# Seagrass collapse due to synergistic stressors is not anticipated by phenological changes

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**running title:** Synergistic stressors cause seagrass collapse

## Abstract

Seagrasses are globally declining and often their loss is due to synergies among stressors. We investigated the interactive effects of eutrophication and burial on the Mediterranean seagrass, *Posidonia oceanica*. A field experiment was conducted to estimate whether shoot survival depends on the interactive effects of three levels of intensity of both stressors and to identify early changes in plants (*i.e.* morphological, physiological and biochemical, and expression of stress-related genes) that may serve to detect signals of imminent shoot density collapse. Sediment burial and nutrient enrichment produced interactive effects on *P. oceanica* shoot survival, as high nutrient levels had the potential to accelerate the regression of the seagrass exposed to high burial (HB). After 11 weeks, HB in combination with either high or medium nutrient enrichment, caused a shoot loss of about 60%. Changes in morphology were poor predictors of the seagrass decline. Likewise, few biochemical variables were associated with *P. oceanica* survival (the phenolics, ORAC and leaf $\delta^{34}S$). By contrast, the expression of target genes had the highest correlation with plant survival: photosynthetic genes (ATPa, psbD and psbA) were upregulated in response to high burial, while carbon metabolism genes (CA-chl, PGK and GADPH) were down-regulated. Therefore, die-offs due to high sedimentation rate in eutrophic areas can only be anticipated by altered expression of stress-related genes that may warn the imminent seagrass collapse. Management of local stressors, such as nutrient pollution, may enhance seagrass resilience in the face of the intensification of extreme climate events, such as floods.



**Key words**: burial, early warnings, eutrophication, multiple-stressors, *Posidonia oceanica*.

## 1. Introduction

Transitions between natural systems with radically different properties can occur abruptly. Important examples can be found in ecology (*e.g*., lake eutrophication and coral reef collapses), where regime shifts have consequences that are often irreversible (Bellwood *et al.* 2004, Carpenter 2011, Perry and Morgan 2017). Predicting and anticipating catastrophic regime shifts represents a timely objective to improve our ability to preserve biodiversity and ecosystem functioning in the face of escalating anthropogenic pressures (Boettiger and Hastings 2013). Among all systems, the coastal marine are experiencing a wide range of human-induced alterations, the magnitude of which increases with the local density of human populations. Generally, the various environmental stressors do not act in isolation (Crain *et al.* 2008); rather, the effects of individual stressors interact to generate cumulative impacts that can be greater or smaller than the sum of their individual impacts (i.e. synergistic or antagonistic effects). Nevertheless, predicting the effects of combinations of stressors is particularly challenging because mechanisms underpinning impact are rarely elucidated and either threshold or nonlinear responses to stressors remain unknown (Griffen *et al.* 2016).

The effects of multiple stressors on slow-growing, habitat-forming species, such as certain seagrasses, are particularly threatening. Seagrass meadows are among the most important and productive coastal systems (Costanza *et al.* 1997). They provide key ecological services, including nursery grounds, habitat (for a review see Heck *et al*., 2003), organic carbon production and export, nutrient cycling, sediment stabilization, trophic transfer to adjacent habitats (Hemminga and Duarte 2000, Larkum *et al.* 2006) and coastal protection from erosion (Fonseca and Cahalan 1992, Fonseca and Koehl 2006). Nonetheless, they are threatened by the rapid environmental changes caused by the expansion of coastal human populations. Rapid, large-scale seagrass loss over relatively

84 short temporal scales has been reported throughout the world (Bulthuis 1983, Orth and
85 Moore 1983, Fourqurean and Robblee 1999, Marbà *et al*. 2005, Walker *et al*. 2006).
86 Stressors, such as sediments and nutrients inputs from terrestrial runoff, physical
87 disturbance (e.g. trawling, anchoring), invasive species, disease, aquaculture, overgrazing,
88 algal blooms and global warming, have been shown to cause seagrass declines at scales
89 ranging from square meters to hundreds of square kilometres (e.g. Munkes 2005, Orth *et*
90 *al*. 2006, Williams, 2007, Waycott *et al*. 2009, Bockelmann *et al.* 2011, Giakoumi *et al.*
91 2015). Overall, enhanced nutrients loading and sedimentation rates are likely the most
92 common and significant causes of seagrass decline (Unsworth *et al.* 2015). Indeed, the
93 current expansion of fish farming and other aquaculture practices (*e.g*., shellfish culture)
94 can have serious consequences on local populations of seagrasses through increased
95 deposition of organic matter and nutrients (Marbà *et al.* 2006). While eutrophication is
96 considered as the main cause of seagrass loss at a regional scale, burial of plants due to
97 anthropogenic-increased sedimentation or natural extreme events like storms or floods,
98 has been identified as an important cause for local die-offs (Short and Wyllie-Echeverria
99 1996, Erftemeijer and Lewis 2006, Orth *et al.* 2006, Cabaço *et al.* 2008, Cabaço and
100 Santos 2014).

101 *Posidonia oceanica* (L.) Delile is a slow-growing seagrass, endemic in the
102 Mediterranean and experiencing a widespread decline throughout the basin (Telesca *et al*.
103 2015). The regression of *P. oceanica* beds and the consequent expansion of alternative
104 habitats (e.g. algal turfs or dead seagrass rhizomes, generally referred to as “dead matte”)
105 is particularly common in the proximity of urban areas (Montefalcone *et al.* 2009,
106 Tamburello *et al*. 2012). In addition to enhanced nutrient loading, *P. oceanica* meadows
107 are exposed to increased sedimentation rates as a consequence of beach nourishment,
108 dredging of waterways, shoreline armouring and severe climatic events (*i.e.* storms and
109 floods). Correlative and experimental studies have assessed the effects of both

eutrophication (Alcoverro *et al*. 1997, Delgado *et al.* 1999, Ruiz *et al*. 2001, Holmer *et al.* 2008) and burial on *P. oceanica* (Manzanera *et al*. 2011, Gera *et al.* 2014), but how organic load levels change the effects of burial is yet to be explored. Within this context, the identification of early warning signals for drastic declines would be a valuable tool for the management of seagrass meadows (McMahon *et al.* 2013, Macreadie *et al.* 2014, Roca *et al*. 2016). This goal has been pursued for pressing disturbances through correlative approaches (e.g., van Katwijk *et al.* 2011) that do not, however, allow estimating signs of imminent collapses.

Here, we investigate the interactive effects of eutrophication and burial on *P. oceanica*. A field experiment was conducted 1) to estimate whether shoot survival depends on the interactive effects of three levels of intensity of both stressors, using a full factorial design, and 2) to identify early changes in plant attributes (*i.e.* morphological/growth, physiological/biochemical, and expression of stress-related genes) that may serve as signals of imminent shoot density collapse (early warnings of degradation). In general, response time and sensitivity to stressors vary with the type of variable examined; thus, understanding how sensitivity to stressors may change according to the level of biological organization is essential to rationalise the choice of indicators of impending seagrass decline and to design monitoring programmes (Roca *et al.* 2016). Indicator specificity is expected to increase when moving towards lower levels of biological organisation (*sensu*, Whitham *et al.* 2006), from the structural metrics to specific physiological and molecular indicators (Adams and Greeley, 2000). Whether this general rule holds for *P. oceanica* remains utterly unexplored.

