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# Response of key stress-related genes of the seagrass *Posidonia oceanica* in the vicinity of submarine volcanic vents

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**Abstract.** Submarine volcanic vents are being used as natural laboratories to assess the effects of increased ocean acidity and carbon dioxide ($CO_2$) concentration on marine organisms and communities. However, in the vicinity of volcanic vents other factors in addition to $CO_2$, which is the main gaseous component of the emissions, may directly or indirectly confound the biota responses to high $CO_2$. Here we used for the first time the expression of antioxidant and stress-related genes of the seagrass *Posidonia oceanica* to assess the stress levels of the species. Our hypothesis is that unknown factors are causing metabolic stress that may confound the putative effects attributed to $CO_2$ enrichment only. We analyzed the expression of 35 antioxidant and stress-related genes of *P. oceanica* in the vicinity of submerged volcanic vents located in the islands of Ischia and Panarea, Italy, and compared them with those from control sites away from the influence of vents. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was used to characterize gene expression patterns.

Fifty-one percent of genes analyzed showed significant expression changes. Metal detoxification genes were mostly down-regulated in relation to controls at both Ischia and Panarea, indicating that *P. oceanica* does not increase the synthesis of heavy metal detoxification proteins in response to the environmental conditions present at the two vents. The up-regulation of genes involved in the free radical detoxification response (e.g., CAPX, SODCP and GR) indicates that, in contrast with Ischia, *P. oceanica* at the Panarea site faces stressors that result in the production of reactive oxygen species, triggering antioxidant responses. In addition, heat shock proteins were also activated at Panarea and not at Ischia. These proteins are activated to adjust stress-accumulated misfolded proteins and prevent their aggregation as a response to some stressors, not necessarily high temperature.

This is the first study analyzing the expression of target genes in marine plants living near natural $CO_2$ vents. Our results call for contention to the general claim of seagrasses as "winners" in a high-$CO_2$ world, based on observations near volcanic vents. Careful consideration of factors that are at play in natural vents sites other than $CO_2$ and acidification is required. This study also constitutes a first step for using stress-related genes as indicators of environmental pressures in a changing ocean.

## 1 Introduction

Seagrass meadows rank amongst the most valuable ecosystems to society in terms of the flow of services and values they support (Costanza et al., 1997; Seitz et al., 2014). They form multidimensional habitats for organisms directly participating in the trophic dynamics (Mazzella et al., 1992) and are a primary food source for herbivores on coral reefs, lagoons, and other shallow habitats (Orth et al., 2006). Seagrasses reduce sediment resuspension and their roots enhance sediment accretion, thus maintaining high water quality. Seagrass ecosystems also represent key sites for carbon storage in the biosphere and are important as $CO_2$ sinks (Mcleod et al., 2011; Fourqurean et al., 2012; Pendleton et al., 2012; Pergent et al., 2012).

There is consensus that increased $CO_2$ will not have negative effects on seagrasses, which have even been predicted to extend their distribution, locally replacing macroalgae (Harley et al., 2006). Nevertheless, there may be indirect effects on seagrasses associated with increased $CO_2$, as loss of phenolic protective substances due to lowered pH (Arnold et al., 2012) and light stress effects due to the modifications in the production and biomass of epiphytic algae on seagrass leaves. Regarding epiphytes, opposing light stress effects may be expected: shading, if non-calcifying epiphytes respond positively to increased $CO_2$ (Martínez-Crego et al., 2014), or high light exposure, if calcifying epiphytes decline (Martin et al., 2008). Experimental evidence for increased seagrass productivity as a response to elevated $CO_2$ levels is also inconclusive, and a recent meta-analysis did not detect significant effects of ocean acidification on seagrass photosynthesis (Kroeker et al., 2010). In a short-term experiment, the seagrass *Zostera marina* was found to grow at increasing rates under $CO_2$ enrichment (Thom, 1996). Similarly, Jiang et al. (2010) found an increase in *Thalassia hemprichii* photosynthesis and leaf growth rate. Nonstructural carbohydrates increased in belowground tissues, whereas in aboveground tissues the carbon content was not affected by $CO_2$ treatments. On the other hand, in a long-term experiment, there was no effect of increasing $CO_2$ levels on the aboveground productivity of *Zostera marina* (Palacios and Zimmerman, 2007), as opposed to belowground. Alexandre et al. (2012) showed that the net photosynthetic rate of *Zostera noltii* was positively affected by the $CO_2$ enrichment of the seawater, but they did not observe an increase in leaf growth rates.

