 17: Constitutive expression level in control plants in C. nodosa (top) and P. oceanica (below)

3.3 REFERENCES

Alberto, F., Correia, L., Arnaud-Haond, S., Billot, C., Duarte, C. M., & Serrao, E. (2003). New microsatellite markers for the endemic Mediterranean seagrass Posidonia oceanica. *Molecular Ecology Notes*, *3*(2), 253–255. http://doi.org/10.1046/j.1471-8286.2003.00415.x

Antao, T., Lopes, A., Lopes, R. J., Beja-Pereira, A., & Luikart, G. (2008). LOSITAN: A workbench to detect molecular adaptation based on a F st -outlier method. *BMC Bioinformatics*, *9*(1), 1–5. http://doi.org/10.1186/1471-2105-9-323

Arnaud-Haond, S., Duarte, C. M., Diaz-Almela, E., Marbà, N., Sintes, T., & Serrão, E. a. (2012). Implications of extreme life span in clonal organisms: millenary clones in meadows of the threatened seagrass Posidonia oceanica. *PloS One*, *7*(2), e30454. http://doi.org/10.1371/journal.pone.0030454

Arnaud-Haond, S., Duarte, C. M., Diaz-Almela, E., Marba, N., Sintes, T., & Serrao, E. A. (2012). Implications of extreme life span in clonal organisms: Millenary clones in meadows of the threatened seagrass posidonia oceanica. *PLoS ONE*, *7*(2), e30454. http://doi.org/10.1371/journal.pone.0030454

Arnaud-Haond, S., Marbà, N., Diaz-Almela, E., Serrão, E. a., & Duarte, C. M. (2009). Comparative Analysis of Stability—Genetic Diversity in Seagrass (Posidonia oceanica) Meadows Yields Unexpected Results. *Estuaries and Coasts*, *33*(4), 878–889. http://doi.org/10.1007/s12237-009-9238-9

Arnaud-Haond, S., Migliaccio, M., Diaz-Almela, E., Teixeira, S., van de Vliet, M. S., Alberto, F., … Serrão, E. a. (2007). Vicariance patterns in the Mediterranean Sea: east-west cleavage and low dispersal in the endemic seagrass Posidonia oceanica. *Journal of Biogeography*, *34*(6), 963–976. http://doi.org/10.1111/j.1365-2699.2006.01671.x

Arnaud-Haond, S., Moalic, Y., Hernández-García, E., Eguiluz, V. M., Alberto, F., Serrão, E. a, & Duarte, C. M. (2014). Disentangling the Influence of Mutation and Migration in Clonal Seagrasses Using the Genetic Diversity Spectrum for Microsatellites. *The Journal of Heredity*, 1–10. http://doi.org/10.1093/jhered/esu015

Arranz, S. E., Avarre, J.-C., Balasundaram, C., Bouza, C., Calcaterra, N. B., Cezilly, F., … Zhuang, Z. (2013). Permanent genetic resources added to molecular ecology resources database 1 December 2012-31 January 2013. *Molecular Ecology Resources*, *13*(3), 546–9. http://doi.org/10.1111/1755-0998.12095

Bailleul, D., Stoeckel, S., Arnaud-Haond, S., & Poisot, T. (2016). RClone: a package to identify MultiLocus Clonal Lineages and handle clonal data sets in r. *Methods in Ecology and Evolution*, *7*(8), 966–970. http://doi.org/10.1111/2041-210X.12550

Bianchi, C. N. (2007). Biodiversity issues for the forthcoming tropical Mediterranean Sea. *Hydrobiologia*, *580*(1), 7–21. http://doi.org/10.1007/s10750-006-0469-5

Bianchi, C. N., Morri, C., Chiantore, M., Montefalcone, M., Parravicini, V., & Rovere, a. (2012). *Mediterranean Sea Biodiversity Between the Legacy From the Past and a Future of Change*. *Life in the Mediterranean Sea : A Look at Habitat Changes*.

Boudouresque, C. F., Bernard, G., Pergent, G., Shili, A., & Verlaque, M. (2009). Regression of

Mediterranean seagrasses caused by natural processes and anthropogenic disturbances and stress: A critical review. *Botanica Marina*, *52*(5), 395–418. http://doi.org/10.1515/BOT.2009.057

Burkholder, J. M., Tomasko, D. a., & Touchette, B. W. (2007). Seagrasses and eutrophication. *Journal of Experimental Marine Biology and Ecology*, *350*(1–2), 46–72. http://doi.org/10.1016/j.jembe.2007.06.024

Cancemi, G., Buia, M. C., & Mazzella, L. (2002). Structure and growth dynamics of Cymodocea nodosa meadows. *Scientia Marina*, *66*(4), 365–373. http://doi.org/10.3989/scimar.2002.66n4365

Celebi, B., Gucu, A. C., Ok, M., Serdar, S., & Akoglu, E. (2007). SURVIVAL OF THE POSIDONIA OCEANICA CUTTINGS TRANSPLANTED INTO THE NORTHEASTERN LEVANT SEA, (May 2006), 33731.

Chefaoui, R. M., Assis, J., Duarte, C. M., & Serrão, E. A. (2016). Large-Scale Prediction of Seagrass Distribution Integrating Landscape Metrics and Environmental Factors: The Case of Cymodocea nodosa (Mediterranean–Atlantic). *Estuaries and Coasts*, *39*(1), 123–137. http://doi.org/10.1007/s12237-015-9966-y

Costanza, R., Arge, R., Groot, R. De, Farber, S., Grasso, M., Hannon, B., … Sutton, P. (1997). The value of the world ' s ecosystem services and natural capital, *387*(May), 253–260.

Dattolo, E., Marín-Guirao, L., Ruiz, J. M., & Procaccini, G. (2017). Long-term acclimation to reciprocal light conditions suggests depth-related selection in the marine foundation species Posidonia oceanica. *Ecology and Evolution*, *7*(4), 1148–1164. http://doi.org/10.1002/ece3.2731

Defeo, O., McLachlan, A., Schoeman, D. S., Schlacher, T. A., Dugan, J., Jones, A., … Scapini, F. (2009). Threats to sandy beach ecosystems: A review. *Estuarine, Coastal and Shelf Science*, *81*(1), 1–12. http://doi.org/10.1016/j.ecss.2008.09.022

Den Hartog, C. (1972). Range extension of Halophila stipulacea (Hydrocharitaceae) in the Mediterranean. *Blumea - Biodiversity, Evolution and Biogeography of Plants*, *20*(1), 154–156. Retrieved from http://www.narcis.nl/publication/RecordID/oai%3Anaturalis.nl%3A525017

Duarte, C. M., Marbà, N., & Santos, R. (2004). What may cause loss of seagrasses ? *European Seagrasses: An Introduction to Monitoring and Management*, 24–32. Retrieved from www.seagrasses.org

Earl, D. A., & vonHoldt, B. M. (2012). STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, *4*(2), 359–361. http://doi.org/10.1007/s12686-011-9548-7

Erftemeijer, P. L. a, & Lewis, R. R. R. (2006). Environmental impacts of dredging on seagrasses: a review. *Marine Pollution Bulletin*, *52*(12), 1553–72. http://doi.org/10.1016/j.marpolbul.2006.09.006

Espino, F., Tuya, F., Blanch, I., & Haroun, R. J. (2008). Los sebadales de Canarias. Oasis de vida en los fondos arenosos. *BIOGES, Universidad de Las Palmas de Gran Canaria*.

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology*, *14*(8), 2611–2620. http://doi.org/10.1111/j.1365-294X.2005.02553.x

Foll, M., & Gaggiotti, O. (2008). A genome-scan method to identify selected loci appropriate for both dominant and codominant markers: A Bayesian perspective. *Genetics*, *180*(2), 977–993. http://doi.org/10.1534/genetics.108.092221

Foyer, C. H., Lopez-Delgado, H., Dat, J. F., & Scott, I. M. (1997). Hydrogen peroxide-and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. *Physiologia Plantarum*, *100*(2), 241–254.

Fyfe, S. K., & Davis, A. R. (2007). Spatial scale and the detection of impacts on the seagrass Posidonia australis following pier construction in an embayment in southeastern Australia. *Estuarine, Coastal and Shelf Science*, *74*(1–2), 297–305. http://doi.org/10.1016/j.ecss.2007.04.022

Gambi, M. C., Barbieri, F., & Bianchi, C. N. (2009). New record of the alien seagrass Halophila stipulacea (Hydrocharitaceae) in the western Mediterranean: a further clue to changing Mediterranean Sea biogeography. *Marine Biodiversity Records*, *2*(January 2016), e84. http://doi.org/doi:10.1017/S175526720900058X

Giantsis, I. A., Mucci, N., Randi, E., Abatzopoulos, T. J., & Apostolidis, A. P. (2014). Microsatellite variation of mussels (Mytilus galloprovincialis) in central and eastern Mediterranean: genetic panmixia in the Aegean and the Ionian Seas. *Journal of the Marine Biological Association of the United Kingdom*, *94*(4), 797–809. http://doi.org/10.1017/S0025315414000174

Gobert, S., Cambridge, M. L., Velimirov, B., Pergent, G., Lepoint, G., Bouquegneau, J. M., … Walker, D. I. (2006). Biology of Posidonia. Seagrasses: Biology, ecology and conservation 17, 387–408, AWD Larkum. ed. Springer, Dordrecht NL.

Goudet, J. (1995). FSTAT (version 1.2): A computer note computer program to calculate F-statistics. *Journal of Heredity*, *86*(August), 485–486. http://doi.org/10.1093/jhered/est020

Green, E. P., & Short, F. T. (2003). *World Atlas of Seagrasses*. Berkeley, USA: UIMEP World Conservation Monitoring Centre. University of California Press.

Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*. http://doi.org/citeulike-article-id:691774

Hastings, K., Hesp, P., & Kendrick, G. A. (1995). Seagrass loss associated with boat moorings at Rottnest Island, Western Australia. *Ocean and Coastal Management*, *26*(3), 225–246. http://doi.org/10.1016/0964-5691(95)00012-Q

Hillebrand, H., Soininen, J., & Snoeijs, P. (2010). Warming leads to higher species turnover in a coastal ecosystem. *Global Change Biology*, *16*(4), 1181–1193. http://doi.org/10.1111/j.1365-2486.2009.02045.x

Jahnke, M., Casagrandi, R., Melià, P., Schiavina, M., Schultz, S. T., Zane, L., & Procaccini, G. (2017). Potential and realized connectivity of the seagrass *Posidonia oceanica* and their implication for conservation. *Diversity and Distributions*, 1–12. http://doi.org/10.1111/ddi.12633

Jahnke, M., Christensen, A., Micu, D., Milchakova, N., Sezgin, M., Todorova, V., … Procaccini, G. (2016). Patterns and mechanisms of dispersal in a keystone seagrass species. *Marine Environmental Research*, *117*, 54–62. http://doi.org/10.1016/j.marenvres.2016.04.004

Jahnke, M., Olsen, J. L., & Procaccini, G. (2015). A meta-analysis reveals a positive correlation between genetic diversity metrics and environmental status in the long-lived seagrass Posidonia oceanica. *Molecular Ecology*. http://doi.org/10.1111/mec.13174

Jordà, G., Marbà, N., & Duarte, C. M. (2012). Mediterranean seagrass vulnerable to regional climate warming. *Nature Climate Change*, *2*(11), 821–824. http://doi.org/10.1038/nclimate1533

Kendrick, G. A., Waycott, M., Carruthers, T. J. B., Cambridge, M. L., Hovey, R., Krauss, S. L., … Verduin, J. J. (2012). The Central Role of Dispersal in the Maintenance and Persistence of Seagrass Populations. *BioScience*, *62*(1), 56–65. http://doi.org/10.1525/bio.2012.62.1.10

Kilminster, K., McMahon, K., Waycott, M., Kendrick, G. A., Scanes, P., McKenzie, L., … Udy, J. (2015). Unravelling complexity in seagrass systems for management: Australia as a microcosm. *Science of the Total Environment*, *534*, 97–109. http://doi.org/10.1016/j.scitotenv.2015.04.061

Kivelä, M., Arnaud-Haond, S., & Saramäki, J. (2015). EDENetworks: A user-friendly software to build and analyse networks in biogeography, ecology and population genetics. *Molecular Ecology Resources*, *15*(1), 117–122. http://doi.org/10.1111/1755-0998.12290

Kopelman, N. M., Mayzel, J., Jakobsson, M., Rosenberg, N. A., & Mayrose, I. (2015). Clumpak: A program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*, *15*(5), 1179–1191. http://doi.org/10.1111/1755-0998.12387

Kuo, J., & Den Hartog, C. (2007). Seagrass morphology, anatomy, and ultrastructure. In *SEAGRASSES: BIOLOGY, ECOLOGYAND CONSERVATION* (pp. 51–87). Springer.

Laubier, L. (2005). Mediterranean Sea and Humans: Improving a Conflictual Partnership BT - The Mediterranean Sea. In A. Saliot (Ed.) (pp. 3–27). Berlin, Heidelberg: Springer Berlin Heidelberg. http://doi.org/10.1007/b107142

Lejeusne, C., Chevaldonné, P., Pergent-Martini, C., Boudouresque, C. F., & Pérez, T. (2010). Climate change effects on a miniature ocean: the highly diverse, highly impacted Mediterranean Sea. *Trends in Ecology and Evolution*, *25*(4), 250–260. http://doi.org/10.1016/j.tree.2009.10.009

Les, D. H., Cleland, M. A., & Waycott, M. (1997). Phylogenetic studies in Alismatidae. *Systematic Botany*, *22*(3), 443–463.