## 2. Materials and Methods

### 2.1. Study site

The study was carried out in a shallow (5-8 m deep) continuous *P. oceanica* meadow, on the north-west coast of Sardinia (40°34.1 N, 09°8.5 E). At this site, *P. oceanica* canopy structure in the inner meadow is well preserved (shoot density mean±SE = 699.4±38.6 $m^{-2}$, *n*=28; canopy height mean±SE = 63.92±1.94 cm, *n*=35). At the site, the grazing sea urchin, *Paracentrotus lividus*, is present at low densities, whilst juveniles of the herbivore fish, *Sarpa salpa*, are common (GC, personal observations).

**2.2. Experimental design and set up**

The experiment started on the 29th of April 2015 and lasted until *P. oceanica* shoot mortality exceeded 60% in some treatments (*i.e.* 11 weeks, see Results). The hypotheses were tested by running a fully-factorial experiment of sediment burial (high, medium and control) and nutrient enhancement (high, medium and ambient) treatments. Twenty-seven circular patches (hereafter referred to as experimental units) were randomly selected across the seagrass meadow, at least 3 m apart one from another. Within each unit, a PVC cylinder (40 cm in height and diameter) was inserted about 10 cm deep into the bottom, thus leaving about 30 cm of the cylinder above the sediment (Fig. S1). Each cylinder enclosed between 81 and 109 *P. oceanica* shoots.

High, medium and control burial (HB, MB and CB) were obtained by adding 12.5 L, 5.0 L and 0 L of sediment (corresponding to a layer 10 cm, 4 cm and 0 cm tick), respectively accordingly with Manzanera *et al*. (2011). We used washed sand for playgrounds in our experiment, as this sand is similar in grain size (coarse sand, 0-1 mm) and mineralogy (carbonate) to sediment at the study site. This ensured that all experimental units were treated with the sand characterized by the same granulometry, devoid of fauna and low in organic content. Depending on the treatment, *P. oceanica* plants were buried with 4 cm of sediment over the ligula in HB, at the ligula height in MB,

158 and unburied in CB. When scuba divers filled experimental units with sand, care was taken
159 not to damage the leaves and to keep the plants upright during the process.

160 Also, high, medium and ambient nutrients (HN, MN and AN) were obtained by adding
161 80 g, 40 g and 0 g of homogenized fish fodder (protein 56.66%, fat 24.88%, cellulose
162 5.53%, ash 9.4%, phosphorous 1.35%, calcium 1.73% and sodium 0.46%) to the sediment
163 in each unit. Treatment levels were decided on the basis of nutrient release from offshore
164 fish farms (Garcìa-Sanz *et al.* 2010). Fish fodder was used to simulate effects of
165 eutrophication related to land and coastal use change (from deforestation to aquaculture),
166 which often increases the organic matter and nutrients input into coastal sediments. The
167 use of fish fodder not only increases ammonium levels through mineralization, but also
168 fuels the production of sulfide (Burkholder *et al*. 2007). Sulfide is a strongly phytotoxic
169 compound, as it blocks the activity of cytochrome oxidase and other metal containing
170 enzymes, which may lead to massive seagrass die-offs (Govers *et al*. 2014).

171 Each unit was randomly assigned to one of the six combinations of treatments ($n$=3).
172 Three extra cylinders with large holes and not exposed to either sediment or fish fodder
173 addition were used as procedural controls. The outer edge of all units was not parted off
174 and a trans-cylinder clonal integration between shoots was not impeded with the aim not
175 to impose further stress on shoots. The effects of the experimental conditions on shoot
176 survival were evaluated every few weeks (see section 2.3.2) with the aim of catching the
177 shoot mortality of about 20% and 60% in the harshest treatments that had corresponded
178 to week 3 and 11. Then, to identify indicators of mortality, shoot survival at week 11 was
179 related with morphological, physiological/biochemical variables and expression of stress-
180 related genes, estimated at week 3.

181 **2.3. Data collection and analyses**

### 2.3.1. Assessment of trophic enrichment

In order to estimate concentrations of inorganic nutrients, samples were taken from the water column at two dates chosen at random during the experiment (week 7 and 11). At each date, two replicate water samples were taken in each unit using a 125-mL sterile bottle about 5 cm above *P. oceanica* rhizomes. Samples were shaken and then filtered (0.45 µm mesh size) as soon as they were brought to the surface. Samples were frozen in liquid nitrogen for transportation to the laboratory, where concentrations of ammonia, nitrate, nitrite and ortophosphate were determined using a continuous-flow AA3 AutoAnalyzer (Bran-Luebbe) and expressed in µmol $l^{-1}$. Concentrations of dissolved inorganic nitrogen (DIN, ammonia+nitrate+nitrite) and phosphorus (DIP as $P\text{-}PO_4^{3-}$) were analysed by means of two one-way analyses of variance (ANOVA) testing the effect of Nutrient enrichment (3 levels, HN, MN and AN) on the pooled data taken in each unit ($n$=18). Cochran's C-test was used before each analysis to check for homogeneity of variance, and data were transformed when necessary. SNK test was used for *a posteriori* means comparisons (Underwood 1997).

### 2.3.2. *P. oceanica* survival

*P. oceanica* shoot density was counted at week 0, 3, 7, 9 and 11 to estimate shoot survival (assessed as the percentage of shoots with leaves) through time. Shoot survival (%) after 3 and 11 weeks was analysed by means of two 2-way analyses of variance (ANOVA), including the factors Burial (3 levels, HB, MB and CB, fixed) and Nutrient addition (3 levels, HN, MN and AN) both fixed and orthogonal ($n$=3). Cochran's C-test was used before each analysis to check for homogeneity of variance and data were transformed when necessary (Underwood 1997).

To evaluate the effects of procedural controls (PC), *P. oceanica* shoot survival at week 3 and 11 was also analysed by two one-way ANOVAs (PC vs. CBAN, $n$=3). Cochran's C-test and SNK test were run as explained above.

### 2.3.3. *P. oceanica* morphological/growth and physiological/biochemical variables

Six morphological variables (epiphyte load, % of leaves with necrosis, maximum leaf length, mean leaf length, number of leaves, shoot biomass), leaf growth rate, and eleven physiological/biochemical variables (leaf N, C and S content and corresponding isotopic signature $\delta^{15}N$, $\delta^{13}C$, $\delta^{34}S$, the antioxidant capacity through the oxygen radical absorbance capacity, ORAC, Trolox equivalent antioxidant capacity, TEAC and phenolics) (Table 1) were estimated after 3 weeks, when shoot mortality was still inconspicuous (see Results). Selection of these variables was based on the review made by Roca *et al*. (2016).

Among the morphological variables, epiphyte load was estimated as the weight of the epiphytes obtained from scraping the shoots with a razor blade (referred to the total weight of scraped leaves). The incidence of leaf necrosis was estimated as the percentage of leaves showing marks of necrosis (black areas or spots). Leaf growth rate was assessed using a modified leaf punching technique. At the beginning of the experiment, three shoots per unit were marked by punching a hole just above the leaf base-leaf blade junction of the outermost leaf with a hypodermic needle, and tagged with a plastic cable tie. After 20 days, the punched shoots were collected and the length of the newly produced tissue in each shoot measured.