A problem with the above experimental approaches is that even the longest experiments do not allow enough time for marine plants to adapt to high $CO_2$ conditions thus making it difficult to forecast how they will perform in a future high $CO_2$ ocean. This is the main argument to use submarine volcanic vents as natural laboratories for the effects of $CO_2$ as this gas is the main component of emissions and the emissions have been happening for a long time (years to hundreds of years; Hall-Spencer et al., 2008).

In the last decade, gene expression approaches have been frequently used to assess the responses of marine organisms to ocean acidification (e.g., sea urchins (Evans et al., 2013; Evans and Watson-Wynn, 2014), corals (Kaniewska et al., 2012; Moya et al., 2012; Moya et al., 2015) and crustaceans (Harms et al., 2014)) both in field or controlled/mesocosm conditions. Research into natural gene expression variations within species in response to environmental changes is growing (Granados-Cifuentes et al., 2013; Oleksiak et al., 2002) due to its central role in the evolutionary adaptation processes which act at population and at species level (Whitehead, 2006).

A highly promising approach is to investigate the expression of specific genes involved in the response to stress (Granados-Cifuentes et al., 2013; Oleksiak et al., 2002; Whitehead and Crawford, 2006). This approach will provide insight for understanding how marine organisms maintain or re-establish homeostatic metabolism in the face of varying physical or chemical environmental variables (Ahuja et al., 2010). Organisms react to environmental pressure by activating a series of conserved stress enzymes/proteins, including redox sensors (e.g., reactive oxygen species (ROS) sensors, antioxidants and detoxification systems), macromolecule damage sensors (e.g., stress-inducible heat shock proteins – HSPs) and/or condition-specific proteins that help to adjust the cellular physiology and metabolism protecting against cell damage or death.

Here we analyzed the expression levels of selected genes of the seagrass *Posidonia oceanica* in the vicinity of submerged volcanic vents located at the islands of Ischia and Panarea (Italy), and compared them with those from control sites away from the influence of the vents. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was used to characterize expression levels of genes involved in stress responses, antioxidant activity, metal-related responses and defense processes in *P. oceanica* in order to understand whether stress defense mechanisms are activated in the vicinity of submarine volcanic vents. Our hypothesis is that unknown factors are causing metabolic stress in *P. oceanica* in the vicinity of natural $CO_2$ vents, which may confound the putative effects attributed to $CO_2$ enrichment only.

In our analysis, target genes have been selected to include possible mechanisms that *P. oceanica* may activate in face of abiotic stressors (Mittler, 2006). We selected heat shock proteins (HSPs), genes from the primary metabolism (the first and second line of defense), antioxidants and other stress-related enzymes. HSPs are molecular chaperones that can be involved in protein folding/unfolding, and degradation of misfolded or aggregated proteins (Sorensen et al., 2003). They are activated in response to various environmental stress factors (e.g., heat, hypoxia, UV radiation, $CO_2$ enrichment, chemical exposure; Feder and Hofmann, 1999; Lauritano et al., 2015; Sorensen et al., 2003). From the first line of defense we analyzed a multixenobiotic resistance transporter or ATP-binding cassette protein (MXR/ABC), which is involved in the efflux of a large number of structurally and functionally diverse, moderately hydrophobic compounds, including anthropogenic pollutants and natural toxins (Bard, 2000). The second line of defense is characterized by detoxification reactions, such as oxidation, reduction, hydrolysis, hydration and de-halogenation of compounds to detoxify (e.g., cytochrome P450, or CYP450; Regoli and Giuliani, 2014). The second line of defense also includes aldehyde dehydrogenases (ALDHs) that detoxify a wide variety of endogenously produced and exogenous aldehydes catalyzing their oxidation to the corresponding acids (Marchitti et al., 2008). Reactive oxygen intermediates (ROIs), such as the superoxide anion, hydrogen peroxide and the hydroxyl radical, are intermediates of many physiological enzymatic reactions

(e.g., mitochondrial respiration and redox enzymes, such as uncoupled nitric oxide synthase, cytochrome P450 isoforms, Lubos et al., 2011). They function as a signal for the activation of stress responses and are rapidly converted to less reactive forms. They are produced in higher amounts under stress conditions, such as drought stress, desiccation, heat shock, heavy metals, air pollutants, nutrient deprivation, mechanical stress and high light stress (Mittler, 2002). The accumulation of high quantities of ROIs can be very damaging to DNA, RNA and proteins and may activate programmed cell death (PDC). The free radical detoxification enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and scavenger molecules such as glutathione were analyzed here.