Marbà, N., & Duarte, C. M. (1998). Rhizome elongation and seagrass clonal growth. *Marine Ecology Progress Series*, *174*, 269–280. http://doi.org/10.3354/meps174269

Marbà, N., Santiago, R., Díaz-Almela, E., Álvarez, E., & Duarte, C. M. (2006). Seagrass (Posidonia oceanica) vertical growth as an early indicator of fish farm-derived stress. *Estuarine, Coastal and Shelf Science*, *67*(3), 475–483. http://doi.org/10.1016/j.ecss.2005.11.034

Marin-Guirao, L., Entrambasaguas, L., Dattolo, E., Ruiz, J. M., & Procaccini, G. (2017). Molecular Mechanisms behind the Physiological Resistance to Intense Transient Warming in an Iconic Marine Plant. *Frontiers in Plant Science*, *8*(June). http://doi.org/10.3389/fpls.2017.01142

Marín-Guirao, L., Ruiz, J. M., Dattolo, E., Garcia-munoz, R., & Procaccini, G. (2016). Physiological and molecular evidence of differential short-term heat tolerance in Mediterranean seagrasses. *Scientific Reports*, (January), 1–13. http://doi.org/10.1038/srep28615

Marín-Guirao, L., Sandoval-Gil, J. M., Bernardeau-Esteller, J., Ruíz, J. M., & Sánchez-Lizaso, J. L. (2013). Responses of the Mediterranean seagrass Posidonia oceanica to hypersaline stress duration and recovery. *Marine Environmental Research*, *84*, 60–75. http://doi.org/10.1016/j.marenvres.2012.12.001

Mateo, M. Á., Sánchez-Lizaso, J. L., & Romero, J. (2003). Posidonia oceanica “banquettes”: A preliminary assessment of the relevance for meadow carbon and nutrients budget. *Estuarine, Coastal and Shelf Science*, *56*(1), 85–90. http://doi.org/10.1016/S0272-7714(02)00123-3

Meinesz, a., Cirik, Ş., Akcali, B., Javel, F., Migliaccio, M., Thibaut, T., … Procaccini, G. (2009). Posidonia oceanica in the Marmara Sea. *Aquatic Botany*, *90*(1), 18–22. http://doi.org/10.1016/j.aquabot.2008.04.013

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. *Aquatic Botany*, *82*(3), 210–221. http://doi.org/10.1016/j.aquabot.2005.03.002

Micheli, F., Halpern, B. S., Walbridge, S., Ciriaco, S., Ferretti, F., Fraschetti, S., … Rosenberg, A. A. (2013). Cumulative human impacts on Mediterranean and Black Sea marine ecosystems: Assessing current pressures and opportunities. *PLoS ONE*, *8*(12). http://doi.org/10.1371/journal.pone.0079889

Millot, C., & Taupier-Letage, I. (2005). Circulation in the Mediterranean Sea. *Environmental Chemistry*, *5*(Part K), 29–66. http://doi.org/10.1007/b107143

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., … Van Breusegem, F. (2011). ROS signaling: The new wave? *Trends in Plant Science*, *16*(6), 300–309. http://doi.org/10.1016/j.tplants.2011.03.007

Neill, S. J., Desikan, R., Clarke, A., Hurst, R. D., & Hancock, J. T. (2002). Hydrogen peroxide and nitric oxide as signalling molecules in plants. *Journal of Experimental Botany*, *53*(372), 1237–1247. http://doi.org/10.1093/jexbot/53.372.1237

Okonechnikov, K., Golosova, O., Fursov, M., Varlamov, A., Vaskin, Y., Efremov, I., … Tleukenov, T. (2012). Unipro UGENE: A unified bioinformatics toolkit. *Bioinformatics*, *28*(8), 1166–1167. http://doi.org/10.1093/bioinformatics/bts091

Olsen, J. L., Rouzé, P., Verhelst, B., Lin, Y.-C., Bayer, T., Collen, J., … Van de Peer, Y. (2016). The genome of the seagrass Zostera marina reveals angiosperm adaptation to the sea. *Nature*, 1–17. http://doi.org/10.1038/nature16548

Olson, D. B., Kourafalou, V. H., Johns, W. E., Samuels, G., & Veneziani, M. (2007). Aegean Surface Circulation from a Satellite-Tracked Drifter Array. *Journal of Physical Oceanography*, *37*(7), 1898–1917. http://doi.org/10.1175/JPO3028.1

Oreska, M. P. J., Mcglathery, K. J., & Porter, J. H. (2017). Seagrass blue carbon spatial patterns at the meadow-scale. *PLoS ONE*, *12*(4), e0176630.

Paetkau, D., Slade, R., Burden, M., & Estoup, A. (2004). Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. *Molecular Ecology*, *13*, 55–65. http://doi.org/10.1046/j.1365-294X.2003.02008.x

Papenbrock, J. (2012). Highlights in Seagrasses' Phylogeny, Physiology, and Metabolism: What Makes Them Special? *ISRN Botany*, *2012*, 1–15. http://doi.org/10.5402/2012/103892

Peakall, R., & Smouse, P. E. (2006). GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, *6*(1), 288–295. http://doi.org/10.1111/j.1471-8286.2005.01155.x

Pérez-Lloréns, J. L., Vergara, J. J., Olivé, I., Mercado, J. M., Conde-Álvarez, R., Pérez-Ruzafa, Á., & Figueroa, F. L. (2014). Autochthonous seagrasses. In *The Mediterranean Sea* (pp. 137–158). Springer.

Pergent G., Bazairi H., Bianchi C.N., Boudouresque C.F., Buia M.C., Clabaut P., Harmelin-Vivien M., Mateo M.A., Montefalcone M., Morri C., Orfanidis S., Pergent- Mar tini C., Semroud R., Serrano O., V. M. (2012). *Mediterranean Seagrass Meadows : Resilience and Contribution to Climate Change Mitigation,A Short Summary /Les herbiers de Magnoliophytes marines de Méditerranée : résilience et contribution à l'atténuation des changements climatiques, Résumé.*

Perkins, S. E., Alexander, L. V., & Nairn, J. R. (2012). Increasing frequency, intensity and duration of observed global heatwaves and warm spells. *Geophysical Research Letters*, *39*(20), 1–5. http://doi.org/10.1029/2012GL053361

Picotti, V., Negri, A., & Capaccioni, B. (2014). *The Mediterranean Sea-Its history and present challenges*. (S. Goffredo & Z. Dubinsky, Eds.), *The Mediterranean Sea: Its History and Present Challenges*. Springer. http://doi.org/10.1007/978-94-007-6704-1_1

Piry, S., Alapetite, A., Cornuet, J. M., Paetkau, D., Baudouin, L., & Estoup, A. (2004). GENECLASS2: A software for genetic assignment and first-generation migrant detection. *Journal of Heredity*, *95*(6), 536–539. http://doi.org/10.1093/jhered/esh074

Por, F. D. (1978). Lessepsian migration. The influx of Red Sea biota into the Mediterranean by way of the Suez Canal. *Springer Verlag Publ., Berlin*, *3*(3), 1–128. http://doi.org/10.1016/0160-9327(79)90119-4

Procaccini, G., Acunto, S., Famà, P., & Maltagliati, F. (1999). Structural, morphological and genetic variability in Halophila stipulacea (Hydrocharitaceae) populations in the western Mediterranean. *Marine Biology*, *135*(1), 181–189. http://doi.org/10.1007/s002270050615

Procaccini, G., Orsini, L., Ruggiero, M. V, & Scardi, M. (2001). Spatial patterns of genetic diversity in Posidonia oceanica, an endemic Mediterranean seagrass. *Molecular Ecology*, *10*(6), 1413–21. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11412364

Procaccini, G., Ruocco, M., Marín-Guirao, L., Dattolo, E., Brunet, C., D'Esposito, D., … Santos, R. (2017). Depth-specific fluctuations of gene expression and protein abundance modulate the photophysiology in the seagrass Posidonia oceanica. *Scientific Reports*, *7*(September 2016), 42890. http://doi.org/10.1038/srep42890

Procaccini, G., & Waycott, M. (1998). Microsatellite loci identified in the seagrass Posidonia oceanica (L.) Delile. *Journal of Heredity*, *89*(6), 562–568. http://doi.org/10.1093/jhered/89.6.562

Raitsos, D. E., Beaugrand, G., Georgopoulos, D., Zenetos, A., Pancucci-Papadopoulou, A. M., Theocharis, A., & Papathanassiou, E. (2010). Global climate change amplifies the entry of tropical species into the eastern Mediterranean Sea. *Limnology and Oceanography*, *55*(4), 1478–1484. http://doi.org/10.4319/lo.2010.55.4.1478

Rannala, B., & Mountain, J. L. (1997). Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Sciences*, *94*(17), 9197–9201. http://doi.org/10.1073/pnas.94.17.9197

Rasheed, M. A. (2004). Recovery and succession in a multi-species tropical seagrass meadow following experimental disturbance: The role of sexual and asexual reproduction. *Journal of Experimental Marine Biology and Ecology*, *310*(1), 13–45. http://doi.org/10.1016/j.jembe.2004.03.022

Ruelland, E., & Zachowski, A. (2010). How plants sense temperature. *Environmental and Experimental Botany*, *69*(3), 225–232. http://doi.org/10.1016/j.envexpbot.2010.05.011

Ruggiero, M. V., Turk, R., & Procaccini, G. (2002). Genetic identity and homozygosity in North-Adriatic populations of Posidonia oceanica : An ancient , post-glacial clone? *Conservation Genetics*, *3*, 71–74. http://doi.org/10.1023/A

Ruiz-Montoya, L., Lowe, R. J., Van Niel, K. P., & Kendrick, G. A. (2012). The role of hydrodynamics on seed dispersal in seagrasses. *Limnol. Oceanogr.*, *57*(5), 1257–1265. http://doi.org/10.4319/lo.2012.57.5.1257

Sayın, E., Eronat, C., Uçkaç, Ş., & Beşiktepe, Ş. T. (2011). Hydrography of the eastern part of the Aegean Sea during the Eastern Mediterranean Transient (EMT), *88*, 502–515. http://doi.org/10.1016/j.jmarsys.2011.06.005

Selles, B. (2013). Oxidative Stress and Redox Regulation, (October 2015). http://doi.org/10.1007/978-94-007-5787-5

Serra, I. a, Innocenti, a M., Di Maida, G., Calvo, S., Migliaccio, M., Zambianchi, E., … Procaccini, G. (2010). Genetic structure in the Mediterranean seagrass Posidonia oceanica: disentangling past vicariance events from contemporary patterns of gene flow. *Molecular Ecology*, *19*(3), 557–68. http://doi.org/10.1111/j.1365-294X.2009.04462.x

Shaltout, M., & Omstedt, A. (2014). Recent sea surface temperature trends and future scenarios for the Mediterranean Sea. *Oceanologia*, *56*(3), 411–443. http://doi.org/10.5697/oc.56-3.411

Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of Botany*, 1–26. http://doi.org/10.1155/2012/217037

Telesca, L., Belluscio, A., Criscoli, A., Ardizzone, G., Apostolaki, E. T., Fraschetti, S., … Salomidi, M. (2015). Seagrass meadows (Posidonia oceanica) distribution and trajectories of change. *Scientific Reports*, *5*, 12505. http://doi.org/10.1038/srep12505

Terrados, J. (1993). Sexual reproduction and seed banks of Cymodocea nodosa (Ucria) Ascherson meadows on the southeast Mediterranean coast of Spain. *Aquatic Botany*, *46*(3–4), 293–299. http://doi.org/10.1016/0304-3770(93)90009-L

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. *Marine Ecology*, *30*(3), 288–300. http://doi.org/10.1111/j.1439-0485.2009.00285.x

Unsworth, R. K. F., & Cullen-Unsworth, L. C. (2017). Seagrass meadows. *Current Biology*, *27*, R443–R445. http://doi.org/10.1016/j.cub.2017.01.021

Waycott, Michelle, Duarte, C. M., Carruthers, T. J. B., Orth, R. J., Dennison, W. C., Olyarnik, S., Calladine, A., … Williams, S. L. (2009). Accelerating loss of seagrasses across the globe threatens coastal ecosystems. *PNAS*, *106*(30), 12377–12381.

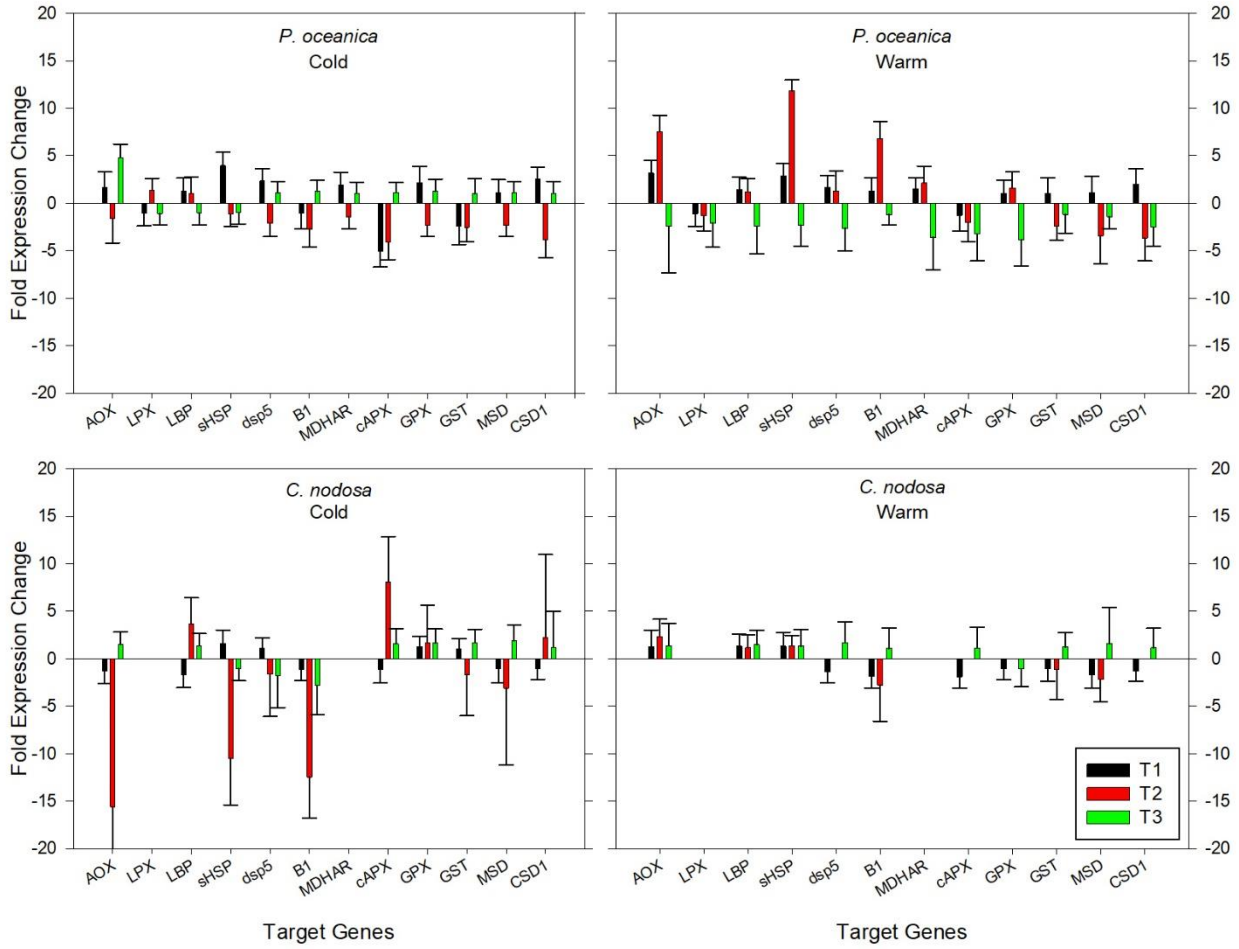
Williams, S. L. (2007). Introduced species in seagrass ecosystems: Status and concerns. *Journal of Experimental Marine Biology and Ecology*, *350*(1–2), 89–110. http://doi.org/10.1016/j.jembe.2007.05.032