Among the physiological/biochemical variables, ORAC, TEAC and phenolics were extracted in frozen leaf tissue samples as described in Costa et al. (2005) and estimated following Huang et al (2002), Re et al. (1999), and Folin-Ciocalteu method (Booker and Miller 1998, Migliore *et al.* 2007), respectively. Leaf N, C, and S (%) were determined in

229 samples of ca. 3.5 mg of dried, finely ground and homogenized material from each unit.
230 Leaf N, C and S isotopic signature, was analysed through elemental analyser combustion
231 for continuous flow isotope ratio mass spectroscopy.

232 Effects of Burial (3 levels, HB, MB and CB, fixed) and Nutrients (3 levels, HN, MN
233 and AN) on morphological/growth and physiological/biochemical variables ($n$=3) at week 3
234 were analysed by means of two 2-way analyses of variance (ANOVA). Cochran's C-test
235 was used before each analysis to check for homogeneity of variance (Underwood 1997).

236 **2.3.4. *P. oceanica* expression of stress-related genes**

237 The expression of ten stress-related genes (GADPH, SHSP, HSP90, PGK, CAB-151,
238 psbD, psbA, CA-chl, rbcl, and ATPa), (Table 1) were estimated after 3 weeks. At this aim
239 three shoots for each experimental unit were collected after 3 weeks of treatment. A 4 cm
240 leaf segment from the youngest fully mature leaves of each shoot (usually the second-rank
241 leaf) was collected and rapidly cleaned from epiphytes with a razor blade, towel-dried and
242 immediately stored in RNAlater® tissue collection solution (Ambion, Life Technologies).
243 Samples were then transported to the laboratory, preserved one night at 4 ◦C and stored
244 at −20 ◦C until RNA extraction.

245 After total RNA extraction (Mazzuca *et al.* 2013), RNA quantity and purity were
246 assessed by Nanodrop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies)
247 and 1% agarose gel electrophoresis. Average Abs260/280 nm and Abs260/230 nm ratios
248 (2.0 and 1.8, respectively) have indicated the absence of protein and solvent
249 contaminations, while gel electrophoresis has showed intact RNA, with sharp ribosomal
250 bands. Total RNA (500 ng) was reverse-transcribed in complementary DNA (cDNA) with
251 the iScript™ cDNA Synthesis Kit (Bio-Rad) using the GeneAmp PCR System 9700 (Perkin
252 Elmer).

The target genes were selected on the basis of the two key events that mandatorily occur in the light and dark reactions of photosynthesis and can be hampered by biotic and abiotic stressful conditions, reducing plant productivity and survival (Ashraf and Harris, 2013). In light reactions, light energy is harvested by antenna pigments, channelled to photosystem II (PSII) to produce photochemistry and converted into chemical energy (*i.e.* ATP) and reducing power (*i.e.* NAPDH) by the flow of electrons along the electron transport chain. Particularly, we have selected and analyzed genes coding for a putative fundamental protein of the photosynthetic antenna complex (CAB-151): the two PSII core proteins D1 and D2 (psbA and psbD) and a putative chloroplastic ATP synthase subunit alpha (ATPa). In dark reactions, $CO_2$ is fixed into carbohydrates in the Calvin-Benson cycle by using ATP and NADPH produced in the light reactions. In relation to this cycle, we have selected several putative genes encoding key enzymes of the plant carbon metabolism: the large subunit of the RuBisCO (rbcl), directly involved in $CO_2$ fixation, and a chloroplast carbonic anhydrase (CA-chl) that catalyzes the conversion of $HCO^-_3$ into $CO_2$. We also selected two major enzymes involved in glycolysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK), for obtaining energy and carbon molecules from glucose. Finally, two general stress genes were also included in the analysis as they are ubiquitous heat stress proteins in environmental stress: the molecular chaperone (HSP90) and one putative small heat shock protein (SHSP). All selected genes were already investigated in other studies (Table S1). Primers sequences and GenBank Accession Numbers are reported in the reference in which the genes have been selected for the first time. The expression stability of a set of three putative reference genes already tested in *P. oceanica* (i.e. EF1A, 18S and L23; Serra *et al.* 2012) was evaluated by running GeNorm (Vandesompele *et al*. 2002) and Normfinder (Andersen *et al*. 2004). According to stability analysis, two genes, namely 18S and L23 (Fig. S2), were used to normalize gene expression data.

After normalizing by each primer efficiency (all >0.92% and $R^2$>0.96), relative treatment gene expression values were calculated as the negative differences in cycles to cross the threshold value (-ΔCT) between the reference genes and the respective target genes ($-\Delta CT = CT_{reference} - CT_{target}$). Subsequently, fold expression changes were calculated for graphical purposes according to the equation: fold expression change = $\pm 2^{(|(-\Delta CTtreatment)-(-\Delta CTcontrol)|)}$.

Effects of Burial (3 levels, HB, MB and CB, fixed) and Nutrients (3 levels, HN, MN and AN) on the relative expression of target genes (-ΔCT values; n=3) were analysed by means of 2-way analyses of variance (ANOVA). Cochran's C-test was used before each analysis to check for homogeneity of variance (Underwood 1997).

**2.3.5. Assessment of the predictive variables**

In order to identify predictors of shoot survival of *P. oceanica* under different combinations of burial and nutrients enhancement levels, separate multiple regressions were run between each of the four groups of response variables included in the study (morphological/growth, antioxidant, isotopes, and gene expression) and shoot survival. More specifically, values of explanatory variables measured at week 3 were used as predictors of shoot survival at week 11. Collinearity among covariates was assessed by means of Variance Inflation Factor (VIF) procedures. Covariates with highest VIF values, calculated using the R car package, were sequentially dropped from the model, until all VIF values were smaller than 3, as recommended by Zuur *et al.* (2010). Linearity and homogeneity of variances was visually checked by means of residual plots. Log transformation of data was effective in enhancing homogeneity of variances. For each group of variables, the best-fit model was selected by means of a stepwise procedure (both directions) using the stepAIC function from the R MASS package (Venables and Ripley 2002). This function selects the best model based on the Akaike Information

304 Criteria. The relationship between plant survival and predictive variables retained by the
305 different best-fit models were visualized by means of partial regression plots, using the
306 function avPlots from the car package. The relative importance (as percentage) and
307 bootstrap confidence intervals of the explanatory variables retained in the best fit models
308 were assessed by means of the Lindemann-Merenda-Gold (lmg) method for calculating
309 sequentially weighted partial $R^2$ (Lindeman *et al.* 1980), using the R "relaimpo" package
310 (Gromping 2006). This method calculates an average coefficient of partial determination
311 for each model permutation using the individual contribution of each explanatory variable.

312 **3. Results**

313 **3.1. Inorganic nutrients and *P. oceanica* survival**

314 The addition of fish fodder to sediments resulted in a significant increase of DIN
315 concentration in the water column above rhizomes ($F_{2,51}$=10.93 $p$=0.0001; Fig. 1); as
316 shown by the SNK tests, the increment in DIN was proportional to the amount of fodder
317 added (HN>MN>AN). By contrast, there were no significant differences in DIP among
318 treatments ($F_{2,51}$=3.08 $p$=0.0547).