The over-accumulation of ROIs when antioxidant/defense systems are not able to cope with stress may induce lipid peroxidation and PDC (Mittler, 2002). This is the reason why we also analyzed the expression of a lipoxygenase (LPX) involved in lipid peroxidation and a death-specific protein (DSP5) involved in cell death (Bidle and Bender, 2008). Other enzymes analyzed here have also antioxidant/protective properties. This is the case of Peroxiredoxin Q, involved in free radical detoxification processes, and germin-like proteins, involved in many different processes such as metal stress response, fungal attack, osmotic regulation, cell wall restructuring and superoxide dismutase activities (Carter and Thombur, 1999; Lamkemeyer et al., 2006). In addition, other proteins/enzymes have been selected for their involvement in heavy metal responses/detoxification (Hussain et al., 2004; Ricachenevsky et al., 2013).

*P. oceanica* is endemic to the Mediterranean, providing a fundamental structural role and key ecological services (Cullen-Unsworth et al., 2014). It is therefore essential to understand which kinds of stresses the species are susceptible to and how it responds to them. This is the first study analyzing key protein activation in this species representing the first step for using stress-related genes of seagrasses as indicators of environmental pressures in a changing ocean.

## 2 Methods

### 2.1 Sampling

The study has been performed in the vicinity of submarine volcanic vents at the islands of Ischia and Panarea, Tyrrhenian Sea, Italy. In both cases, the hydrothermal vents are characterized by the emission into sea water of thermal waters and gases, mainly $CO_2$, inducing changes in the chemical composition of the water column and associated community (Italiano and Nuccio, 1991; Kerrison et al., 2011).

*Ischia*: The study was performed in a very small fringe of a *Posidonia oceanica* meadow close to vent areas off the Castello Aragonese isle (Ischia; 40°43.849′ N, 13°57.089′ E; Naples, Italy). At this site, underwater $CO_2$ vents occur in the shallowest rocky bottoms, and a pH gradient is formed (Hall-Spencer et al., 2008). Archaeological evidence suggests that vent sites around Castello Aragonese in Ischia were above sea level in the fourth century BC, but that the region underwent a tectonic lowering (bradyseism) and was flooded by about AD 130–150 (de Alteriis and Toscano, 2003; Zucco, 2003). Thus, at these sites, subsurface vent activity can be dated back to about 1800–1900 years (Lombardi et al., 2011). Three individual shoots of *P. oceanica* were randomly collected at a control site at ambient pH in Ischia (S1, about 8.14 pH) and at a site of low pH (S2, about 7.83 pH) in dense and continuous meadows. Three additional shoots were collected from a very isolated and confined site (about 10 $m^2$) of *P. oceanica* in conditions of extremely low pH (S3, about 6.57 pH). The depth range varies from 3.5 to 1 m along the pH gradient.

*Panarea*: The $CO_2$ vents of Panarea originated from recent volcanic activity that occurred in 2002, which resulted in a series of gas bursts (Tassi et al., 2009). Sampling was conducted at two separate sites off the Island (38°38′00″ N, 15°04′00″ E) – a control site with pH 8.17 (the islet of Bottaro) and a relatively acidified site with pH 7.91 (Formiche shoals), both at 12 m depth. At each sampling site, six adult shoots of *P. oceanica* were collected.

For both sites, tissue from the youngest fully mature leaves of the shoots (usually the second-rank leaf) was collected and rapidly cleaned from epiphytes with a razor blade, towel-dried and immediately stored in RNAlater® tissue collection solution (Ambion, Life Technologies). Samples were then transported to the laboratory, preserved one night at 4 °C and stored at −20 °C until RNA extraction.