York, P. H., Smith, T. M., Coles, R. G., Mckenna, S. A., Connolly, R. M., Irving, A. D., … Whitehead, S. (2017). Identifying knowledge gaps in seagrass research and management : An Australian perspective. *Marine Environmental Research*, *127*, 163–172.

http://doi.org/10.1016/j.marenvres.2016.06.006

	cAPX	GPX	MSD	LBP	GST	sHSP	dsp5	CSD1	AOX	B1	MDHAR	LPX
PC-T1	-5.1	2.2	1.1	1.3	-2.5	4.0	2.4	2.5	1.7	-1.0	1.9	-1.1
PC-T2	-4.1	-2.4	-2.3	1.1	-2.5	-1.2	-2.1	-3.8	-1.6	-2.7	-1.4	1.4
PC-T3	1.1	1.3	1.1	-1.0	1.0	-1.0	1.1	1.1	4.8	1.3	1.0	-1.1
PW-T1	-1.3	1.0	1.1	1.4	1.1	2.9	1.7	2.0	3.2	1.3	1.5	-1.1
PW-T2	-2.0	1.6	-3.4	1.2	-2.4	11.8	1.3	-3.7	7.5	6.8	2.1	-1.3
PW-T3	-3.3	-3.8	-1.4	-2.4	-1.2	-2.3	-2.6	-2.5	-2.4	-1.2	-3.6	-2.1
CW-T1	-1.9	-1.1	-1.7	1.3	-1.1	1.3	-1.4	-1.3	1.3	-1.8		
CW-T2	-56.9	-69.3	-2.2	1.2	-1.2	1.4	-31.1	-183.1	2.3	-2.8		
CW-T3	1.1	-1.1	1.6	1.5	1.3	1.3	1.7	1.2	1.4	1.1		
CC-T1	-1.2	1.3	-1.1	-1.7	1.1	1.6	1.1	-1.0	-1.3	-1.1		
CC-T2	8.1	1.7	-3.1	3.6	-1.7	-10.5	-1.7	2.2	-15.6	-12.5		
CC-T3	1.6	1.7	1.9	1.4	1.7	-1.0	-1.8	1.2	1.5	-2.8		

Appendix 3: Fold expression change values of each gene for both species and thermal origin.



Appendix 4:Relative level of expression (fold expression change) of selected target genes in P. oceanica plants from cold and warm origins and C. nodosa plants from cold and warm origins.

Marine Environmental Research 132 (2017) 94–102

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Antioxidant response to heat stress in seagrasses. A gene expression study

O. Tutar[a,b], L. Marín-Guirao[a,*], J.M. Ruiz[c], G. Procaccini[a]

[a] Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy
[b] Department of Earth and Environmental Sciences, University Milano-Bicocca, Piazza della Scienza, 4-20126 Milano, Italy
[c] Seagrass Ecology Group, Oceanographic Center of Murcia, Spanish Institute of Oceanography, C/ Varadero, 30740 San Pedro del Pinatar, Murcia, Spain

ARTICLE INFO

Keywords:
Seagrass
Temperature
Global change
Oxidative stress
Antioxidant defense
Heat stress
Gene expression

ABSTRACT

Seawater warming associated to the ongoing climate change threatens functioning and survival of keystone coastal benthic species such as seagrasses. Under elevated temperatures, the production of reactive oxygen species (ROS) is increased and plants must activate their antioxidant defense mechanisms to protect themselves from oxidative damage. Here we explore from a molecular perspective the ability of Mediterranean seagrasses to activate heat stress response mechanisms, with particular focus on antioxidants. The level of expression of targeted genes was analyzed in shallow and deep plants of the species *Posidonia oceanica* and in shallow plants of *Cymodocea nodosa* along an acute heat exposure of several days and after recovery. The overall gene expression response of *P. oceanica* was more intense and complete than in *C. nodosa* and reflected a higher oxidative stress level during the experimental heat exposure. The strong activation of genes with chaperone activity (heat shock proteins and a luminal binding protein) just in *P. oceanica* plants, suggested the higher sensitivity of the species to increased temperatures. In spite of the interspecific differences, genes from the superoxide dismutase (SOD) family seem to play a pivotal role in the thermal stress response of Mediterranean seagrasses as previously reported for other marine plant species. Shallow and deep *P. oceanica* ecotypes showed a different timing of response to heat. Shallow plants early responded to heat and after a few days relaxed their response which suggests a successful early metabolic adjustment. The response of deep plants was delayed and their recovery incomplete evidencing a lower resilience to heat in respect to shallow ecotypes. Moreover, shallow ecotypes showed some degree of pre-adaptation to heat as most analyzed genes showed higher constitutive expression levels than in deep ecotypes. The recurrent exposure of shallow plants to elevated summer temperatures has likely endowed them with a higher basal level of antioxidant defense and a faster responsiveness to warming than deep plants. Our findings match with previous physiological studies and supported the idea that warming will differently impact Mediterranean seagrass meadows depending on the species as well as on the depth (i.e. thermal regimen) at which the meadow grows. The increase in the incidence of summer heat waves could therefore produce a significant change in the distribution and composition of Mediterranean seagrass meadows with considerable consequences for the functioning of the whole ecosystem and for the socio-economic services that these ecosystems offer to the riverine populations.

1. Introduction

Human-induced climate changes cause regime shift in the marine environment (Wernberg et al., 2016) and, directly and indirectly, trigger seagrass regression. While summer heatwaves are getting stronger (Garrabou et al., 2009) and more frequent (Perkins et al., 2012), the number of reports relating climate changes to seagrass mortality are increasing. Heat represents one of the most important challenges for marine plants (Repolho et al., 2017) but climate changes do not affect the seagrass ecosystem only altering sea water temperature. Climate changes, in fact, also increase sea level, atmospheric CO_2 and storm frequency (Brierley and Kingsford, 2009) which can combine with other environmental stressors (e.g. eutrophication, sedimentation), exacerbating the pressure on the plants.

Heat stress affects plants by causing protein unfolding, changes in membrane fluidity and disassembly of cytoskeleton (Ruelland and Zachowski, 2010). Plants continuously produce reactive oxygen species (ROS) as a byproduct of different metabolic pathways due to their aerobic nature, but at the same time use them as key regulators (Mittler et al., 2011). Under normal physiological conditions, ROS are detoxified by antioxidants; when plants are under environmental stress, the defense system cannot counterbalance the ROS accumulation and

* Corresponding author.
E-mail address: maringuirao@gmail.com (L. Marín-Guirao).

http://dx.doi.org/10.1016/j.marenvres.2017.10.011
Received 19 July 2017; Received in revised form 16 October 2017; Accepted 22 October 2017
Available online 27 October 2017
0141-1136/ © 2017 Elsevier Ltd. All rights reserved.

oxidative stress occurs. Under oxidative stress, ROS start to give damage to membranes, DNA and protein structure which drives the cell to homeostatic imbalance and eventually death (Sharma et al., 2012).

Studies to heat stress response on model or economically important plant species showed differences in the response, thermotolerance and signaling of different ecotypes (Charng et al., 2007; Swindell et al., 2007; Saidi et al., 2011), with heat affecting photosynthesis (Havaux and Tardy, 1996; Allakhverdiev et al., 2008; Zhao et al., 2014), and inducing oxidative stress (Lin et al., 2006; Volkov et al., 2006; Yin et al., 2008; Larkindale and Knight, 2002; Hawkins et al., 2015).

Oxidative stress might be triggered by various factors, all of which affect gene regulation through different mechanisms (Mittler et al., 2004). Response to oxidative stress is achieved by a complicated cascade of signaling molecules including ROS by themselves in the form of hydrogen peroxide (Hung et al., 2005). The antioxidant system starts to work from signaling molecules, which sense the heat and trigger the defense mechanism (Knight and Knight, 2001; Saidi et al., 2011; Mullineaux et al., 2000). When proteins are damaged, the repairing system acts through unfolded proteins and lipid peroxidation; non-enzymatic and enzymatic antioxidants scavenge the ROS and the enzymes regenerate the active forms of antioxidants. In the case of excessive stress, apoptosis is activated. The glutathione-ascorbate cycle is the center of the oxidative stress response (Foyer and Noctor, 2011).

First concerns about possible effects of global warming on seagrasses date back to the 1990's (Short and Neckles, 1999). Studies both in natural and in controlled conditions of the differential response to heat of putative natural ecotypes, are giving interesting results. Different species and ecotypes distributed along latitudinal and bathymetric gradients showed differences in the photophysiological and molecular response and different transcriptomic resilience to summer heatwaves (e.g. Seddon and Cheshire, 2001; Ehlers et al., 2008; Massa et al., 2009, 2011; Bergmann et al., 2010; Franssen et al., 2011, 2014; Winters et al., 2011; García et al., 2013; Jueterbock et al., 2016; Marín-Guirao et al., 2016, 2017). Genotypic diversity has also been shown to play a role to recover from a heat-shock (Reusch et al., 2005; Ehlers et al., 2008).

A few of these studies report on the specific response to oxidative stress and on the activity of antioxidants in seagrasses. The MnSOD gene has been shown to play and important role in the response to heat stress tolerance in *Zostera marina* (Winters et al., 2011; Liu et al., 2016). Massa et al., 2011 suggest that Glutathione-S-transferase (GST) participates to heat stress response in *Zostera noltii*.

The most abundant seagrass species in the Mediterranean basin are *P. oceanica* and *C. nodosa*. Australian species of the same two genera have been classified as persistent and opportunistic, respectively (Kilminster et al., 2015), and the same can apply to co-generic representatives from the north hemisphere. *C. nodosa* is an opportunistic species, considering its relatively fast shoot turnover rate and time to reach sexual maturity, physiological resistance and response to disturbance (Cancemi et al., 2002). *P. oceanica* is a persistent species with slow shoot turnover (Marbà and Duarte, 1998), long life time (Arnaud-Haond et al., 2012), longer life cycle, and slower ability to recover (Kilminster et al., 2015). The species showed a higher sensitivity to short term exposure to acute heat stress (Marín-Guirao et al., 2016).

In this study, we aimed to analyze the stress response of the seagrasses *P. oceanica* and *C. nodosa* in controlled conditions to enhanced heat. We analyzed the changes in the expression of genes involved in different steps of the stress response, with particular focus to antioxidants. We selected genes coding for proteins involved in the regeneration of glutathione or ascorbate, the ROS scavengers, heat-shock proteins, an apoptosis-related gene, and other generic stress responsive genes. Through our approach, we also aimed to identify target genes that can be utilized as indicators of plant response to heat stress.

2. Material and methods

2.1. Study area, sample collection and experimental design

Results presented in this study have been obtained on the same plant material utilized in Marín-Guirao et al. (2016) and maintained in a mesocosm system with experimental conditions as explained therein. In synthesis, *P. oceanica* and *C. nodosa* samples were collected from a large well preserved meadow in the SE part of Mediterranean Spanish coastline (Isla Grosa; 37°43′N, 00°42′W). *P. oceanica* ramets were collected from −5 m (POS) and −25 m (POD) depth, and *C. nodosa* (CYM) samples were collected from −5 m depth by SCUBA-diving. The plant fragments, which consist of the apical meristem and a large number of connected orthotropic shoots, were transported to the lab and immediately transplanted in the mesocosm system within 2 h after uprooting. Eight *P. oceanica* plant portions from each depth were individually planted in eight independent 120 L tanks without sediment to prevent toxicity caused by heat-enhanced sulfide accumulation (García et al., 2013). *C. nodosa* fragments were divided into portions, each including 3–5 shoots, and randomly planted in plastic trays with washed and dried sediments from the collection site. Eight trays were assembled and randomly allocated in eight independent 120-L experimental tanks. Each tank had their own illumination and temperature control system as previously described in detail (Garrote-Moreno et al., 2015), representing independent replicates.