319 The comparison between CBAN and PC shows that there was no artefact of PVC
320 cylinders on shoot mortality at both week 3 ($F_{1,4}$=0.25 p=0.6430) and week 11 ($F_{1,4}$=0.04
321 p=0.8554) (Fig. 2). *P. oceanica* shoot survival was significantly affected by sand burial, as
322 about the 25% of shoots died by week 3 in the HB units (Fig. 2 and Table 2, HB<MB=CB).
323 Burial was the only stressor affecting mortality of the seagrass in the short-term (3 weeks).
324 After that, shoot survival decreased through time and, after 11 weeks, it was regulated by
325 the interactive effects of nutrient addition and burial (Table 2; Fig. 2). In units exposed to
326 high burial, shoot survival under high and intermediate nutrients addition was lower than at
327 ambient nutrient levels (HBHN=HBMN<HBAN). In contrast, shoot survival was not affected

by nutrients levels in units exposed to either medium or ambient burial (SNK tests in Table 2). At high nutrients levels, shoot survival was lower in units exposed to high than medium or ambient burial levels (HBHN<MBHN=CBHN). At medium nutrients levels, the survival decreased with increasing severity of burial (HBMN<MBMN<CBMN), while at ambient nutrient levels, there was no difference among burial treatments (Fig. 2 and Table 2, CBAN=MBAN=HBAN).

Synergistic effects of both high and medium nutrients addition and high burial were further assessed by comparing the average response of *P. oceanica* shoot survival (%), both at each stressor level and at each combination of stressor levels, with those obtained for the theoretical additive response of stressors levels in combination (Fig. 3). In fact, for the average response given at high nutrient addition (HN), medium nutrient addition (MN), and high burial (HB) when applied individually, the cumulative effects under additive conditions (HN+HB and MN+HB) on shoot survival would have been about two fold higher than that observed (Fig. 3). The difference in shoot survival between the average experimental evidence and the theoretical additive estimates provides evidence for the synergism between nutrient (high and medium) and burial (high) stressors.

### 3.2. *P. oceanica* predictive variables

After three weeks since treatments, nutrient addition changed epiphyte load, necrosis and leaf growth rate, among morphological/growth variables, phenolics and the antioxidant response, ORAC and TEAC, among the physiological variables. However, there was no significant effect of nutrient enrichment on isotopes or gene expression, except for the stress gene HSP90, which experienced a slight inactivation (Fig. 4, 5, 6 and Table 3). In contrast, burial significantly increased epiphyte load and affected gene expression of GADPH, PGK, psbA, CA-chl and ATPa (Table 3).

352 The only variables that responded to the interactive effects of nutrient addition and
353 burial were the percentage of leaf necrosis and phenolics content, being one the mirror
354 pattern of the other (Table 3): the first was significantly greater at high nutrient levels when
355 plants were exposed to high burial (HN>MN=AN) and at high burial levels if plants were at
356 ambient nutrients levels (HB>MB=CB), while the latter was smaller at high nutrients levels
357 condition only when plants were exposed to HB (HN<MN=AN), and in high buried plants
358 only at HN (HB<MB=CB).

359 The multiple regressions conducted with genetic, isotope, antioxidant and
360 morphological covariates explained the 60.6%, 33.2%, 36.4% and 27.8% (Adjusted $R^2$
361 values) of the variance in shoot survival, respectively (Table 4). After simplification through
362 the step-wise procedure, the best fit model at the level of gene expression included two
363 explanatory variables, CA-chl and ATPa, although only the effects of the former was
364 significant, contributing nearly for 84% of the variation in shoot mortality explained by the
365 model (Table 5; Fig. 6). The positive estimate of CA-chl suggests that overexpression of
366 this gene at week 3 is positively correlated with shoot survival at week 11 (Fig. S3). CA-chl
367 is directly related with carbon metabolism (correlated to PGK and GADPH) and was, in
368 fact, down-regulated in treatments, such as HBHN and HBMN (SNK test, Table 6), where
369 *P. oceanica* had the lowest survival. ATPa is a gene involved in the photosynthesis
370 (correlated to psbA and psbD) and was up-regulated in high burial treatments (Fig. 6 and
371 Table 6).

372 Four explanatory variables were retained in the best fit isotope model and only one of
373 these, namely leaf $\delta^{34}S$, was significantly related with shoot survival at 11 weeks and
374 accounted for about 50% of the total variation in shoot survival explained by the model
375 (Table 4). However, although leaf $\delta^{34}S$ was positively (Fig. S4) related with shoot survival,
376 the ANOVA did not identify any significant effect neither of nutrient addition nor of burial on

leaf $\delta^{34}S$ content (Table 3). The best fit antioxidant model retained two variables, ORAC and phenolic content, both of which were significantly correlated with shoot survival. Phenolic contents accounted for a greater proportion (~63%) than ORAC (~37%) of the total variability in shoot mortality explained by the model (Table 4). Coefficient estimates were negative for ORAC and positive for phenolics (Fig. S5).

The morphological/growth best fit model retained 3 variables, epiphyte load, shoot biomass and growth (Table 4). The former variable accounted for most of the total variation in shoot survival explained by the model (~77%, Table 5). The negative estimate of the relationship coefficients (Table 4) indicates that increasing levels of epiphytic loading at week 3 were associated with lower shoot survival at week 11 (Fig. S6). Plants that had grown more in the first three weeks had greater chances of be alive at week 11.

## DISCUSSION

### 4.1. Synergistic effects of nutrients and burial on *P. oceanica* mortality

*P. oceanica* shoot survival was affected by synergistic effects of burial and nutrient addition over a short time. High sediment load (HB), corresponding to the complete burial of meristems, increased seagrass mortality independently of the organic load in just three weeks. Apparently, HB was the only level strong enough to cause rapid seagrass mortality.

Over a longer term, burial and nutrient enrichment had interactive, negative effects on *P. oceanica* survival. In particular, high nutrients levels (HN) had the potential to accelerate the regression of the seagrass subjected to high burial. The use of a gradient of both stressors, manipulated in a crossed design, has also allowed detecting the rapid threshold responses to their combinations. Thus, by week 11, HB in combination with either HN or MN, caused a shoot loss of about 60%. In addition, the slow decrease in

401 shoot survival under high burial without organic loading (HBAN), suggests that mortality at
402 HB was determined by the level of nutrient load, from the fastest at HN to the slowest at
403 AN.

404 Total shoot mortality did not occur within 11 weeks since the start of the experiment,
405 even under the most stressful conditions and it is unknown whether it would have occurred
406 on a longer term. However, several mechanisms may underpin the survival of some
407 shoots within experimental plots. First, the clonal integration with neighbouring shoots
408 immediately outside the unit edge: continuity between shoots was not prevented to avoid
409 further stress that would have biased the response of the plants. Thus, transfer of
410 metabolites (*e.g.* carbohydrates) from non-treated plants may have sustained the survival
411 of plants experimentally exposed to stressful conditions. Second, burial effect might have
412 not been homogeneous among shoots within each experimental unit, as rhizome height
413 varied among them and samples were only three-replicated. Third, individual response to
414 stress can differ. Differences in resilience can be related to individual characteristics (*e.g*.
415 age) of single ramets or to genetic peculiarities of single genets. More resilient genotypes
416 could respond better to the synergistic action of the applied stressors (Hughes and
417 Randall 2004).