### 2.2 Sample genotyping

Samples collected for RT-qPCR analysis were also genotyped using species-specific microsatellite markers. About 50–70 mg of dried tissue from individual samples was ground in a Mixer Mill MM300 (Qiagen). Subsequent DNA extraction was carried out using the NucleoSpin® 96 Plant II kit (Macherey-Nagel) as in Tomasello et al. (2009). Individual multilocus genotypes were assessed by a total of 29 microsatellites (SSRs): 13 *P. oceanica*-specific anonymous loci that are putatively neutral and widely employed to assess neutral genetic variation (e.g., Procaccini and Waycott, 1998; Alberto et al., 2003; Migliaccio et al., 2005; Serra et al., 2010) and 16 loci representing a subset of the new EST-linked microsatellites developed from two existing *P. oceanica* EST libraries (Arranz et al., 2013). PCR conditions were designed based on Arranz et al. (2013). Multiplex amplification reactions were performed using multiplex PCR buffer (Qiagen Multiplex PCR Master Mix).

PCR products were analyzed on an automated capillary electrophoresis sequencer (3730 DNA analyzer, Applied Biosystems). Electropherogram profiles were visualized and analyzed using the software PeakScanner (Applied Biosys-

tems). Individual multilocus genotypes were determined using the software Gimlet (Valière, 2002).

### 2.3 RNA extraction and cDNA synthesis

Portions of seagrass leaf tissue were ground into a fine powder using a mortar and pestle and liquid nitrogen. About 100 mg of powered tissue was used for the RNA extraction using an Aurum™ Total RNA Mini Kit (BIO-RAD) as in Mazzuca et al. (2013). After lysis solution, samples were homogenized using a Qiagen TissueLyser and tungsten carbide beads (3 mm) (Qiagen) for 3 min at 20.1 Hz. RNA quantity was assured by a NanoDrop ND-1000 UV–visible spectrophotometer (NanoDrop Technologies), monitoring the absorbance at 260 nm; purity was determined by monitoring the 260 / 280 nm and 260 / 230 nm ratios using the same instrument. Both ratios were about 2.0. All samples were free of protein and organic solvents used during RNA extraction. RNA quality was evaluated by agarose gel electrophoresis that showed intact RNA, with sharp ribosomal bands. Total RNA (500 ng) was retro-transcribed into cDNA with the iScript™ cDNA synthesis kit (BIO-RAD) following the standard protocol, using the GeneAmp PCR System 9700 (Perkin Elmer). The reaction was carried out in 20 µL final volume with 4 µL 5× iScript reaction mix, 1 µL iScript reverse transcriptase and DNase-free $H_2O$. The mix was first incubated for 5 min at 25 °C, followed by 30 min at 42 °C and finally heated to 85 °C for 5 min.

### 2.4 Oligo design and PCR (polymerase chain reaction) optimization

Primers for genes of interest (GOI) were designed considering sequences from the seagrass EST database Dr. Zompo (Wissler et al., 2009), unpublished sequences from the transcriptome of *P. oceanica* (D'Esposito et al., 2015) or from the generic online database GenBank (http://www.ncbi.nlm.nih.gov/genbank/; Table 2). Primers were designed using the software Primer3 v0.4.0 (http://frodo.wi.mit.edu/primer3/). Table 1 lists selected GOI, their functions, primers' sequences and amplicon sizes. Primers were optimized as in Serra et al. (2012). The sequences are deposited in GenBank under the accession numbers shown in Table 1.

### 2.5 Best reference gene (RG) assessment

In order to analyze the expression levels of specific GOI, a panel of seven putative reference genes (RGs) was first screened to find the most stable genes in the seagrass *P. oceanica* at both natural $CO_2$-enriched sampling sites. The screened panel included the eukaryotic initiation factor-4A (eIF4A) (F 5'-TTCTGCAAGGGTCTTGACGT-3' and R 5'-TCACACCCAAGTAGTCACCAAG-3'; $E = 1.85$; $R^2 = 0.99$) as well as the ones already published in Serra et al. (2012): ubiquitin (UBI), ribosomal protein L23 (L23), elongation factor 1-alpha (EF1A), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal RNA 18S (18S) and ubiquitin-conjugating enzyme (NTUBC2). Three different algorithms were utilized to identify the best RGs in our experimental design: BestKeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004).