Plants were acclimated for 10 days under their respective salinity (37.5, Practical Salinity Scale), temperature (24 °C) and irradiance levels (250 ± 20 and 65 ± 5 μmol photons m^{-2} s^{-1} for shallow and deep plants, respectively; photoperiod of 8:16) as determined at the moment of sampling. Temperature and salinity in experimental tanks were accurately maintained constant (± 0.2) and verified on a daily basis with mercury thermometers and a WTW® conductivity meter. The temperature of half of the tanks (four randomly selected tanks for each species or ecotype) was subsequently increased at a rate of 0.5 °C $h^{-1}$to 32 °C. The rate of temperature increase did not reflect natural conditions but was selected to induce a heat shock response on plants. The maximum temperature for the experiment is chosen by considering the projected maximum summer seawater temperature for the Mediterranean Sea by the end of the twenty-first century (IPCC, 2007). For five days, plants were exposed to heat stress and thereafter the temperature was decreased to the initial level to assess the resilience capacity of plants after a five-day recovery period. Water quality was maintained by partial water renewal each 2–3 days.

Leaf samples (i.e. a 4-cm^2 leaf segment from the middle part of the first fully mature leaf) for gene expression analysis were collected from one randomly selected shoot in each experimental tank (n = 8 for each species or ecotype). Tissue sampling was done after 24 h (T1) and 5 d (T2) of heat exposure to characterize early and mid-term response and at the end of the recovery period (T3) to assess plant resilience. Immediately after collection, leaf samples were cleaned of epiphytes and store in RNAlater© stabilization solution (Invitrogen™) at −80 °C until RNA extraction. Apical shoots were not used in our analysis due to their different metabolism in respect to orthotropic shoots (Marbá et al., 2002).

2.2. RNA extraction and cDNA synthesis

RNA was obtained from RNAlater® fixed samples using the BIORAD Aurum™ Total RNA mini kit. During RNA extraction samples were treated with DNase solution, to remove possible DNA contamination. The purity and concentration of the total RNA was checked using NanoDrop (ND-1000 UV–Vis spectrophotometer; NanoDrop Technologies) and 1% agarose gel electrophoresis. Five hundred nanograms of total RNA were reverse transcribed with BIORAD iScript™ cDNA synthesis kit, according to the manufacturer.

Table 1
List of genes of interest analyzed in *P. oceanica* (5 and 25 m depth) and *C. nodosa*.

Category	Abbrev.	Gene full name	Primers sequence 5'- > 3' (F/R)	Reference or Contig name[b]	% Efficiency	Tm
ROS scavengers	GST[a]	Glutathione S-transferase	P: ACCTTCGCTAGCTTCCCTTC/AGATGGCAACAATGGGGATA	P: Lauritano et al., 2015	P: 100	P:58.5/58.6
			C: ATGCCGACTCTCCAGGTCTA/GCTGACGCCTTGCGAGATCA	C: HADH01013300	C: 95	C: 63.8/71,2
	GPX[a]	Glutathione peroxidase	P: TTGGAAAGGGGAAGTTGTTG/CGGGTCAAACCCTGAGATAA	P: Lauritano et al., 2015	P: 100	P: 63.7/63.7
			C: CGTGGGAAAGATGTGAGCTT/GCTCTTGACCACCGAATTGG	C: HADH01002324	C: 100	C:64.2/66.8
	GLUSYN[a]	Glutathione synthase	TAGGTTTGGCCAATTCTTGC/AAGGGGTGGTTCTTCCAGAT	P & C: Lauritano et al., 2015	100	63.8/63.6
	GLURED[a]	Glutathione reductase	P: AGTCCACACCAAATGGAAGC/AAGGGGAGGGAAGGGTTATT	P: Lauritano et al., 2015	P: 100	P:63.8/63.8
			C: TCCTCCAAGCTTAGTGCTTCA/ACACAACCAGACGGTGTCAA	C: Olivé et al., 2017	C: 100	C:63.9/63.8
	MDHAR[a]	Monodehydro ascorbate reductase	P: CTGCTGATTGGAGCTTAGCC/AGGGGATTTTCCTTGAAAGTG	P: Lauritano et al., 2015	P: 96.5	P:63.9/63.2
			C: GAGGGCCTTCGTGTACGTTA/TTTGCTTAATGCAGGACGTT	C: HADH01006111	C: 100	C:63.9/62.2
	CAT[a]	Catalase	CATCACATGCTGGGTTTCAC/ACCGATCCTGGACATCTGAC	P & C: Lauritano et al., 2015	100	63.8/63.7
	FSD	Iron superoxide dismutase	TCATGACTTCTTTTGGGAATCA/CCCAACCAGATCCAAACAGT	P: Lauritano et al., 2015	100	63.3/63.6
	MSD[a]	Manganese superoxide dismutase	P: GGCGGAGGTCATATAAACCA/ATAAGCAAGCCACACCCATC	P: Lauritano et al., 2015	P: 95	P:63.6/63.7
			C: CCGACCTCCCCTACGACTAC/CTTGGACATGGCGCCTTCAA	C: HADH01006348	C: 95	C:64.7/70.5
	APX 3[a]	Ascorbate peroxidase, microsomal	P: TCAGCTTGCTGGAGTTGTTG/CCCATGCGGTAAAAGATGTC	P: Lauritano et al., 2015	P: 97.5	P:63.6/63.7
			C: CTCCGAGGTCTCATCTCCAG/CATTGGCCGCGTGCCTCAGC	C: HADH01005988	C: 99	C:64/77.3
	cAPX[a]	Ascorbate peroxidase,chloroplastic (stromal)	P: GCATGATGCTGGAACGTATG/AATTTTGGGACCTCCAGCTT	P: Lauritano et al., 2015	P: 100	P:63.9/63.7
			C: AAGAGGGGAGGAGCTAATGG/GCTGGCAAGCTGAAACAAGT	C: Olivé et al., 2017	C: 100	C:63.4/64.4
	Prx Q[a]	Peroxiredoxin Q	P: AGGTGTCCAAGGGAGATGTG/TTGTGCGAAGCAGAATCATC	P: Lauritano et al., 2015	P: 100	P:63.8/63.8
			C: ATGATCCTGCTTCCCACAAG/AGGTCTCATCTACATGCTTC	C: HADH01004691	C: 94	C: 64/55.8
Chaperons	LBP[a]	Luminal binding protein	ACCAGAGCTCGGTTTGAAGA/ATTCTGGTGCTTCCACCAAC	P & C: Lauritano et al., 2015	97.5	63.8/63.8
	HSP90[a]	HSP90	CTCCATCTTGCTTCCCTCAG/TCAGTTTGGAGGAACCGAAC	P & C: Lauritano et al., 2015	100	63.7/63.9
	sHSP	Small heatshock protein	ACCGGAGGATGTGAAGATTG/AGCTTGCTGGACAAGGTGAT	P: Lauritano et al., 2015	95	63.7/63.7
Respiration	AOX[a]	Alternative oxidase 1a	P: TGCTGCATTGCAAGTCTCTAC/GTTGTGACACCTCCATGAAGGTC	P: Procaccini et al., 2017	P: 100	P:63/66.8
			C: GTTTCCAAGCCAAGGTGGTA/ACATCCTTAAGCGTGGCATC	C: HADH01001408	C: 92	C:63.8/63.9
Apoptosis	DSP5[a]	Death specific protein 5	P:TCTCAGGTCCGGCACTAATC/GAAAGGCTTGCTCGTATTGC	P: Lauritano et al., 2015	P: 100	P:64/63.8
			C: AGAGGAGAGTGCAGGAGCTG/ACAGCCCTGATAACAACATC	C: HADH01012884	C: 95	C:63.9/59
Lipid peroxidation	LPX	Lipoxygenase	TGTAGCCACCGAAGGGATAC/GGTTGGTGGGATGAGGTAAA	P: Lauritano et al., 2015	100	63.7/63.4

[a] Genes analyzed in both species; P and C: primers utilized for the PCR amplification in *P. oceanica* and *C. nodosa*, respectively.
[b] Contigs obtained from Ruocco et al. (2017) are accessible at the ENA (European Nucleotide Archive) website.

2.3. Target and reference gene selection and primer design

Genes of interest (GOIs) were selected considering the most affected cell compartments, key stress enzymes and proteins (Table 1). More in detail, Glutathione reductase (GLURED), Monodehydroascorbate reductase (MDHAR), Glutathione synthetase (GLUSYN), Glutathione-S-transferase (GST) and two isoforms of Ascorbate peroxidase (APX3 and chloroplastic APX) are ascorbate-glutathione cycle enzymes involved in hydrogen peroxide (H_2O_2) detoxification. Glutathione peroxidase (GPX) is an important enzyme that also contributes to H_2O_2 removal in plants (Rouhier et al., 2008). Superoxide dismutase (SOD), Catalase (CAT) and Peroxiredoxin Q (PrxQ) are ROS scavengers. The former directly converts the superoxide radical (O_2^-) into O_2 and H_2O_2 and the latter two catalyze the decomposition of H_2O_2 into water and oxygen. Two isoforms of SOD, Fe-SOD (FSD) and Mn-SOD (MSD), located at chloroplast and mitochondria respectively, were selected. Alternative oxidase (AOX) is a respiratory protein of the mitochondrial electron transport chain that increases its activity under stress conditions to protect cells from oxidative damage by reducing respiratory ROS formation (Vanlerberghe, 2013). Lipoxygenase (LPX) is an indicator of lipid peroxidation and its activity responds to oxidative damage in plant tissues under temperature stress (Ali et al., 2005). Heat shock proteins (sHSP and HSP90) are stress messengers and chaperones, which repair and protect unfolded proteins. The luminal binding protein (LBP) is also a molecular chaperon that participates in the constitutive function of the endoplasmic reticulum (ER) and protects the cell against stresses (Liu and Howell, 2010). The death-specific protein 5 (dsp5) has a suggested role in the signal transduction of the stress to the cell death machinery (Chung et al., 2008).

Putative reference genes (RGs) were selected on the basis of previous studies conducted with both Mediterranean species under heat stress and other abiotic stresses (e.g. Serra et al., 2012; Dattolo et al., 2014, 2017; Lauritano et al., 2015; Marín-Guirao et al., 2016; Olivé et al., 2017; Ruocco et al., 2017). They included the Eukaryotic initiation factor 4A (eIF4A), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Ubiquitin (UBI), 60S ribosomal protein L23 (L23), Ubiquitin-conjugating enzyme (NTUBC).

All GOIs and RGs considered in the analysis were initially tested for their expression stability in our experimental conditions by using three different algorithms BestKeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004). GAPDH, eIF4A and UBI were identified as the most stable genes for *C. nodosa*, and L23 and NTUBC for *P. oceanica*. For normalization of RT-qPCR results, their average was utilized.

Some of the primers were already available (Table 1). New *C. nodosa* primers were designed on conserved regions of aligned sequences of the target genes, obtained in a recently published transcriptome for the species (Ruocco et al., 2017), selecting amplicons between 100 and 250bp long. Alignments were performed by using BioEdit Software version 7.2.5 (Hall, 1999), primers were designed using Unipro UGENE v1.16.2 (Okonechnikov et al., 2012). Amplification efficiency of the primer pairs was calculated from the slopes of standard curves of the threshold cycle (CT) of five cDNA dilutions using the equation $E = 10^{-1/slope}$. Amplicons of the newly designed primers were sequenced for assessing primers' specificity. Primer's sequences, percent efficiencies (E) and melting temperatures (Tm) of RGs and GOIs are reported in Table 1.

2.4. RT-qPCR

After testing the efficiency of the primers, RT-qPCR reactions were assembled manually and reactions were performed using Fast SYBR® Green Real-Time PCR Master Mix in the ViiA7 384 wells format RT-qPCR machine (Applied Biosystems™). For each RT-qPCR reaction, three replicates per sample were run and three no-template negative controls were included for each primer pair. The RT-qPCR data were analyzed at ViiA7™ Software v1.0 (Applied Biosystems™) to obtain cycles (CT values) for each gene and sample. The CT values were normalized with primer efficiency value for each sample. Subsequently, -ΔCT values were calculated as the negative difference in CT values between RGs and each GOI (-ΔCT = CT (RGs) – CT (GOIs)). Fold expression change values were calculated according to the equation Fold expression change = $\pm 2^{(|(-\Delta CTtreatment)-(-\Delta CTcontrol)|)}$.

2.5. Statistical analysis

Permutational multivariate analysis of variance (PERMANOVA) with all analyzed GOIs ($-\Delta CT$ values) was performed to identify significant differences in the overall gene expression patterns among control and heated plants at the different time points. A two-way PERMANOVA (permutations: 9999) was conducted on the PRIMER 6 & PERMANOVA + software package (Anderson et al., 2008) with 'Treatment' (T, two levels: control vs. heated) and 'Species/ecotypes' (S, three levels: POS, POD and CYM) as fixed factors. Pair-wise comparisons were subsequently run to search for significant differences when any of the factors or their combination displayed significant effects. In addition, a similarity percentage (SIMPER) analysis based on Bray-Curtis similarities was carried out to compare similarities among heated POS, POD and CYM plants in each time point and to identify genes most responsible for differences between groups.

Two-way ANOVA analysis with the same factors was conducted to search for differences in the level of expression of each selected GOIs in each sampling time with STATISTICA 7 software (StatSoft, v. 7.0). Data normality and homocedasticity was previously checked through graphic visualization and Cochran's test, respectively. To explore subsequent pair wise comparisons Newman-Keuls test *post hoc* analyses were performed. Heatmap analysis was performed on RStudio Version 0.99.483 (RStudio Team, 2015) by using package 'ggplot2' version 1.0.

3. Results

The overall response of all GOIs was significantly different among species/ecotypes (CYM, POS and POD) and treatments (control and heated) in the three time points, and almost significant ($p < 0.08$) in the interaction of both factors in T1 and T3 (Table 2). *C. nodosa* plants were significantly different than both *P. oceanica* ecotypes at all time points, while shallow and deep *Posidonia* plants did not show significant differences in any time point (Table 2).

The expression values, relative to the controls, for all the analyzed GOIs and for the two species are shown in Supplementary figure S1 and synthesized in the heatmaps of Fig. 1. The two *Posidonia* ecotypes showed a stronger response in respect to *Cymodocea*, as evident from the intensity of colors (level of expression).