418 **4.2. Predictive variables of *P. oceanica* collapse**

419 The protraction of the experiment for 11 weeks allowed detecting widespread
420 mortality in experimental units exposed to HBHN and HBMN. Thus, besides the
421 identification of lethal effects of burial and eutrophication on *P. oceanica*, our study
422 enabled the identification of the plant attributes (morphological/growth,
423 physiological/biochemical and transcriptomic) at week 3, prior to mortality, related to
424 survival at week 11.

425 **4.2.1. Morphological/growth variables**

426 Except for the increase in epiphyte load, changes in morphological variables and
427 growth rate were little effective in predicting seagrass loss (Table 6). An increase in
428 epiphyte biomass, especially macroalgae, is a common response to eutrophic conditions
429 (Piazzi *et al*. 2016) and the ratio between epiphyte and leaf biomass in *P. oceanica* is one
430 of the descriptors used to assess the ecological quality of Mediterranean water bodies
431 under the European Water Framework Directive (Gobert *et al*. 2009, Oliva *et al*. 2012).
432 Our study shows that the epiphyte load can be used also as an indicator of burial stress,
433 as probably a consequence of lower phenolics content in the buried plants (Costa *et al*.
434 2015). Furthermore, signals of imminent mortality can also be gained by the percent of
435 leaves with necrosis; leaf necrosis has been previously suggested to increase with
436 eutrophication (Roca *et al.* 2016). Here, a higher proportion of leaves exposed to both high
437 burial and both high and medium nutrient addition had necrotic spots which were not
438 uniformly distributed along the blades, being mostly concentrated on the basal part of the
439 leaf. Finally, leaf growth was accelerated by nutrient addition, but was not related to burial,
440 suggesting that this variable is inadequate for predicting imminent seagrass degradation
441 due to the combination of stressors.

442 **4.2.2. Physiological/biochemical variables**

443 Based on the multiple regressions, the biochemical/physiological variables
444 associated with the *P. oceanica* survival, were the phenolic content, ORAC and $\delta^{34}S$.
445 Among these, only phenolics are likely to be a useful predictor (positive coefficient), since
446 differences in their concentration were also related to the interactive effect of nutrient
447 addition and burial (Table 6). Phenolics can have multiple biological functions mainly
448 related to the reproductive strategy, adaptation and survival to environmental
449 disturbances, antimicrobial and antifouling properties. Their deposition as lignin in cell

walls increases their mechanical strength and improves plant response against pathogens and wounding. Variations in phenolics content have been observed in *P. oceanica* as a response to changes in water quality and when competing with invasive species (Pergent *et al.* 2008, Migliore *et al.* 2007, Rotini *et al.* 2013).

Furthermore, ORAC, which reflects the ability of the plant metabolism to scavenge oxygen reactive species (ROS, Mittler, 2002) through hydrogen atom donation (both enzymatically and non-enzymatically), was negatively associated with *P. oceanica* survival, and shoots with higher ORAC will have higher probability of mortality for the high concentrations of ROS. However, it only responded to nutrient addition (Table 6). Furthermore, the $\delta^{34}S$ content (positive coefficient) would suggest that the presence of sulphide intrusion in leaf tissue, lower leaf $\delta^{34}S$ signature, could predict greater shoot survival (Table S4). However, this isotopic signal did not respond uniformly to any treatment, probably due to the sediment type (*i.e.* coarse-carbonate sediments, Oliva 2012): indeed, the relationship between sediment sulfide and the $\delta^{34}S$ of *P. oceanica* tissues is known to be rather complex being controlled both by the sediment sulfide concentrations and the oxygen status of the plants (Borum *et al*. 2005). Because of the high variability, the use of $\delta^{34}S$ as an indicator for fish farm effects on *P. oceanica* has already not been supported (Frederiksen *et al*. 2007). Therefore, among the biochemical/physiological variables only results in leaf phenolics content are promising enough to promote it as an indicator of nutrient and burial stressors of *P. oceanica* (Table 6).

**4.2.3. Expression of selected genes**

Gene expression of target genes had the highest correlation with shoot survival. Photosynthetic genes (ATPa, psbD and psbA) were up-regulated in response to high burial suggesting that high sediment load tends to increase the number of PSII and ATP

475 synthase, probably to compensate for high energy consumption. By contrast, carbon
476 metabolism genes (CA-chl, PGK and GADPH) were down-regulated, being associated
477 with plant survival. In particular, the experimental treatments significantly affected the
478 ability of plants to fix C and recycle ATP through glucolysis or gluconeogenesis, as
479 suggested by the significant down-regulation of PGK. This response is likely to result in a
480 long-term starvation of the plants, likely contributing to the high mortality observed in these
481 treatments. In other words, burial produced a strong effect on the carbon metabolism of
482 plants, reducing carbon fixation and the production of energy: buried plants invested
483 energy to increase the amount of PSII and ATP synthase to sustain the thylacoid proton
484 gradient necessary to produce more ATP. Further, because HSP90 is an ATP-dependent
485 molecular chaperone it is possible that a strong ATP reduction experienced by plants from
486 the most intense treatments resulted in lower expression levels of this gene. It is also
487 known that inhibition of HSP90 induced cell death in plants (Nishizawa-Yokoi *et al.* 2010;
488 Moshe *et al.* 2016) and, likely, the observed down-regulation is promoting and anticipating
489 plant mortality two months in advance.

490 **4.3**. **Conclusions**

491 This study has estimated that *P. oceanica* shoot survival strongly depends on the
492 interactive effects of levels of intensity of nutrients and burial and it has identified some
493 early changes in plant attributes. Within the whole set of possible predictors of seagrass
494 collapse, only a few were associated with shoot survival (with different contribution to the
495 total variability) and these did not necessarily correspond to those affected by the
496 treatments (Table 6). Furthermore, they were attributed to a different reliability level based
497 on the association to shoot survival. Overall, the evaluation of the candidate indicators of
498 *P. oceanica* regression for the consequences of high organic load and sediment burial has
499 produced some very promising evidence for the gene CA-chl, and secondly of ATPa, while

500 phenolics and epiphyte load are likely to be less reliable predictors. Thus, the sensitivity of
501 *P. oceanica* attributes to these stressors has increased as the level of biological
502 organization decreases (according to expectations, Roca *et al.* 2016), underlining the
503 importance of implementing monitoring protocols with biochemical and molecular
504 indicators. However, high-replicated studies that include different field conditions (*e.g.*
505 sediment type, wave exposure) are needed so that consistency of variables' response
506 would be estimated and solid predictors incorporation promoted in any kind of
507 management program (*i.e.* assessment of ecosystem status, environmental quality,
508 impacts or the results of mitigation actions).