### 2.6 Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Expression level analyses were then performed for specific GOIs related to antioxidant activity, stress and detoxification processes (Table 1 and Table 1S). Primer efficiencies were calculated for each oligo pair generating standard curves with five dilution points by using the cycle threshold (Ct) value versus the logarithm of each dilution factor and using the equation $E = 10^{-1/\text{slope}}$. RT-qPCR was performed as in Dattolo et al. (2014). Control sites (S1 pH 8.14 and 8.17, for Ischia and Panarea, respectively) were used as reference conditions. Statistical analyses were performed using the statistical software Prism v4.00 (GraphPad Software). Statistical significant gene regulation was considered at $p < 0.05$.

## 3 Results

### 3.1 Best reference gene (RG) assessment for Ischia and Panarea

According to the mathematical approach of BestKeeper, the most stable genes were L23, GAPDH and UBI for Ischia and 18S, L23 and UBI for Panarea (Figs. S1a and S2a in the Supplement). NormFinder indicated elF4a, NTUBC2 and UBI for Ischia and L23, NTUBC2 and EF1A for Panarea as best candidate reference genes (Figs. S1b and S2b in the Supplement). According to geNorm analysis, the two most stable genes were eIF4A and NTUBC2 in Ischia (Fig. S1c in the Supplement) and L23 and UBI in Panarea (Fig. S2c in the Supplement). All these genes were below the threshold M value of 1.5, which indicates that a gene can be considered suitable as a RG (Figs. S1c and S2c in the Supplement). The approach implemented in geNorm also allowed inferring the minimum number of necessary genes to be used as RGs in given data set. Pair-wise variation values were always < 0.15 at both sampling sites ($V$ value; Figs. S1d and S2d in the Supplement), indicating that only two genes were sufficient for the analysis. Nevertheless, when results were not consistent among the different approaches utilized, we also included a third RG in the analysis. The best RGs identified for each statistical approach and utilized for normalizing GOI expression levels at the two sampling sites were L23, elF4a and NTUBC2 in Ischia and L23, 18S and UBI in Panarea (Table 2).

**Table 1.** List of selected genes of interest, with their abbreviations and functions.

| Abbreviation | Gene name | Function |
|---|---|---|
| HSP90 | HSP90 | Stress protein |
| DNAJ | Chaperone protein DNAJ | Stress protein |
| HSP83 | HSP83 | Stress protein |
| OzSP | Ozone stress protein | Stress protein |
| DehSP | Dehydra stress protein | Stress protein |
| SHSP | SH stress protein | Stress protein |
| HSFA5 | Heat shock factor A5 | Heat shock protein transcription factor |
| LBP | Luminal binding protein LBP | Stress protein |
| ABC | ABC_MDH | Transporter protein |
| CYP | Cytochrome P450 | Primary metabolism/detoxification |
| ALDH | Aldehyde dehydrogenase | Primary metabolism/detoxification |
| CAT | Catalase | Free radical detoxification |
| SODCP | Superoxide dismutase [Cu–Zn], chloroplastic | Free radical detoxification |
| CSD1 | Cu–Zn superoxide dismutase, cytosolic | Free radical detoxification |
| FSD | Chloroplast iron superoxide dismutase | Free radical detoxification |
| MSD | Manganese superoxide dismutase | Free radical detoxification |
| GST | Glutathione S-transferase | Antioxidants |
| GPX | Glutathione peroxidase | Antioxidants |
| GSH-S | Glutathione synthase | Antioxidants |
| GR | Glutathione reductase | Antioxidants |
| AR | Ascorbate reductase | Antioxidants |
| APX | Ascorbate peroxidase, microsomal | Antioxidants |
| CAPX | Ascorbate peroxidase, chloroplastic (stromal) | Antioxidants |
| Prx Q | Peroxiredoxin Q | Antioxidants |
| GLP | Germin-like protein | Antioxidants |
| DSP5 | Death-specific protein 5 | Apoptosis |
| LPX | Lipooxygenase | Lipid metabolism |
| FtsH2 | ATP-dependent zinc metalloprotease | Metal-related gene |
| HMA | Heavy metal transport detoxification domain | Heavy metal domain |
| NRAMP1 | Root-specific metal transporter | Heavy metal transporter |
| HMATPase | Heavy metal p-type ATPase | Heavy metal ATPase |
| HMATPase5 | Heavy metal ATPase 5 protein gene | Heavy metal ATPase |
| MT3 | Metallothionein-3 | Heavy metal stress response |
| Fe-SP | Iron-stress-related protein | Heavy metal stress response |
| MTP | Metal tolerance protein | Heavy-metal-related gene |

### 3.2 Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

*P. oceanica* samples collected for gene expression analyses were previously genotyped using microsatellite markers, assuring that there were at least three distinct genotypes for each gene expression replicate. Results obtained from all distinct genotypes, using the site with normal pH as control, show that a different gene category or specific gene functions have different behavior at the two sampling sites.