In order to evaluate the general pattern of activation of selected genes, in Fig. 2 we plotted the ratio between the number of upregulated and the number of downregulated genes in the different species and ecotypes, regardless if their expression was significant or not. In the shallow *P. oceanica* plants (POS), fourteen genes were up-regulated at T1, while three genes (GPX, PrxQ, and CAT) were down-regulated (up/down ratio = 4.6; Fig. 2). HSP90 and LBP show the highest expression with average fold change values (± SE) of 4.6 ± 1.4 and 4.7 ± 1.6, respectively. In T2, the up/down ratio was reversed in respect to T1 and only seven genes were still over-expressed (up/down ratio = 0.7), with the small Heat Shock Protein, sHSP, having the highest expression value (4.6 ± 1.2 fold change). At the end of the recovery (T3), the number of overexpressed genes was even lower, with an up/down ratio of 0.55. Most of the genes, including the two HSPs (HSP90 and sHSP), were down-regulated. Only LBP, MDHAR and GLUSYN were constantly over-expressed along the whole experimental phases.

In the deep *P. oceanica* samples (POD), two HSPs (HSP90 and sHSP) were up-regulated in the three experimental phases, and their over-expression was significant in T2 (Fig. 1). The two genes did not show a

Table 2
Results of the two-way PERMANOVA test to assess the effects of species/ecotypes (CYM, POS and POP) and experimental treatments (control and heat stress) on the overall gene expression response of experimental plants after 24 h (T1) and five days (T2) of heat exposure and after five days of heat recovery (T3). PERMANOVA analyses and subsequent pair-wise comparisons for the factors showing significant effects were checked using 9999 permutations.

2-way PERMANOVA						
T1	df	SS	MS	Pseudo-F	P(perm)	*Pair-wise*
Species/ ecotypes (S)	2	705.3	352.65	32.05	< **0.001**	POS = POD ≠ CYM
Treatment (T)	1	46.63	46.63	4.24	< **0.001**	Control ≠ Heat
SxT	2	34.49	17.25	1.57	0.070	
Residual	18	198.05	11.00			
T2						
Species/ ecotypes (S)	2	748.91	374.46	29.33	< **0.001**	POS = POD ≠ CYM
Treatment (T)	1	45.74	45.74	3.58	**0.006**	Control ≠ Heat
SxT	2	38.44	19.22	1.51	0.135	
Residual	18	229.81	12.77			
T3						
Species/ ecotypes (S)	2	730.37	365.18	38.74	< **0.001**	POS = POD ≠ CYM
Treatment (T)	1	32.33	32.33	3.43	< **0.001**	Control ≠ Heat
SxT	2	29.95	14.97	1.59	0.078	
Residual	18	169.69	9.43			

reduction of their activity in the recovery phase, when expression levels were still similar to T1 but lower than in T2. The same was true for FSD (significant in T2) and AOX (not significant). A series of genes not reacting at the beginning of the treatment, resulted over-expressed at the end of the recovery, although only MSD was significant. The ratio between up- and down-expressed genes showed a different pattern to POS plants and progressively increased with time from an up/down ratio of 0.88 in T1, 1.12 in T2 and 1.43 in T3, after the recovery period. At this time, the most highly upregulated gene was the depth-specific protein 5 (dsp5) with 3.49 ± 3.00-fold expression change, but not significantly due to its high variability.

In *C. nodosa* (CYM), HSP90, MSD and cAPX were up-regulated all along the experiment, although only MSD was significant in T2. Four out of the seven genes that were over-expressed during the heat exposure (both in T1 and T2) recovered their expression at the end of the recovery phase. Only GLUSYN was slightly up-regulated in T3 and down-regulated in T1 and T2. The ratio between up- and down-expressed genes was similar to POS plants, in T1 (2.50), but differed in T2 (1.80) and in T3 (1.00), showing a decreasing pattern over time.

The three experimental times cluster in the same way in POS and in CYM (Fig. 1), where the two heat treatments branch in a separate group in respect to the recovery (T3). POD showed a different clustering (i.e. T2 clusters with T3), indicative of a later response and inefficient recovery.

Dissimilarity values, as assessed by a SIMPER analysis (Table 3 and Supplementary Table S1), confirmed the higher similarity in the early response (T1) between POS and CYM (Aver. Dissimilarity = 4.2; Table 3) when both species activated the higher number of genes. *Cymodocea* plants maintained a more active response after few days of exposure, when POD plants activate their late response, reflecting in the lower dissimilarity value between POD and CYM in T2 and T3 (T2, Aver. Dissimilarity = 6.00; T3, Aver. Dissimilarity = 4.13).

The two ROS scavenger genes, cAPX and PrxQ are among the ones with the higher contribution in the dissimilarity between species/ecotypes, with values > 10% in six and four of the nine pairwise comparisons, respectively (Table 3). The respiration gene AOX also appears

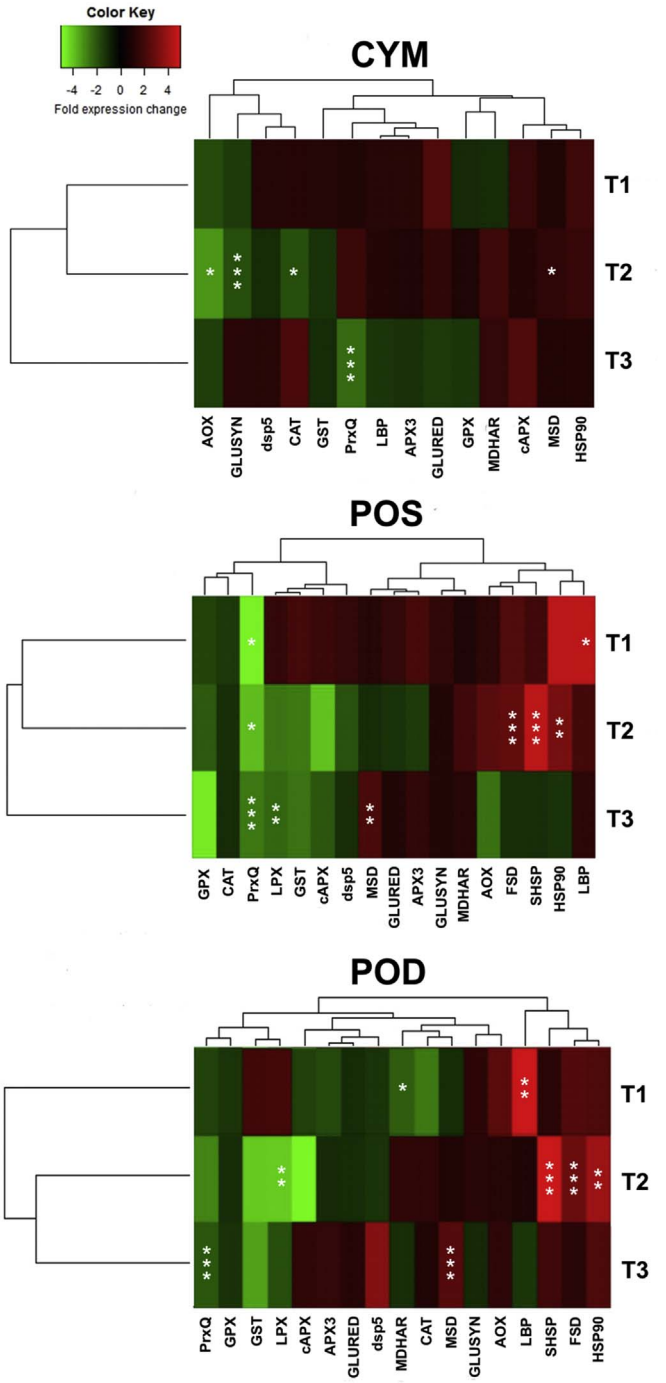


Fig. 1. Heatmaps representing expression levels of target genes in *C. nodosa* -5 m (CYM), *P. oceanica* -5 m (POS) and *P. oceanica* −25 m (POD) after 24 h (T1) and 5 days (T2) of heat exposure and after 5 days (T3) of heat recovery. Asterisks indicate significant differences between control and heated plants: *p < 0.05; **p < 0.01; p < 0.001.

with values > 10% in six comparisons. It was always down-regulated in CYM (p < 0.05 in T2; n.s. in T1 and T3), while it was upregulated in T1 (n.s.) and T2 (n.s.) in POS and in all three time points in POD (always n.s.). Among the two ROS scavenger genes PrxQ and cAPX, PrxQ was one of the two genes always down-regulated in both *Posidonia* ecotypes (POD: p < 0.001 in T1, p < 0.05 in T2, n.s. in T3; POS: n.s. in T1 and T2, p < 0.01 in T3), while it was up-regulated both at T1 (n.s.) and T2 (n.s.) in *Cymodocea*. It is remarkable that PrxQ was significantly down-expressed (p < 0.01) by all species/ecotypes during recovery (Fig. 1; Fig. S2). Reversely, cAPX was always up-regulated but not significant in CYM and was down-regulated, although no

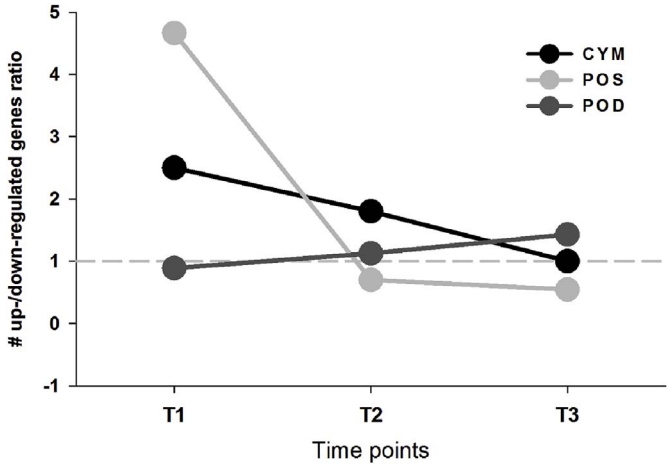


Fig. 2. Ratio between the number of upregulated and the number of downregulated genes in *C. nodosa* -5 m (CYM), *P. oceanica* -5 m (POS) and *P. oceanica* −25 m (POD) after 24 h (T1) and 5 days (T2) of heat exposure and after 5 days (T3) of heat recovery.

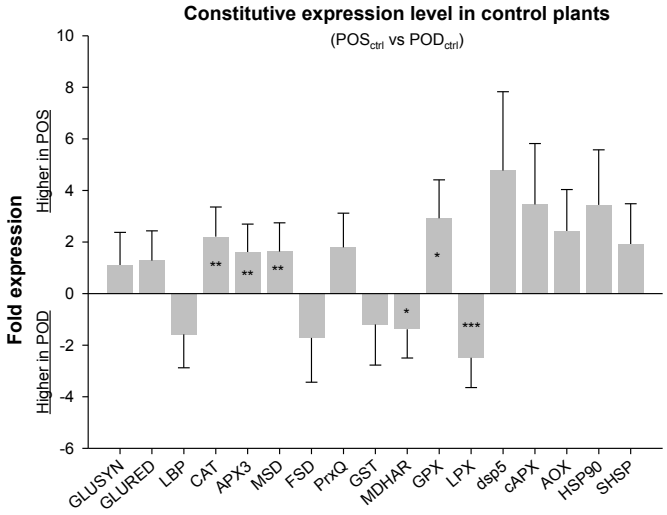


Fig. 3. Expression levels of target genes in POS control plants against POD control plants. Positive and negative expression levels indicate higher constitutive levels in POS and in POD controls, respectively. Bars represent average fold expression values ± standard errors.

significantly, in T1 and T2 and in T2 and T3 in POD and POS, respectively (Fig. 1; Fig. S1).

All heatmaps suggest a close relationship between molecular chaperone HSP90 and one of the SOD family genes (MSD or FSD) which are important ROS scavengers. MSD was the only gene significantly upregulated in CYM during the heat exposure. In both *P. oceanica* ecotypes FSD was significantly upregulated at T2 ($p < 0.001$) and lowered its expression at T3, when MSD was significantly upregulated ($p < 0.01$). At Fig. 1, molecular chaperones LBP, sHSP and HSP90 cluster with FSD in POS and POD. Their upregulation during heat treatment and downregulation at recovery period indicates the activation of an antioxidant response during the exposure. In both *Posidonia* ecotypes LBP shows an early up-regulation at T1 ($p < 0.01$) and decreases its expression in T2, when the other two molecular chaperons are up-regulated (HSP90, $p < 0.01$; sHSP, $p < 0.001$) (Fig. 1; Fig. S2).

POS showed an opposite trend with a strong activation during the first day of heat stress that is relaxed after a few days. The constitutive expression of the selected genes was also compared between the two *P. oceanica* ecotypes. We observed that twelve out of the 17 analyzed genes were upregulated in POS controls with respect to POD controls with an average FC of 2.38 (max = 4.77; Fig. 3). From this set of genes four showed significantly higher transcripts concentrations in POS plants and included the key ROS scavengers CAT, APX3, MSD and GPX. The five genes with higher transcripts concentration in POD plants showed an average expression level 1.48 folds higher than POS levels. From these only monodehydroascorbate reductase (MDHAR) and the indicator of lipid peroxidation (LPX) were significant. This reflects that most of the selected genes, including ROS scavengers and molecular chaperons, have higher constitutive expression levels in plants from shallow depths than in plants growing on deeper meadow areas (Fig. 3).

4. Discussion

In our analysis, we evaluated the expression of antioxidant-defense genes against short-term acute heat stress in two different seagrass species, *Posidonia oceanica* and *Cymodocea nodosa*, and in two *P. oceanica* ecotypes, collected above and below the summer thermocline. Overall, results show differences between the two species, with a more intense response of *P. oceanica*, and also differences between the two ecotypes. Deep *P. oceanica* plants have a delayed response and lower resilience relative to shallow ones, that show higher constitutive expression levels of selected genes.