509 Nevertheless, no morphological change of shoot or leaf size should be expected after
510 a severe storm or flood that accumulates high loads of sediments, rich in organic matter,
511 over a *P. oceanica* bed; these attributes are likely to be reliable warnings only of pressing
512 disturbances over a longer temporal scale (Roca *et al*. 2016). Consequently, if other levels
513 of biological organization besides the morphological are not considered, the seagrass
514 collapse without early warning signals (Pace *et al*. 2015). Our results indicate that the
515 interaction between two of the main regional anthropogenic stressors in temperate coastal
516 ecosystems, eutrophication and high sediment loads (the deposition of organic detritus
517 likely brings with it increased sediments, Terrados *et al.* 1999), can trigger the fast collapse
518 of seagrass meadows without any major phenological change.

519 In the growing interest of identifying the type and role of interactions among local
520 anthropogenic stressors in driving habitat shifts in marine ecosystems (Russell and
521 Connell 2012), this controlled factorial experiment provides evidence that eutrophication
522 and burial have non-additive consequences on seagrass beds. The input of excess
523 nutrients (primarily nitrate and phosphate) to the marine environment is a global problem
524 associated with a range of human activities, but coastal eutrophication through organic

matter dispersal represents a further source of disturbance. In fact, the addition of a detrital layer to a seagrass bed will not just result in increased dissolved inorganic nutrients to the sediments, it will have a whole stimulatory impact upon the microbial, fungal and detrital feeding community (Danovaro *et al.* 1994). A likely additional effect of increased organic detritus is that of increasing the levels of sulphide stress within the sediments with follow-up negative effects upon the seagrass (Marbà *et al.* 2006). Indirectly, our results provide strong evidence suggesting that development of coastal areas and their associated human activities will have major impacts on the seagrass meadows and such information should be used to identify appropriate local management actions to halt the global loss of seagrasses in favour of alternative habitats composed by either macroalgae or anoxic mud (Unsworth *et al.* 2015).

Finally, local anthropogenic stressors are thought to negatively interact with global climatic stressors resulting in decline of many habitats, such as coral reefs, macroalgal forests, mangroves and seagrasses. These findings suggest that shifts from seagrass systems to dead rhizomes (*i.e.* dead matte) in areas characterized by poor water quality, may become more common under future scenarios of climate change (Garcia *et al*. 2013), as frequency and intensity of storms and floods are expected to increase.

Because our study has demonstrated that the effects of nutrients and sediment burial on seagrasses are synergistic, strategies for reducing nutrient levels, a widely advocated strategy for seagrass conservation, should be pursued bearing in mind the need to control also sedimentation rates. Local management of nutrient loading can represent a valid tool for mitigating the impacts of global stressors on marine macrophytes (Falkenberg *et al.* 2010). Our study shows that reducing nutrient loading may be not sufficient to enhance the resistance of seagrasses to global stressors, such as seawater warming (NOAA 2016). Given the cumulative nature of human impacts and the large small-scale variation in life-

traits occurring in coastal environments, one size fits all strategies are unlikely be successful for sustaining the functioning of marine ecosystems in the face of climate changes. In addition, our study makes a first step towards the identification of response variables that may function as early warning signals of imminent seagrass collapse. It also provides evidence for increased indicator specificity at lower levels of biological organisation, promoting the need of implementing monitoring with molecular analyses. As a final note of caution, the robustness of the response variables identified as most promising must be assessed against variations in stressor intensity and background abiotic and biotic conditions before their implementation in monitoring programs.

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821

**Table 1.** The *P. oceanica* response variables classified in the three levels of biological organization.

| **Morphological/growth** | | **Physiological and biochemical** | **Gene expression** |
|---|---|---|---|
| Epiphyte load (mg/sh) | antioxidant | ORAC (µmol EQT/g DW) | GADPH |
| Necrosis (% leaf) | | TEAC (µmol/g DW) | SHSP |
| Max leaf length (cm) | | Phenolics (mg/g DW) | HSP90 |
| Mean leaf length (cm) | | | PGK |
| Num of leaves/shoot | | | CAB-151 |
| Shoot biomass (g DW) | isotopes | Leaf N (%) | psbD |
| Leaf growth rate (cm/sh·day) | | Leaf $\delta^{15}$N (‰) | psbA |
| | | Leaf C (%) | CA-chl |
| | | Leaf $\delta^{13}$C (‰) | rbcl |
| | | Leaf S (%) | ATPa |
| | | Leaf $\delta^{34}$S (‰) | |

**Table 2.** ANOVAs on the effects of Nutrients (three levels: high, medium, and ambient; HN, MN, AN), and Burial (three levels: high, medium, and none; HB, MB, CB) on shoot survival after 3 and 11 weeks after the start of the experiment. *n*=3. SNK tests results are reported below.

| | | **shoot survival 3 weeks** | | | **shoot survival 11 weeks** | | |
|---|---|---|---|---|---|---|---|
| **Source** | **df** | **MS** | **F** | **P** | **MS** | **F** | **P** |
| Nutrients = (N) | 2 | 168.8 | 0.90 | 0.4240 | 631.1 | 7.6 | 0.0041 |
| Burial = (Bu) | 2 | 1181.9 | 6.30 | **0.0084** | 4430.8 | 53.3 | 0.0000 |
| N×Bu | 4 | 278.1 | 1.48 | 0.2488 | 836.5 | 10.1 | **0.0002** |
| Residual | 18 | 187.5 | | | 83.1 | | |
| | | C = 0.3185 (ns) | | | C = 0.3214 (ns) | | |
| | | Transform: None | | | Transform: None | | |
| **SNK test** | | **Bu,** SE=4.561 | | | **N×Bu,** SE=5.263 | | |
| | | HB<MB=CB | | | HB: HN=MN<AN | | |
| | | | | | MB: HN=MN=AN | | |
| | | | | | CB: HN=MN=AN | | |
| | | | | | HN: HB<MB=CB | | |
| | | | | | MN: HB<MB<CB | | |
| | | | | | AN: HB=MB=CB | | |

**Table 3.** ANOVAs on the effects of Nutrient addition (three levels: high, medium, and ambient; HN, MN, AN) and Burial (three levels: high, medium, and none; HB, MB, CB) on morphological/growth, physiological/biochemical and gene expression response variables. Significant *F* values are reported in bold; * = p<0.05.

| Variable | Nutrients $F_{2,18}$ | Burial $F_{2,18}$ | Nutrients x Burial $F_{4,18}$ |
|---|---|---|---|
| **Morphological/growth** | | | |
| Epiphyte load | **6.23 *** | **4.10 *** | 2.76 |
| Necrosis | **5.83 *** | 2.91 | **3.06 *** |
| Max leaf length | 1.48 | 2.60 | 1.33 |
| Mean leaf length | 1.58 | 2.41 | 1.45 |
| Num of leaves/shoot | 1.06 | 0.72 | 0.22 |
| Shoot biomass | 0.48 | 0.89 | 2.07 |
| Leaf growth rate | **3.90 *** | 0.39 | 2.14 |
| **Physiological and biochemical** | | | |
| ORAC | **5.01 *** | 3.11 | 2.84 |
| TEAC | **5.11 *** | 0.26 | 2.11 |
| Phenolics | 0.34 | 3.29 | **4.07 *** |
| Leaf N | 0.53 | 0.05 | 1.70 |
| Leaf $\delta^{15}N$ | 0.46 | 1.68 | 2.28 |
| Leaf C | 0.74 | 0.86 | 1.31 |
| Leaf $\delta^{13}C$ | 2.64 | 0.32 | 0.63 |
| Leaf S | 3.44 | 0.16 | 0.99 |
| Leaf $\delta^{34}S$ | 2.91 | 2.66 | 0.20 |
| **Gene expression** | | | |
| GADPH | 1.04 | **4.21 *** | 1.17 |
| SHSP | 3.29 | 0.89 | 2.47 |
| HSP90 | **4.06*** | 0.26 | 0.75 |
| PGK | 2.39 | **8.00 *** | 2.19 |
| CAB-151 | 0.45 | 1.60 | 1.31 |
| psbD | 0.19 | 2.52 | 2.87 |
| psbA | 0.37 | **4.58 *** | 2.44 |
| CA-chl | 2.25 | **9.25 *** | 2.03 |
| rbcl | 0.10 | 0.67 | 1.45 |
| ATPa | 0.95 | **5.75 *** | 2.88 |