Opposite patterns of expression levels between the two sites were observed for many HSPs (Fig. 1a). At Ischia, many HSPs were significantly down-regulated. In particular, HSP90, HSP83 and the transcription factor HSFA5 were 2-fold down-regulated at both site S2 and S3 ($p < 0.001$), while DNAJ was significantly down-regulated only at S3 ($p < 0.001$). However, HSP83 ($p < 0.05$) and DehSP ($p < 0.01$) were significantly up-regulated at the Panarea site (Fig. 1a). The other HSPs did not show significant changes.

For the primary metabolism genes, ABC and CYP were significantly up-regulated at the Panarea site ($p < 0.01$ for both), while CYP was down-regulated only at the Ischia S2 site ($p < 0.01$). ABC did not show significant expression level changes in Ischia, and ALDH did not show significants changes at both Ischia and Panarea (Fig. 1b).

Regarding genes involved in the antioxidant response (Fig. 2a), CAT did not show significant changes in either Ischia or Panarea, while among the SOD isoforms analyzed (SODCP, CSD1, FSD and MSD), only the Cu–Zn chloroplastic one (SODCP) was down-regulated at the Ischia S3 site ($p < 0.001$) and up-regulated at Panarea ($p < 0.01$). For the glutathione-related enzymes (GST, GPX, GSH-S and GR), GST was significantly down-regulated only at the Ischia S3 site ($p < 0.001$), GPX was up-regulated at all sites

**Table 2.** Best reference genes as given by BestKeeper, NormFinder and geNorm analyses, for each sampling location (Ischia and Panarea). Genes are ranked from the most stable (in bold) to the least stable.

| Rank | BestKeeper | NormFinder | geNorm |
|---|---|---|---|
| Ischia (S2 and S3) | | | |
| 1 | **L23** | **elF4a** | **elF4a-NTUBC2** |
| 2 | GAPDH | NTUBC2 | UBI |
| 3 | UBI/elF4a/NTUBC2 | EF1A | EF1A |
| 4 | EF1A | UBI | GAPDH |
| 5 | 18S | L23 | L23 |
| 6 | | GAPDH | 18S |
| 7 | | 18S | |
| Panarea | | | |
| 1 | **18S** | **L23** | **L23/UBI** |
| 2 | L23 | NTUBC2 | NTUBC2 |
| 3 | UBI/NTUBC2 | EF1A/UBI | 18S |
| 4 | elF4A | 18S | EF1A |
| 5 | GAPDH | elF4A | elF4A |
| 6 | EF1A | GAPDH | GAPDH |

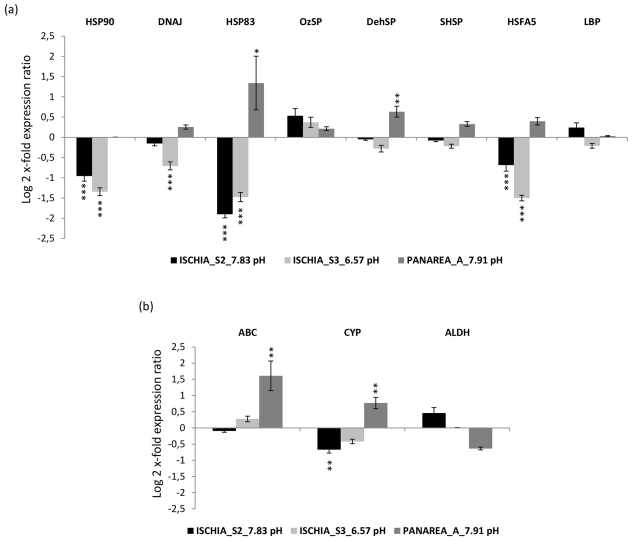


**Figure 1.** Expression levels of *P. oceanica* heat shock protein **(a)** and primary metabolism genes **(b)** from plants collected at Ischia (S2 and S3 sites, with pH 7.83 and 6.57, respectively) in relation to the control site (pH 8.14), and at Panarea (pH 7.91) in relation to its control site (pH 8.17).