4.1. Species-level comparison

During the heat stress exposure, the overall gene expression response of *P. oceanica* was more intense and more complete than in *C. nodosa*. This is reflected by the higher number of genes showing notable changes in expression but also by the higher gene expression levels in *P. oceanica*. The latter species activates gene expression during heat stress, with a different response in timing and intensity between the two ecotypes. The stronger activation of the antioxidant defensive system indicates that in *P. oceanica* the experimental temperature caused higher oxidative stress level than in *C. nodosa*. Physiological and demographic studies where the two species were exposed to thermal conditions similar to the ones experienced in our study, confirm the higher tolerance to warming of *C. nodosa* (Olsen et al., 2012; Marín-Guirao et al., 2016). These contrasting responses are associated to the different ecology of the two species. *P. oceanica* is stenohaline and stenothermal and dominates open coastal waters with relatively stable

Table 3
Results of the similarity percentage (SIMPER) analysis assessing the average dissimilarity in the overall gene expression response (ΔΔCT values) among the three species/ecotypes (CYM, POS and POD) at the three-time sampling points (T1, T2 and T3). The three genes most responsible (contribution > 10%) for the similarity in each comparison are also shown.

SIMPER						
Comparison	T1		T2		T3	
	Aver. dissimilarity	Contribution (> 10%)	Aver. dissimilarity	Contribution (> 10%)	Aver. dissimilarity	Contribution (> 10%)
POS vs POD	4.67	PrxQ/APX3/cAPX	6.46	cAPX/dsp5/GPX	4.70	GPX/AOX/cAPX
POS vs CYM	4.20	PrxQ/LBP/AOX	6.93	cAPX/PrxQ/AOX	4.52	cAPX/GPX/AOX
POD vs CYM	4.90	LBP/AOX/CAT	6.00	CAPX/PrxQ/GST	4.13	GST/AOX/dsp5

environmental conditions, while the opportunistic *C. nodosa* also grows in estuaries and coastal lagoons, subjected to wide environmental fluctuations. Similarly, the seagrass *Zostera noltii* showed a weaker and less complete antioxidant response to heat stress than *Z. marina* (Franssen et al., 2014). The former dominates intertidal habitats, where temperature highly fluctuates, whereas the latter dominates subtidal habitats with more stable thermal regimes. The success of a plant acclimative response relies not only on the strength but also on the timing of the response (Bjornson et al., 2016). The antioxidant and HSPs response of the intertidal *Z. noltii* is very fast and tend to stop just few hours after the beginning of a heat shock (Massa et al., 2011). Similarly, it is possible that *C. nodosa* responds much faster and more efficiently to heat than *P. oceanica* although our timing of sampling has not caught its prompt and fleeting response. The higher tolerance to warming of *C. nodosa* could also derive from the evolutionary origin of the species, the only temperate one of a tropical genus (Green and Short, 2003). The higher heat tolerance of *C. nodosa* in respect to *P. oceanica*, could strongly affect species distribution as predicted by future changes in climate scenario. The lower heat-stress level experienced by *C. nodosa* could provide the species with higher resistance and resilience to warming, replacing *P. oceanica* where it can get extinct due to sea water warming. Recent projections predict that *P. oceanica* meadows will reach densities 10% of the present levels by year 2049, considering also the additive effects of anthropogenic pressure (Jordà et al., 2012). The possibility that a more dynamic species, better equipped to face sea water warming, such as *C. nodosa*, can take advantage from the reduction of *P. oceanica* meadows, has to be taken seriously in consideration. Moreover, the competition would be even stronger with invasive seaweed and seagrass species, naturally living in warmer climates.

In spite of the discrepancies in the antioxidant response to heat between *P. oceanica* and *C. nodosa*, SOD-related genes seem to play an active role in the protection of cells from ROS damage during heat stress in both species. SOD is a key enzyme protecting organisms from oxidative damage and the compartmentalization of different forms of the enzyme in plants makes it a very efficient ROS scavenger (Das and Roychoudhury, 2014). Photosynthesis and respiration were identified as particularly affected by heat in plants utilized in our experiment (Marín-Guirao et al., 2016). These two processes are the main ROS producers in chloroplasts and mitochondria. Among the SOD genes considered in the present analysis, the chloroplastic gene FSD and the mitochondrial gene MSD can be considered as potential candidates for early warning detection of heat stress in marine plants. The central role of SOD in the thermal stress response of seagrasses has previously been reported for *Z. marina* (Winters et al., 2011; Liu et al., 2016).

In *P. oceanica*, a luminal binding protein (LBP) and two HSPs (sHSP and HSP90) showed the higher responsiveness, as revealed by high fold changes. LBP is highly and significantly activated early in T1 but it is relaxed in T2 coinciding with a sharp significant activation of HSPs. The small heat shock protein (sHSP) is the one that reached the higher expression levels in our study. This is in agreement with the fact that sHSPs are the most prevalent genes involved in thermal tolerance in terrestrial plants (e.g. Wang et al., 2004), but contrasts with the low responsiveness reported for members of this gene family in the seagrass *Z. marina* (Bergmann et al., 2010). The more intense and the shorter duration of our heat stress exposure relative to that of Bergmann et al. (2010) could be the reason of such differences. The genes showing the higher sensitivity to heat in *Z. marina* were LBP and a HSP, in particular HSP80. These two genes were also the ones contributing most to the differences in the overall stress response among *Z. marina* populations (Bergmann et al., 2010). In our case, however, LBP was also responsible of the early differences in the overall response of the two Mediterranean species (SIMPER analysis, Table 3), but played a minor role in discriminating *P. oceanica* plants from the two depths.

4.2. Ecotype-level comparison

Most of the analyzed target genes (71%) showed higher constitutive expression in shallow than in deep *Posidonia* plants, although differences are not always significant. This may be reflecting a degree of pre-adaptation that confers higher thermal tolerance to plants from shallow areas, since it can serve as a preparative defense against frequent heat stress. Exposure to mild stress levels are known to stimulate higher stress tolerance in plants (e.g. Durrant and Dong, 2004). *P. oceanica* was collected in the late summer when shallow portions of the meadow were previously exposed to higher and more fluctuant temperatures than deep ones, due to the summer water column thermal stratification (Marín-Guirao et al., 2016). It is therefore likely that they have acquired heat stress resistance during summer and hence, that they were pre-adapted to the heat stress experimentally induced here. The front-loaded genes in shallow plants included the two analyzed HSPs (HSP90 and sHSP). Higher constitutive expression levels of HSPs in thermally tolerant populations or individuals is reported in many marine taxa including crustaceans, molluscs, echinoderms and corals (e.g. Barshis et al., 2013; Gleason and Burton, 2015; Dong and Somero, 2008; Dong et al., 2010) and has also been recently described in *P. oceanica* (Marín-Guirao et al., 2017). We also showed higher constitutive expression levels in shallow plants for most of the antioxidant genes, supporting that the shallow portions of the meadow experience oxidative stress more often than the deep portions, in particular during summer. This set includes genes that catalyze the dismutation of the superoxide radical into oxygen and hydrogen peroxide (e.g. MSD) and genes involved in the reduction of hydroperoxides (e.g. CAT, APX, GPX). Curiously, the gene dsp5 involved in the activation of cell apoptotic processes also showed a clear pattern of front-loading in shallow plants, with more than four-fold expression level, although not statistically significant. Death-specific proteins are apparently associated with cell death induced by the accumulation of ROS in marine diatoms under abiotic stress (Chung et al., 2008; Gallina et al., 2015). Higher constitutive expression levels of cell death signaling related genes have also been reported in thermally tolerant coral populations than in more sensitive ones, as a likely response to shift the degree of stress triggering apoptosis (Barshis et al., 2013).

As hypothesized previously for the difference between the two species, a different timing of response to the imposed heat stress was observed between the two *P. oceanica* ecotypes. Marín-Guirao et al. (2016) showed that respiratory rate strongly increases at the beginning of the heating treatment in both ecotypes, but only the shallow plants achieved respiratory homeostasis after a few days at the end of the treatment, while deep plants, instead, do not adjust their respiration during the heat treatment (Marín-Guirao et al., 2016). In general, the early response of shallow plants, just 24 h from the beginning of warming, included the activation of the majority of the selected genes (i.e. 14 genes with at least doubled average expression levels). On that moment, the overall gene expression response of shallow plants was more similar to *C. nodosa* than their co-specific deep plants (SIMPER results). The deep *P. oceanica* plants showed a clear stress warning response late in T2, when they activate the expression of a higher number of antioxidants, and their response became more similar to *C. nodosa*. The deep *P. oceanica* plants activated the higher number of genes in the recovery, when the lower number of genes were still induced in shallow *P. oceanica* plants and in *C. nodosa*. This is again an indication of a lower resilience and reflects the lower thermal tolerance of plants living at depths with colder and more stable thermal environments. Franssen et al. (2011) have demonstrated that the recovery of gene expression patterns after heat stress in the seagrass *Z. marina* is linked to its thermal adaptation. Plants from warmer latitudes recovered their transcriptomic profile and better tolerated stress than plants from colder latitudes (Franssen et al., 2011; Bergmann et al., 2010). Although at the physiological level deep plants seems to have recovered from the acute and short heat exposure (Marín-Guirao et al., 2016), the

expression responses here observed points to an incomplete recovery from the stress level they experienced during the heat exposure.

Species or ecotypes are adapted to the environment where they live and are not always capable to perform well, when environmental conditions change. Dattolo et al. (2017) have shown that shallow and deep *Posidonia* plants are adapted to their natural light environmental conditions and are not able to perform a fully plastic response in crossed environmental conditions. Moreover, deep and shallow portions of the same meadow show a different daily timing in the activation of metabolic pathways, both at the photophysiology, proteomic and transcriptomic levels (Procaccini et al., 2017). Considering that water transparency and temperature along the water column are strongly affected by undergoing climatic changes (Short and Neckles, 1999), the strong adaptation and relatively scarse plasticity of the species is cause of concern. The delayed antioxidant response and lower resilience to transient warming of deep *P. oceanica* ecotypes suggest a higher susceptibility of deep meadows to the effects of global warming in accordance with previous findings (Marín-Guirao et al., 2016). Deep portions of *P. oceanica* meadows are also more sensitive to light reduction along the water column, which is another important threat associated to global warming (Short and Neckles, 1999). It is therefore expected that impact caused by synergistic effects of factors related to global climatic changes will be exacerbated and occur earlier at the lower limits of meadow bathymetric distribution in comparison to shallow meadows.

Temperatures as high as 32 °C are expected to occur with increasing frequency in the Mediterranean over the XXI century as a result of global warming, potentially threatening the distribution and composition of seagrass meadows. *P. oceanica* is at a higher risk of oxidative damage during extreme thermal events and its populations could be replaced in the future by the more tolerant *C. nodosa*, at least at shallow depths where the two species co-exist. Deep *P. oceanica* meadows, more sensitive to drivers of climate change than shallow ones, may be overcompeted by more dynamic and low-light/high-temperature tolerant invasive species, such as the seaweed *Caulerpa cylindracea* and the seagrass *Halophila stipulacea*, both expanding their areal in the Mediterranean. This means that the possible species substitution in the coming decades could result in a loss of the valuable ecosystem services offered by *P. oceanica* meadows.

Acknowledgements

Ozge Tutar is the recipient of a Ph.D. grant from the Italian flagship project RITMARE (MIUR) to the University Milano Bicocca (Italy). This research was funded by the European Union FP7 Project HEATGRASS (Tolerance to heat stress induced by climate change in the seagrass Posidonia oceanica, FP7-PEOPLE-IEF-2013 #624035) and Science and Innovation of the Spanish Government through the project RECCAM (Seagrass Meadows resilience to global warming: an analysis based on responses at ecophysiological, population and ecosystem levels CTM2013-48027-C3-2-R).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2017.10.011.

References

Ali, M.B., Hahn, E.-J., Paek, K.-Y., 2005. Effects of temperature on oxidative stress defense systems, lipid peroxidation and lipoxygenase activity in Phalaenopsis. Plant Physiology Biochem. 43, 213–223.

Allakhverdiev, S.I., Kreslavski, V.D., Klimov, V.V., Los, D.A., Carpentier, R., Mohanty, P., 2008. Heat stress: an overview of molecular responses in photosynthesis. Photosynth. Res. 98, 541–550.

Andersen, C.L., Jensen, J.L., Ørntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245–5250. http://dx.doi.org/10.1158/0008-5472.can-04-0496.

Arnaud-Haond, S., Duarte, C.M., Diaz-Almela, E., Marba, N., Sintes, T., Serrao, E.A., 2012. Implications of extreme life span in clonal organisms: millenary clones in meadows of the threatened seagrass *Posidonia oceanica*. PloS one 7, e30454.

Barshis, D.J., Ladner, J.T., Oliver, T.A., Seneca, F.O., Traylor-Knowles, N., Palumbi, S.R., 2013. Genomic basis for coral resilience to climate change. Proc. Natl. Acad. Sci. 110, 1387–1392.

Bergmann, N., Winters, G., Rauch, G., Eizaguirre, C., Gu, J., Nelle, P., Fricke, B., Reusch, T.B., 2010. Population-specificity of heat stress gene induction in northern and southern eelgrass *Zostera marina* populations under simulated global warming. Mol. Ecol. 19, 2870–2883.

Bjornson, M., Dandekar, A., Dehesh, K., 2016. Determinants of timing and amplitude in the plant general stress response. J. Integr. Plant Biol. 58, 119–126.

Brierley, A.S., Kingsford, M.J., 2009. Impacts of climate change on marine organisms and ecosystems. Curr. Biol. 19, R602–R614.

Cancemi, G., Buia, M.C., Mazzella, L., 2002. Structure and growth dynamics of *Cymodocea nodosa* meadows. Sci. Mar. 66, 365–373.

Charng, Y.-y., Liu, H.-c., Liu, N.-y., Chi, W.-t., Wang, C.-n., Chang, S.-h., Wang, T.-t., 2007. A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in arabidopsis. Plant physiol. 143, 251–262.

Chung, C.C., Hwang, S.P.L., Chang, J., 2008. Nitric oxide as a signaling factor to upregulate the death-specific protein in a marine diatom, *Skeletonema costatum*, during blockage of electron flow in photosynthesis. Appl. Environ. Microbiol. 74, 6521–6527.