**Table 4.** Multiple regressions of *P. oceanica* shoot survival against different groups of response variables. Coefficient estimates (Estimate), standard errors (SE), t-values, and significance level (*P*-value) for variables retained in the best-fit model are reported.

| **Morphological/growth** | | | | |
|---|---|---|---|---|
| ***effect*** | **Estimate** | **SE** | **t-value** | ***P*-value** |
| Epiphyte load | -0.00365 | 0.00104 | -3.50 | **0.00193** |
| Shoot biomass | -0.17576 | 0.10531 | -1.66 | 0.10869 |
| Leaf growth | 0.06651 | 0.03766 | 1.766 | 0.09066 |
| Adjusted $R^2$=0.2779 | | $F_{3,23}$=4.33 P-value 0.0146 | | |

| **Physiological and biochemical (antioxidant)** | | | | |
|---|---|---|---|---|
| ***effect*** | **Estimate** | **SE** | **t-value** | ***P*-value** |
| ORAC | -0.0004 | 0.00013 | -3.045 | **0.00557** |
| Phenolics | 0.0007 | 0.00021 | 3.697 | **0.00113** |
| Adjusted $R^2$=0.3644 | | $F_{2,24}$=8.45 P-value 0.0016 | | |

| **Physiological and biochemical (isotopes)** | | | | |
|---|---|---|---|---|
| ***effect*** | **Estimate** | **SE** | **t-value** | ***P*-value** |
| Leaf N | -0.4591 | 0.2735 | -1.678 | 0.1075 |
| Leaf $\delta^{15}$N | -0.1782 | 0.1195 | -1.491 | 0.1501 |
| Leaf S | -0.5854 | 0.4482 | -1.306 | 0.2050 |
| Leaf $\delta^{34}$S | 0.0627 | 0.0266 | 2.356 | **0.0278** |
| Adjusted $R^2$=0.3321 | | $F_{2,22}$=4.23 P-value 0.0108 | | |

| **Gene expression** | | | | |
|---|---|---|---|---|
| ***effect*** | **Estimate** | **SE** | **t-value** | ***P*-value** |
| CA-chl | 0.0546 | 0.00972 | 5.623 | **0.00000** |
| ATPa | -0.0216 | 0.01205 | -1.796 | 0.08510 |
| Adjusted $R^2$=0.6056 | | $F_{2,24}$=20.96 P-value 0.0000 | | |

**Table 5.** Rank of variables contributing most to the *P. oceanica* shoot survival (% contribution to $R^2$).

| | % contribution | | | |
|---|---|---|---|---|
| **Morphological/growth** | **Epiphyte load** 76.7 | **Leaf growth** 12.6 | **Shoot biomass** 10.7 | |
| **Physiological and biochemical (antioxidant)** | **Phenolics** 63.0 | **ORAC** 37.0 | | |
| **Physiological and biochemical (isotopes)** | **$\delta^{34}S$** 49.5 | **Leaf S** 19.2 | **$\delta^{15}N$** 16.9 | **Leaf N** 14.4 |
| **Gene expression** | **CA-chl** 83.8 | **ATPa** 16.1 | | |

**Table 6.** List of variables evaluated as possible indicators of imminent collapse of *P. oceanica* due to Nutrient (= N) and Burial (= B) stressors. For each of the variables included in the study are reported the direction of stressor effects (↑ =increase and ↓ =decrease effect and 'no Ha' = no alternative hypothesis detected by the SNK tests), the sign and strength of their association with *P. oceanica* shoot survival on the basis of their relative contribution to the total variability explained by the best-fit model (arbitrary scale: 0-25%: low; 25-50%: medium; 50-75%: high; 75-100%: very high) and reliability based on the total variability explained by the regression model the variables belongs to (arbitrary scale: poor $R^2$<40, good 60>$R^2$>40, very good $R^2$>60).

| Variable | Stressor effect | Association to survival | indicator reliability |
|---|---|---|---|
| Epiphyte load | ↑N ↑B | very high | poor |
| Necrosis | ↑N ↑B | - | |
| Leaf growth rate | ↑N | low | |
| ORAC | no Ha | medium | poor |
| TEAC | ↑N | - | |
| Phenolics | ↓N ↓B | high | |
| Leaf $\delta^{34}S$ | no effect | medium | poor |
| GADPH | no Ha | - | very good |
| HSP90 | no Ha | - | |
| PGK | ↓B | - | |
| psbA | no Ha | very high | |
| CA-chl | ↓ B | low | |
| ATPa | ↑ B | | |

**Fig. 1** Water nutrients. Change (respect to controls) in mean water DIN (left axis) and P-$PO_4^{3-}$ (right axis) due to experimental nutrient addition. Dots are averages of two sampling times (n=6). Black, grey, and light grey colours indicate high, medium, and control burial (HB, MB, CB, respectively). Nutrient addition levels are separated by dashed lines

**Fig. 2** *P. oceanica* shoot survival. Effect of experimental treatments (nutrient addition and burial) after 3, 7, 9 and 11 weeks (mean±SE, n=3). Black, grey, and light grey colours denote high, medium, and control burial treatments, respectively (HB, MB, CB, respectively). PC corresponds to Procedural controls

**Fig. 3** *P. oceanica* survival. Average (n=3) shoot change in controls (CBAN), in each level of each single stressor (MB, HB, MN and HN), and in each combination of levels for multiple stressors interactions (MBMN, MBHN, HBMN, and HBHN). Grey and striped bars represent the experimental data and the theoretical additive response (calculated by summing the response to the single stressor levels), respectively

**Fig. 4** Morphological/growth variables (mean+SE, n=9) change due to nutrient addition (H=high, M=medium and A=ambient) and burial (high, medium, and control)

**Fig. 5** Physiological/biochemical variables (mean+SE, n=9) change due to nutrient addition (H=high, M=medium and A=ambient) and burial (high, medium, and control)

**Fig. 6** Stress-related gene expression (mean+SE, n=9) change due to nutrient addition (H=high, M=medium and A=ambient) and burial (high, medium, and control)