($p < 0.05$ for Ischia S2, $p < 0.01$ for Ischia S3 and Panarea), GSH-S did not show significant variations, and GR was down-regulated at both Ischia sites ($p < 0.001$ for both) and up-regulated at the Panarea site ($p < 0.01$). Regarding the ascorbate-related enzymes (AR, APX3 and CAPX), AR did not show significant changes, APX3 was only significantly down-regulated at the Ischia S2 site ($p < 0.001$), and CAPX was down-regulated at the Ischia S2 site ($p < 0.001$) and up-regulated at Panarea ($p < 0.01$). Finally, Prx Q was up-

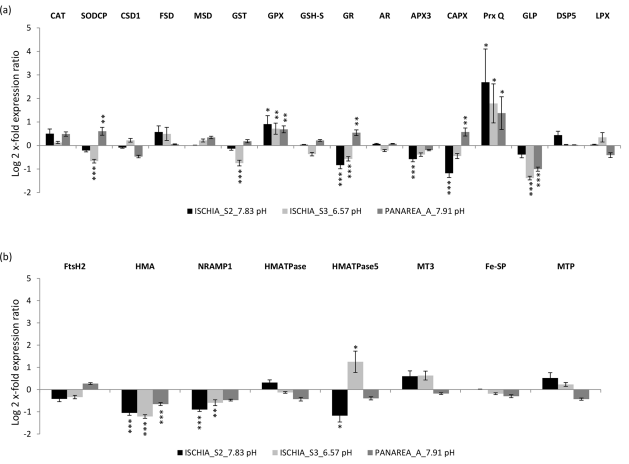


**Figure 2.** Expression levels of *P. oceanica* antioxidant **(a)** and heavy-metal-related genes **(b)** from plants collected at Ischia (S2 and S3 sites with pH 7.83 and 6.57) in relation to the control site (pH 8.14), and at Panarea (pH 7.91) in relation to its control site (pH 8.17). Control sites are represented by $x$ axis.

regulated at all the sites ($p < 0.05$), while GLP was down-regulated at both the Ischia S3 site and the Panarea site ($p < 0.001$). DSP5 and LPX did not change significantly.

For the metal-related genes (Fig. 2b), HMA was down-expressed at both Ischia and Panarea ($p < 0.001$) and NRAMP1 only at the Ischia sites ($p < 0.001$ for S2 and $p < 0.01$ for S3), while HMATPase5 was down-regulated at S2 and up-regulated at S3 ($p < 0.05$ for both). The other genes did not change significantly.

## 4 Discussion

To our knowledge, there are no published data on gene expression patterns in seagrasses in the vicinity of submarine volcanic vents. Here we analyzed the expression of 35 genes of the Mediterranean engineering seagrass species *Posidonia oceanica*, in high-$CO_2$, low-pH sites, in relation to control sites. Genes involved in different phases of plant response to stress were selected. Fifty-one percent of genes analyzed in this study showed significant expression changes at either the two sites of Ischia, at Panarea, or at both locations (summarized in Fig. 2). A consistent gene response at the three sites was observed for three genes – heavy-metal-associated (HMA) domain, glutathione peroxidase (GPX) and peroxiredoxin Q (Prx Q). HMA was significantly down-regulated at both Ischia and Panarea, showing that plants do not increase the synthesis of heavy metal detoxification proteins in proximity to volcanic emissions compared to the control site. This was further supported by the consistent pattern observed at both Ischia and Panarea of the down-regulation of most metal detoxification genes examined, suggesting that the putative heavy metal emissions from the vents at Ischia

and Panarea do not cause stress on *P. oceanica* plants. The bioavailability of heavy metals, which depends on pH and redox potential, may be low at the sites where plants grow, as Vizzini et al. (2013) pointed out for the volcanic vents of the island of Vulcano, Italy.