Das, K., Roychoudhury, A., 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front. Environ. Sci. 2, 53. http://dx.doi.org/10.3389/fenvs.2014.00053.

Dattolo, E., Marín-Guirao, L., Ruiz, J.M., Procaccini, G., 2017. Long-term acclimation to reciprocal light conditions suggests depth-related selection in the marine foundation species *Posidonia oceanica*. Ecol. Evol. 7, 1148–1164.

Dattolo, E., Ruocco, M., Brunet, C., Lorenti, M., Lauritano, C., D'Esposito, D., De Luca, P., Sanges, R., Mazzuca, S., Procaccini, G., 2014. Response of the seagrass Posidonia oceanica to different light environments: insights from a combined molecular and photo-physiological study. Mar. Environ. Res. 101, 225–236.

Dong, Y.-W., Ji, T.-T., Meng, X.-L., Dong, S.-L., Sun, W.-M., 2010. Difference in thermotolerance between green and red color variants of the Japanese sea cucumber, *Apostichopus japonicus Selenka*: hsp70 and heat-hardening effect. Biol. Bull. 218, 87–94.

Dong, Y., Somero, G.N., 2008. Temperature adaptation of cytosolic malate dehydrogenases of limpets (genus Lottia): differences in stability and function due to minor changes in sequence correlate with biogeographic and vertical distributions. J. Exp. Biol. 212, 169–177.

Durrant, W.E., Dong, X., 2004. Systemic acquired resistance. Annu. Rev. Phytopathology 42, 185–209.

Ehlers, A., Worm, B., Reusch, T.B.H., 2008. Importance of genetic diversity in eelgrass *Zostera marina* for its resilience to global warming. Mar. Ecol. Prog. Ser. 355, 1–7.

Foyer, C.H., Noctor, G., 2011. Ascorbate and glutathione: the heart of the redox hub. Plant physiol. 155, 2–18.

Franssen, S.U., Gu, J., Bergmann, N., Winters, G., Klostermeier, U.C., Rosenstiel, P., Bornberg-Bauer, E., Reusch, T.B., 2011. Transcriptomic resilience to global warming in the seagrass *Zostera marina*, a marine foundation species. Proc. Natl. Acad. Sci. U. S. A. 108, 19276–19281.

Franssen, S.U., Gu, J., Winters, G., Huylmans, A.K., Wienpahl, I., Sparwel, M., Coyer, J.A., Olsen, J.L., Reusch, T.B., Bornberg-Bauer, E., 2014. Genome-wide transcriptomic responses of the seagrasses *Zostera marina* and *Nanozostera noltii* under a simulated heatwave confirm functional types. Mar. genomics 15, 65–73.

Gallina, Alessandra A., Chung, Chih-Ching, Casotti, Raffaella, 2015. Expression of death-related genes and reactive oxygen species production in *Skeletonema tropicum* upon exposure to the polyunsaturated aldehyde octadienal. Adv. Oceanogr. Limnol. 6, 12–19. doi.org/10.4081/aiol.2015.5466.

García, R., Holmer, M., Duarte, C.M., Marbà, N., 2013. Global warming enhances sulphide stress in a key seagrass species (NW Mediterranean). Glob. Change Biol. 19, 3629–3639.

Garrabou, J., Coma, R., Bensoussan, N., Bally, M., ChevaldonnÉ, P., Cigliano, M., Diaz, D., Harmelin, J.G., Gambi, M.C., Kersting, D.K., Ledoux, J.B., Lejeusne, C., Linares, C., Marschal, C., PÉRez, T., Ribes, M., Romano, J.C., Serrano, E., Teixido, N., Torrents, O., Zabala, M., Zuberer, F., Cerrano, C., 2009. Mass mortality in Northwestern Mediterranean rocky benthic communities: effects of the 2003 heat wave. Glob. Change Biol. 15, 1090–1103.

Garrote-Moreno, A., Sandoval-Gil, J., Ruiz, J., Marín-Guirao, L., Bernardeau-Esteller, J., Muñoz, R., Sánchez-Lizaso, J., 2015. Plant water relations and ion homoeostasis of Mediterranean seagrasses (*Posidonia oceanica* and *Cymodocea nodosa*) in response to hypersaline stress. Mar. Biol. 162, 55–68.

Gleason, L.U., Burton, R.S., 2015. RNA-seq reveals regional differences in transcriptome response to heat stress in the marine snail *Chlorostoma funebralis*. Mol. Ecol. 24, 610–627. http://dx.doi.org/10.1111/mec.13047.

Green, E.P., Short, F.T., 2003. World Atlas of Seagrasses. University of California Press, Berkeley, USA.

Hall, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Havaux, M., Tardy, F., 1996. Temperature-dependent adjustment of the thermal stability of photosystem II in vivo: possible involvement of xanthophyll-cycle pigments. Planta 198, 324–333.

Hawkins, T.D., Krueger, T., Wilkinson, S.P., Fisher, P.L., Davy, S.K., 2015. Antioxidant responses to heat and light stress differ with habitat in a common reef coral. Coral

Reefs 34, 1229–1241.
Hung, S., Yu, C., Lin, C.H., 2005. Hydrogen peroxide functions as a stress signal in plants. Botanical Bull. Acad. Sinica 46, 1–10.
IPCC, 2007. Climate Change 2007: the Physical Science Basis. Cambridge University Presshttp://dx.doi.org/10.1017/CBO9781107415324.004.
Jordà, G., Marbà, N., Duarte, C.M., 2012. Mediterranean seagrass vulnerable to regional climate warming. Nat. Clim. Change 2, 821–824. http://dx.doi.org/10.1038/nclimate1533.
Jueterbock, A., Franssen, S.U., Bergmann, N., Gu, J., Coyer, J.A., Reusch, T.B.H., Bornberg-Bauer, E., Olsen, J.L., 2016. Phylogeographic differentiation versus transcriptomic adaptation to warm temperatures in *Zostera marina*, a globally important seagrass. Mol. Ecol. 25, 5396–5411.
Kilminster, K., McMahon, K., Waycott, M., Kendrick, G.A., Scanes, P., McKenzie, L., O'Brien, K.R., Lyons, M., Ferguson, A., Maxwell, P., Glasby, T., Udy, J., 2015. Unravelling complexity in seagrass systems for management: Australia as a microcosm. Sci. Total Environ. 534, 97–109.
Knight, H., Knight, M.R., 2001. Abiotic stress signaling pathways: specificity and cross-talk. Trends plant Sci. 6, 262–267.
Lauritano, C., Ruocco, M., Dattolo, E., Buia, M.C., Silva, J., Santos, R., Olivé, I., Costa, M.M., Procaccini, G., 2015. Response of key stress-related genes of the seagrass *Posidonia oceanica* in the vicinity of submarine volcanic vents. Biogeosciences 12, 4185–4194.
Larkindale, J., Knight, M.R., 2002. Protection against heat stress-induced oxidative damage in arabidopsis involves calcium, abscisic acid, ethylene, and Salicylic acid. Plant physiol. 128, 682–695.
Lin, H., Decuypere, E., Buyse, J., 2006. Acute heat stress induces oxidative stress in broiler chickens. Comp. Biochem. Physiology Part A Mol. Integr. Physiology 144, 11–17.
Liu, J., Tang, X., Wang, Y., Zang, Y., Zhou, B., 2016. A *Zostera marina* manganese superoxide dismutase gene involved in the responses to temperature stress. Gene 575, 718–724.
Liu, J.-X., Howell, S.H., 2010. Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants. Plant Cell 22, 2930–2942.
Marbá, N.r., Hemminga, M.A., Mateo, M.A., Duarte, C.M., Mass, Y.E.M., Terrados, J., Gacia, E.a., 2002. Carbon and nitrogen translocation between seagrass ramets. Mar. Ecol. Prog. Ser. 226, 287–300.
Marbà, N., Duarte, C.M., 1998. Rhizome elongation and seagrass clonal growth. Mar. Ecol. Prog. Ser. 174, 269–280.
Marín-Guirao, L., Ruiz, J.M., Dattolo, E., Garcia-Munoz, R., Procaccini, G., 2016. Physiological and molecular evidence of differential short-term heat tolerance in Mediterranean seagrasses. Sci. Rep. 6, 28615.
Marín-Guirao, L., Entrambasaguas, L., Dattolo, E., Ruiz, J.M., Procaccini, G., 2017. Mechanisms of resistance to intense warming events in an iconic seagrass species. Front. Plant Sci. 8, 1142.
Massa, S.I., Arnaud-Haond, S., Pearson, G.A., Serrão, E.A., 2009. Temperature tolerance and survival of intertidal populations of the seagrass *Zostera noltii* (hornemann) in southern europe (Ria Formosa, Portugal). Hydrobiologia 619, 195–201.
Massa, S.I., Pearson, G.A., Aires, T., Kube, M., Olsen, J.L., Reinhardt, R., Serrao, E.A., Arnaud-Haond, S., 2011. Expressed sequence tags from heat-shocked seagrass *Zostera noltii* (Hornemann) from its southern distribution range. Mar. Genomics 4, 181–188.
Mittler, R., Vanderauwera, S., Gollery, M., Van Breusegem, F., 2004. Reactive oxygen gene network of plants. Trends Plant Sci. 9, 490–498.
Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V.B., Vandepoele, K., Gollery, M., Shulaev, V., Van Breusegem, F., 2011. ROS signaling: the new wave? Trends Plant Sci. 16, 300–309.
Mullineaux, P., Ball, L., Escobar, C., Karpinska, B., Creissen, G., Karpinski, S., 2000. Are diverse signalling pathways integrated in the regulation of arabidopsis antioxidant defence gene expression in response to excess excitation energy? Philos. Trans. Biol. Sci. 355, 1531–1540.
Okonechnikov, K., Golosova, O., Fursov, M., 2012. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics 28, 1166–1167.
Olivé, I., Silva, J., Lauritano, C., Costa, M.M., Ruocco, M., Procaccini, G., Santos, R., 2017. Linking gene expression to productivity to unravel long- and short-term responses of seagrasses exposed to CO2 in volcanic vents. Sci. Rep. 7, 42278. http://dx.doi.org/10.1038/srep42278.
Olsen, Y., Sánchez-Camacho, M., Marbà, N., Duarte, C., 2012. Mediterranean seagrass growth and demography responses to experimental warming. Estuaries Coasts 35, 1205–1213.
Perkins, S.E., Alexander, L.V., Nairn, J.R., 2012. Increasing frequency, intensity and duration of observed global heatwaves and warm spells. Geophys. Res. Lett. 39, 1–5.
Pfaffl, M., Tichopad, A., Prgomet, C., Neuvians, T., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–515. http://dx.doi.org/10.1023/B:BILE.0000019559.84305.47.
Procaccini, G., Ruocco, M., Marín-Guirao, L., Dattolo, E., Brunet, C., D'Esposito, D., Lauritano, C., Mazzuca, S., Serra, I.A., Bernardo, L., Piro, A., Beer, S., Björk, M., Gullström, M., Buapet, P., Rasmusson, L.M., Felisberto, P., Gobert, S., Runcie, J.W., Silva, J., Olivé, I., Costa, M.M., Barrote, I., Santos, R., 2017. Depth-specific fluctuations of gene expression and protein abundance modulate the photophysiology in the seagrass *Posidonia oceanica*. Sci. Rep. 7, 42890.
Repolho, T., Duarte, B., Dionísio, G., Paula, J.R., Lopes, A.R., Rosa, I.C., Grilo, T.F., Caçador, I., Calado, R., Rosa, R., 2017. Seagrass Ecophysiological Performance under Ocean Warming and Acidification, vol. 7. pp. 41443.
Reusch, T.B., Ehlers, A., Hammerli, A., Worm, B., 2005. Ecosystem recovery after climatic extremes enhanced by genotypic diversity. Proc. Natl. Acad. Sci. U. S. A. 102, 2826–2831.
Rouhier, N., Lemaire, S.D., Jacquot, J.-P., 2008. The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation. Annu. Rev. Plant Biol. 59, 143–166.
RStudio Team, 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA. http://www.rstudio.com/.
Ruelland, E., Zachowski, A., 2010. How plants sense temperature. Environ. Exp. Bot. 69, 225–232.
Ruocco, M., Musacchia, F., Olivé, I., Costa, M.M., Barrote, I., Santos, R., Sanges, R., Procaccini, G., Silva, J., 2017. Genome-wide transcriptional reprogramming in the seagrass *Cymodocea nodosa* under experimental ocean acidification. Mol. Ecol. 26, 4241–4259. http://dx.doi.org/10.1111/mec.14204.
Saidi, Y., Finka, A., Goloubinoff, P., 2011. Heat perception and signalling in plants: a tortuous path to thermotolerance. New Phytol. 190, 556–565.
Seddon, S., Cheshire, A.C., 2001. Photosynthetic response of *Amphibolis Antarctica* and *Posidonia australis* to temperature and desiccation using chlorophyll fluorescence. Mar. Ecol. Prog. Ser. 220, 119–130.
Serra, I., Lauritano, C., Dattolo, E., Puoti, A., Nicastro, S., Innocenti, A., Procaccini, G., 2012. Reference genes assessment for the seagrass *Posidonia oceanica* in different salinity, pH and light conditions. Mar. Biol. 159, 1269–1282.
Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot. 1–26. http://dx.doi.org/10.1155/2012/217037. 2012.
Short, F.T., Neckles, H.A., 1999. The effects of global climate change on seagrasses. Aquat. Bot. 63, 169–196.
Swindell, W.R., Huebner, M., Weber, A.P., 2007. Plastic and adaptive gene expression patterns associated with temperature stress in *Arabidopsis thaliana*. Heredity 99, 143–150.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, 1–12. http://dx.doi.org/10.1186/gb-2002-3-7-research0034.
Vanlerberghe, G., 2013. Alternative oxidase: a mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. Int. J. Mol. Sci. 14, 6805.
Volkov, R.A., Panchuk, I.I., Mullineaux, P.M., Schöffl, F., 2006. Heat stress-induced H2O2 is required for effective expression of heat shock genes in Arabidopsis. Plant Mol. Biol. 61, 733–746.
Wang, W., Vinocur, B., Shoseyov, O., Altman, A., 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci. 9, 244–252.
Wernberg, T., Bennett, S., Babcock, R.C., de Bettignies, T., Cure, K., Depczynski, M., Dufois, F., Fromont, J., Fulton, C.J., Hovey, R.K., Harvey, E.S., Holmes, T.H., Kendrick, G.A., Radford, B., Santana-Garcon, J., Saunders, B.J., Smale, D.A., Thomsen, M.S., Tuckett, C.A., Tuya, F., Vanderklift, M.A., Wilson, S., 2016. Climate-driven regime shift of a temperate marine ecosystem. Science 353, 169–172.
Winters, G., Nelle, P., Fricke, B., Rauch, G., Reusch, T.B.H., 2011. Effects of a simulated heat wave on photophysiology and gene expression of high- and low-latitude populations of *Zostera marina*. Mar. Ecol. Prog. Ser. 435, 83–95.
Yin, H., Chen, Q., Yi, M., 2008. Effects of short-term heat stress on oxidative damage and responses of antioxidant system in *Lilium longiflorum*. Plant Growth Regul. 54, 45–54.
Zhao, X.X., Huang, L.K., Zhang, X.Q., Li, Z., Peng, Y., 2014. Effects of heat acclimation on photosynthesis, antioxidant enzyme activities, and gene expression in orchardgrass under heat stress. Molecules 19, 13564–13576.