Glutathione peroxidase (GPX) and Peroxiredoxin Q (Prx Q), involved in free radical detoxification, were significantly up-regulated at both sites in Ischia and Panarea in relation to control sites, suggesting that *P. oceanica* plants are activating similar antioxidant protective mechanisms. Peroxiredoxins are ubiquitous thioredoxin- or glutaredoxin-dependent peroxidases, the function of which is to destroy peroxides (Rouhier et al., 2004), while GPX is important for reducing cytotoxic hydroperoxides (Lubos et al., 2011). In contrast, the activity of another antioxidant gene, the germin-like protein (GLP; Gucciardo et al., 2007), was down-regulated at both sites in Ischia (although in S2, pH 7.83, it was not significant) as well as in Panarea, indicating that this antioxidant defence system was not active in plants at the vicinity of vents.

Many contrasting patterns in the expression of the studied genes were observed between Ischia and Panarea, indicating that different environmental stressors are at play. Fourteen out of the 18 genes exhibiting significant expression changes were different between Panarea and Ischia. Unlike in the control, plants collected in the Panarea acidified site activated antioxidant enzymes such as SODCP, GR, CAPX, and detoxification proteins, such as CYP and ABC. Moreover, and by contrast with the Panarea plants, for the plants collected in the Ischia acidified sites these enzymes were down-expressed or did not show any significant change. Most of these genes are also activated after various types of biotic and abiotic stressors in different plants species (see Vranovà et al., 2002, for a review). Our results indicate that, in contrast with Ischia, *P. oceanica* at Panarea faces stressors near the vents that result in the production of reactive oxygen species that trigger antioxidant responses. There are only few published studies on the occurrence of antioxidant responses in seagrasses, mostly based on indirect observations of photosynthetic parameters derived from chlorophyll *a* fluorescence (Ralph et al., 1998; Campbell et al., 2006), and our work is the first one to show the expression of genes associated with the antioxidant responses in *P. oceanica*.

The activation of heat shock protein genes such as HSP83 and DehSP in Panarea and the significant down-regulation of HSP90, DNAJ, HSP83 and HSFA5 in Ischia plants is also worthy of attention. HSPs play an essential role as molecular chaperones by assisting the correct folding of nascent and stress-accumulated misfolded proteins, and by preventing their aggregation. HSPs' induction and synthesis are not only a response to high temperature, which does not occur in Panarea, but also to many different types of stress, including exposure of cells to toxins or nitrogen deficiency (Santoro, 2000). As HSPs are very sensitive to even minor damage, they are suitable as an early-warning bio-indicator of cellular hazard (Bergmann et al., 2010; Gupta et al., 2010). Our observation that HSPs were down-regulated at Ischia and up-regulated at Panarea supports the overall finding that relevant environmental differences exist between the two volcanic sites. An alternative hypothesis is that *P. oceanica* gene expression responses at Panarea are still going through the initial phase of acclimation, whereas at Ischia the species has already adapted to existing environmental conditions. Thus, the natural gene expression differences revealed in this work may be a component of the species homeostatic evolutionary compensation. The volcanic vent in Ischia could in fact be as old as about 2000 years, as indicated by archaeological evidence (Lombardi et al., 2011), whereas the vent in Panarea is only about 10 years old. It is quite possible that some of the Ischia genotypes of *P. oceanica* have been there since the onset of the volcanic vents as it has been recently revealed that the longevity of this species can be up to thousands of years (Arnaud-Haond et al., 2012).

This is the first time that a gene expression study has been performed in marine plants in the vicinity of submarine volcanic vents, which are generally assumed to be good natural laboratories for investigating the effects of increased $CO_2$ and ocean acidification on marine organisms. In our analysis, we identified a subset of genes that were coherently expressed at both sites and that could be further explored for suggesting their use as early-warning indicators of low-pH conditions in photosynthetic marine organisms. Nevertheless, caution should be taken when using only natural volcanic vents as a proxy for future ocean acidification scenario, and experimental work in controlled laboratory conditions is necessary to unambiguously test organismal response to increased $CO_2$ and low-pH conditions. Our results call for careful consideration of other factors that can cause stress to seagrasses and other organisms near the vents and that may confound the effects of $CO_2$ and acidification. In order to clarify/predict seagrass stress responses to environmental stimuli, the study of general stress-coping, stress-avoiding, and tolerance mechanisms is needed, as is the analysis of more than one gene category.

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