Supplementary data

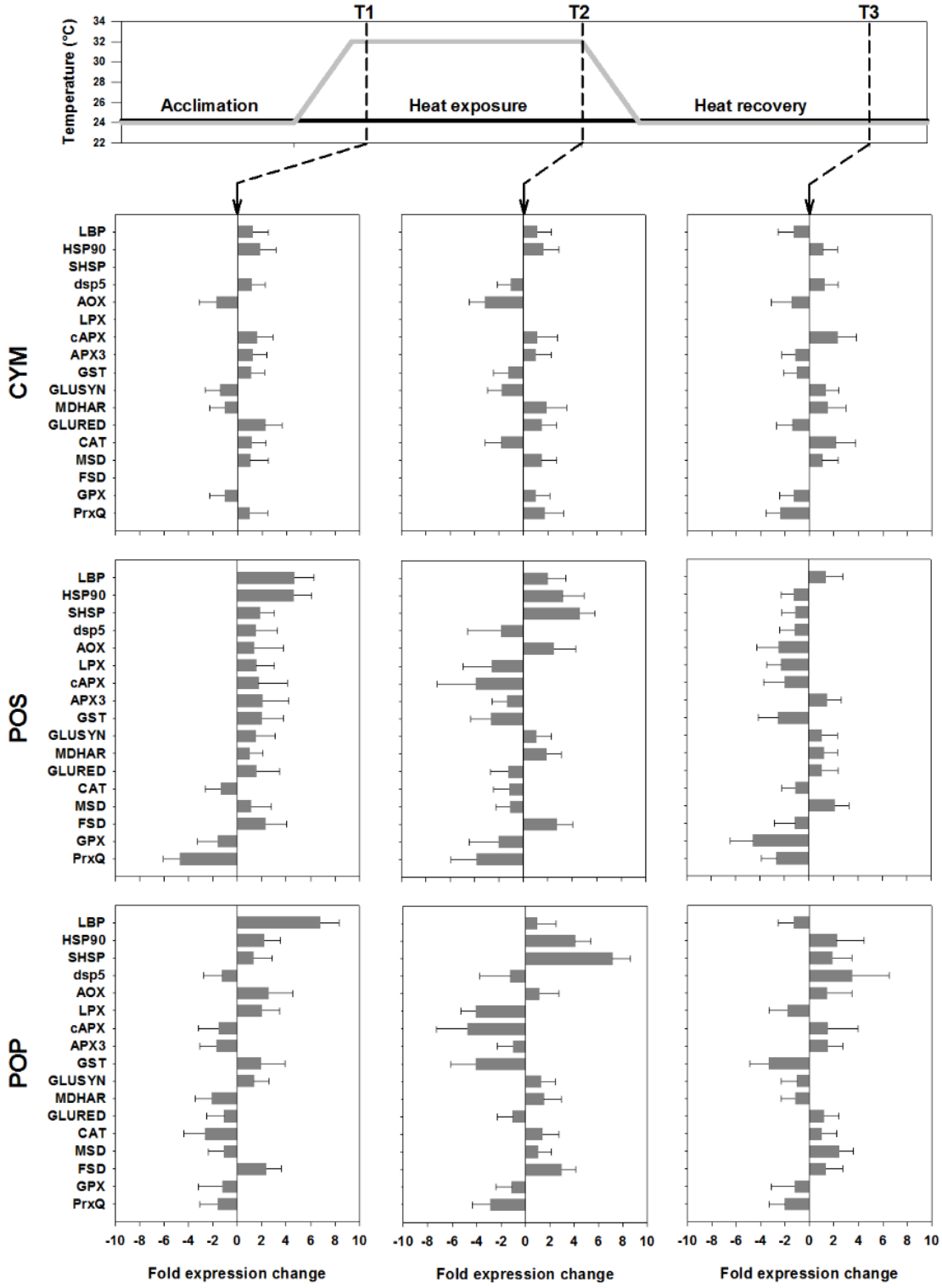


Figure S1. Relative level of expression (fold expression change) of selected target genes in *C. nodosa* -5m (CYM), *P. oceanica* -5m (POS) and *P. oceanica* -25m (POD) along the experiment. Gene expression was quantified at 24 hours (T1) and 5 days (T2) of heat exposure and after 5 days (T3) of heat recovery. For full genes name see Table 1. Bars represent average fold expression changes ± standard error.

Table S1. SIMPER analysis identifying which target genes contribute most strongly towards differences in the response to heat in *C. nodosa* -5m (CYM), *P. oceanica* -5m (POS) and *P. oceanica* -25m (POD) after 24 hours (T1) and 5 days (T2) of heat exposure and after 5 days (T3) of heat recovery.

T1 (24 hours of heat exposure)

Groups POS & POD
Average dissimilarity = 4.67

Species	Group POS Av.Abund	Group POD Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
PrxQ	2.16	2.30	0.58	2.25	12.47	12.47
APX3	2.66	2.28	0.54	4.30	11.55	24.02
cAPX	2.60	2.31	0.51	1.50	10.95	34.97
AOX	2.54	2.71	0.35	1.41	7.46	42.43
GST	2.65	2.62	0.35	1.29	7.40	49.84
MDHAR	2.45	2.22	0.34	2.68	7.20	57.04
CAT	2.36	2.13	0.32	1.57	6.96	64.00
GLURED	2.57	2.42	0.31	1.25	6.62	70.62
dsp5	2.57	2.38	0.28	1.79	6.05	76.67
HSP90	2.87	2.67	0.28	3.92	5.94	82.61
MSD	2.48	2.42	0.23	1.73	4.94	87.55
GPX	2.30	2.38	0.23	1.44	4.92	92.47

Groups POS & CYM
Average dissimilarity = 4.20

Species	Group POS Av.Abund	Group CYM Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
PrxQ	2.16	2.45	0.68	2.26	16.25	16.25
LBP	2.87	2.51	0.51	2.28	12.07	28.32
AOX	2.54	2.28	0.40	1.39	9.47	37.79
HSP90	2.87	2.63	0.34	2.30	8.12	45.90
GLUSYN	2.57	2.34	0.32	1.74	7.60	53.51
cAPX	2.60	2.58	0.31	2.40	7.35	60.86
GST	2.65	2.49	0.30	2.57	7.22	68.08
GLURED	2.57	2.68	0.29	1.35	6.86	74.93
GPX	2.30	2.43	0.25	1.56	6.07	81.00
MSD	2.48	2.46	0.20	1.45	4.81	85.81
APX3	2.66	2.52	0.20	2.77	4.71	90.52

Groups POD & CYM
Average dissimilarity = 4.90

Species	Group POD Av.Abund	Group CYM Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
LBP	2.96	2.51	0.65	2.85	13.28	13.28
AOX	2.71	2.28	0.63	2.55	12.78	26.06

CAT	2.13	2.50	0.52	2.33	10.69	36.75
cAPX	2.31	2.58	0.42	1.36	8.47	45.22
GST	2.62	2.49	0.38	2.19	7.80	53.02
GLURED	2.42	2.68	0.37	1.47	7.52	60.54
APX3	2.28	2.52	0.34	2.44	7.02	67.56
MDHAR	2.22	2.43	0.31	2.19	6.37	73.93
PrxQ	2.30	2.45	0.31	1.55	6.37	80.30
GLUSYN	2.54	2.34	0.30	1.61	6.05	86.35
dsp5	2.38	2.50	0.21	2.04	4.28	90.64

T2 (five days of heat exposure)

Groups POS & POD
Average dissimilarity = 6.46

Species	Group POS Av.Abund	Group POD Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
cAPX	1.80	1.83	1.35	1.32	20.95	20.95
dsp5	2.14	2.31	0.99	0.89	15.25	36.20
GPX	2.16	2.41	0.70	0.98	10.79	46.99
GST	2.13	1.94	0.60	1.09	9.35	56.34
PrxQ	1.96	2.11	0.57	1.06	8.84	65.18
AOX	2.70	2.49	0.48	1.62	7.38	72.56
LBP	2.64	2.45	0.37	1.69	5.69	78.24
HSP90	2.77	2.83	0.27	1.40	4.25	82.49
CAT	2.41	2.55	0.25	1.18	3.94	86.44
MDHAR	2.63	2.56	0.25	1.96	3.81	90.24

Groups POS & CYM
Average dissimilarity = 6.93

Species	Group POS Av.Abund	Group CYM Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
cAPX	1.80	2.47	1.19	0.87	17.22	17.22
PrxQ	1.96	2.61	0.98	1.33	14.20	31.42
AOX	2.70	2.08	0.91	2.31	13.10	44.53
dsp5	2.14	2.43	0.73	0.74	10.59	55.12
GPX	2.16	2.45	0.69	0.93	9.94	65.06
GST	2.13	2.39	0.39	1.58	5.67	70.73
HSP90	2.77	2.59	0.35	1.67	5.00	75.73
GLURED	2.38	2.57	0.32	1.22	4.63	80.36
LBP	2.64	2.48	0.31	2.09	4.48	84.84
GLUSYN	2.47	2.27	0.30	3.08	4.36	89.20
CAT	2.41	2.26	0.25	1.45	3.63	92.83

Groups POD & CYM
Average dissimilarity = 6.00

Species	Group POD Av.Abund	Group CYM Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
cAPX	1.83	2.47	1.08	1.23	18.06	18.06
PrxQ	2.11	2.61	0.74	3.72	12.34	30.40
GST	1.94	2.39	0.73	1.13	12.14	42.55
dsp5	2.31	2.43	0.66	1.06	10.92	53.46
AOX	2.49	2.08	0.60	2.20	10.04	63.51
CAT	2.55	2.26	0.44	1.80	7.28	70.78
GLUSYN	2.52	2.27	0.38	4.77	6.34	77.13
HSP90	2.83	2.59	0.36	1.83	5.95	83.08
MDHAR	2.56	2.63	0.23	2.14	3.80	86.87
GLURED	2.43	2.57	0.21	2.10	3.54	90.42

T3 (five days of heat recovery)

Groups POS & POD
Average dissimilarity = 4.70

Species	Group POS Av.Abund	Group POD Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
GPX	1.90	2.37	0.84	1.31	17.83	17.83
AOX	2.14	2.54	0.71	1.56	15.17	33.00
cAPX	2.22	2.56	0.58	1.39	12.24	45.25
dsp5	2.40	2.79	0.58	4.04	12.24	57.48
HSP90	2.39	2.68	0.42	2.82	8.97	66.46
GST	2.15	2.06	0.31	1.25	6.63	73.09
LBP	2.54	2.37	0.24	0.98	5.20	78.29
GLURED	2.46	2.50	0.20	1.20	4.16	82.46
MDHAR	2.51	2.40	0.17	1.50	3.68	86.13
GLUSYN	2.46	2.44	0.17	1.48	3.67	89.80
PrxQ	2.14	2.23	0.15	1.41	3.10	92.90

Groups POS & CYM
Average dissimilarity = 4.52

Species	Group POS Av.Abund	Group CYM Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
cAPX	2.22	2.69	0.70	1.57	15.43	15.43
GPX	1.90	2.37	0.70	1.02	15.42	30.85
AOX	2.14	2.32	0.51	1.51	11.28	42.13
GST	2.15	2.43	0.43	1.38	9.43	51.56
CAT	2.42	2.67	0.36	2.49	8.02	59.58
MSD	2.66	2.47	0.29	3.21	6.51	66.09
LBP	2.54	2.37	0.26	1.02	5.77	71.86
APX3	2.57	2.40	0.26	2.84	5.68	77.54
GLURED	2.46	2.35	0.25	1.71	5.48	83.02
MDHAR	2.51	2.57	0.19	2.34	4.25	87.27
dsp5	2.40	2.52	0.17	1.71	3.83	91.10

Groups POD & CYM
Average dissimilarity = 4.13

Species	Group POD Av.Abund	Group CYM Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
GST	2.06	2.43	0.54	2.44	13.02	13.02
AOX	2.54	2.32	0.50	1.39	12.16	25.19
dsp5	2.79	2.52	0.40	3.48	9.56	34.74
GPX	2.37	2.37	0.35	1.41	8.49	43.24
MSD	2.70	2.47	0.34	2.94	8.20	51.44
CAT	2.46	2.67	0.30	1.93	7.30	58.74
HSP90	2.68	2.48	0.29	1.85	6.96	65.69
MDHAR	2.40	2.57	0.28	1.85	6.78	72.47
APX3	2.57	2.40	0.25	2.06	6.12	78.59
GLURED	2.50	2.35	0.24	1.62	5.85	84.44
cAPX	2.56	2.69	0.24	1.09	5.72	90.16