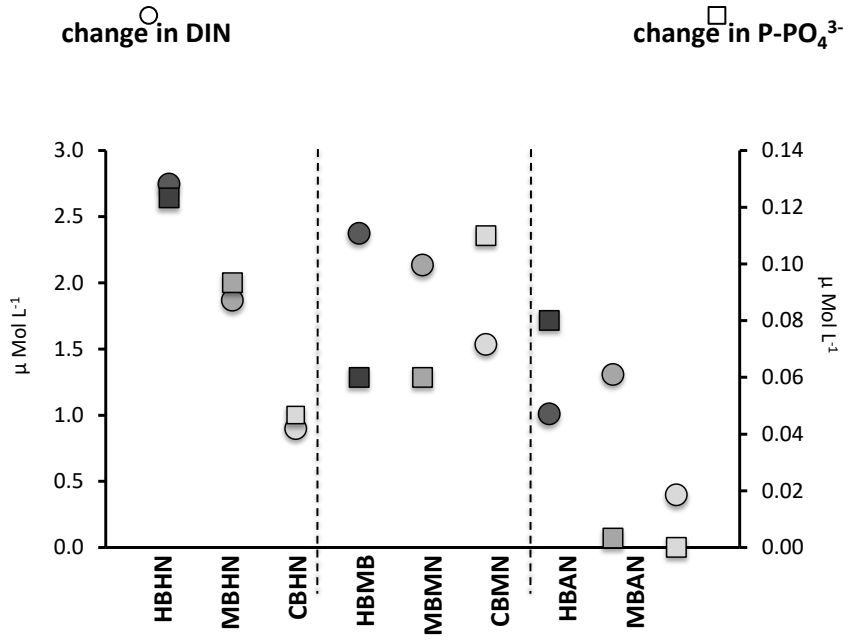

change in DIN
change in P-PO4 3-
µ Mol L-1
µ Mol L-1
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
HBHN
MBHN
CBHN
HBMB
MBMN
CBMN
HBAN
MBAN

**Fig 1**

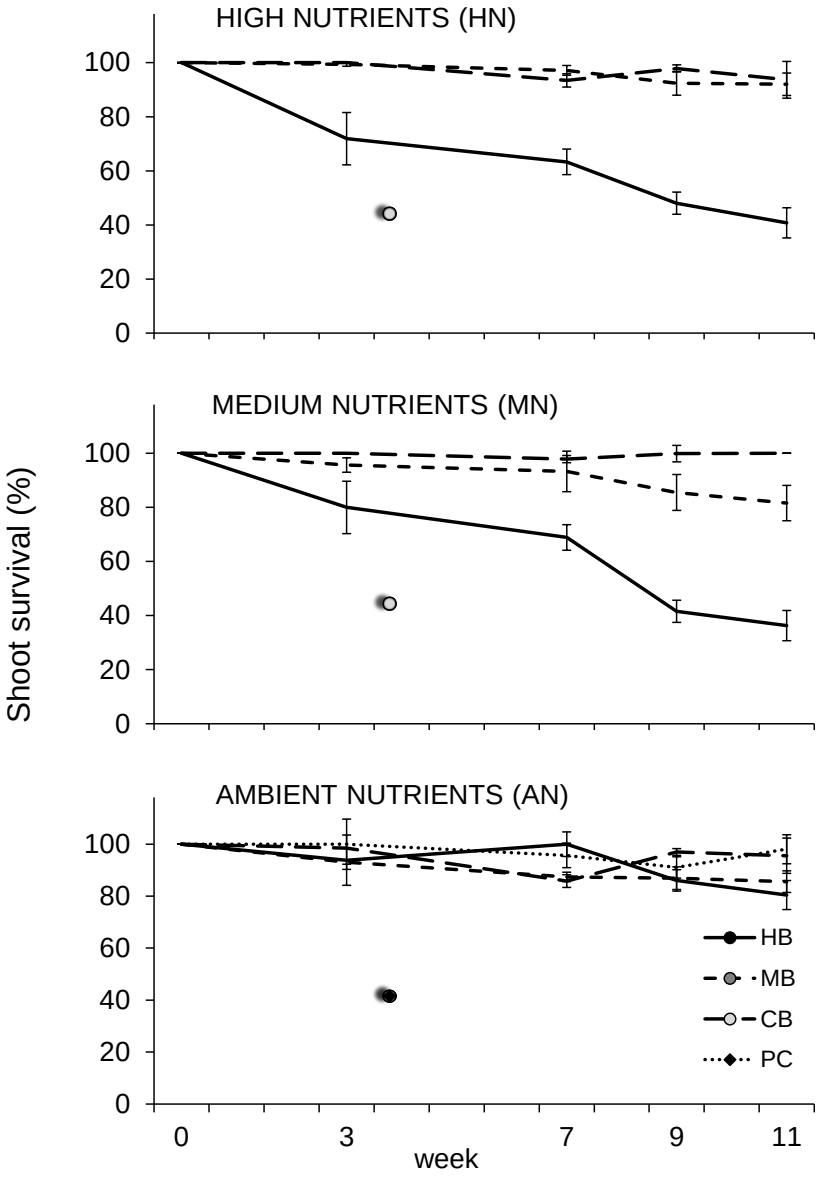

HIGH NUTRIENTS (HN)
MEDIUM NUTRIENTS (MN)
AMBIENT NUTRIENTS (AN)
Shoot survival (%)
100
80
60
40
20
0
0
3
7
9
11
week
HB
MB
CB
PC

**Fig 2**

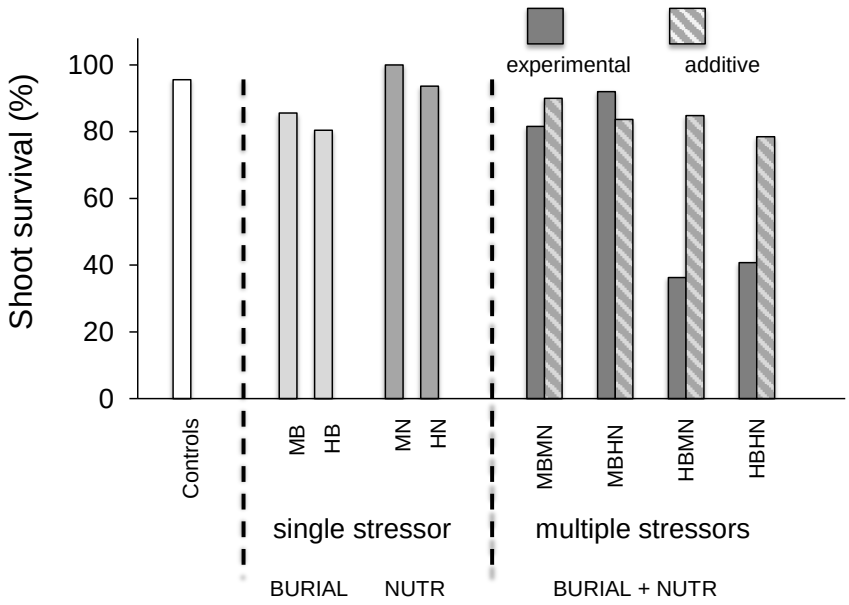

Shoot survival (%)
100
80
60
40
20
0
experimental
additive
Controls
MB
HB
MN
HN
MBMN
MBHN
HBMN
HBHN
single stressor
multiple stressors
BURIAL
NUTR
BURIAL + NUTR

Fig 3

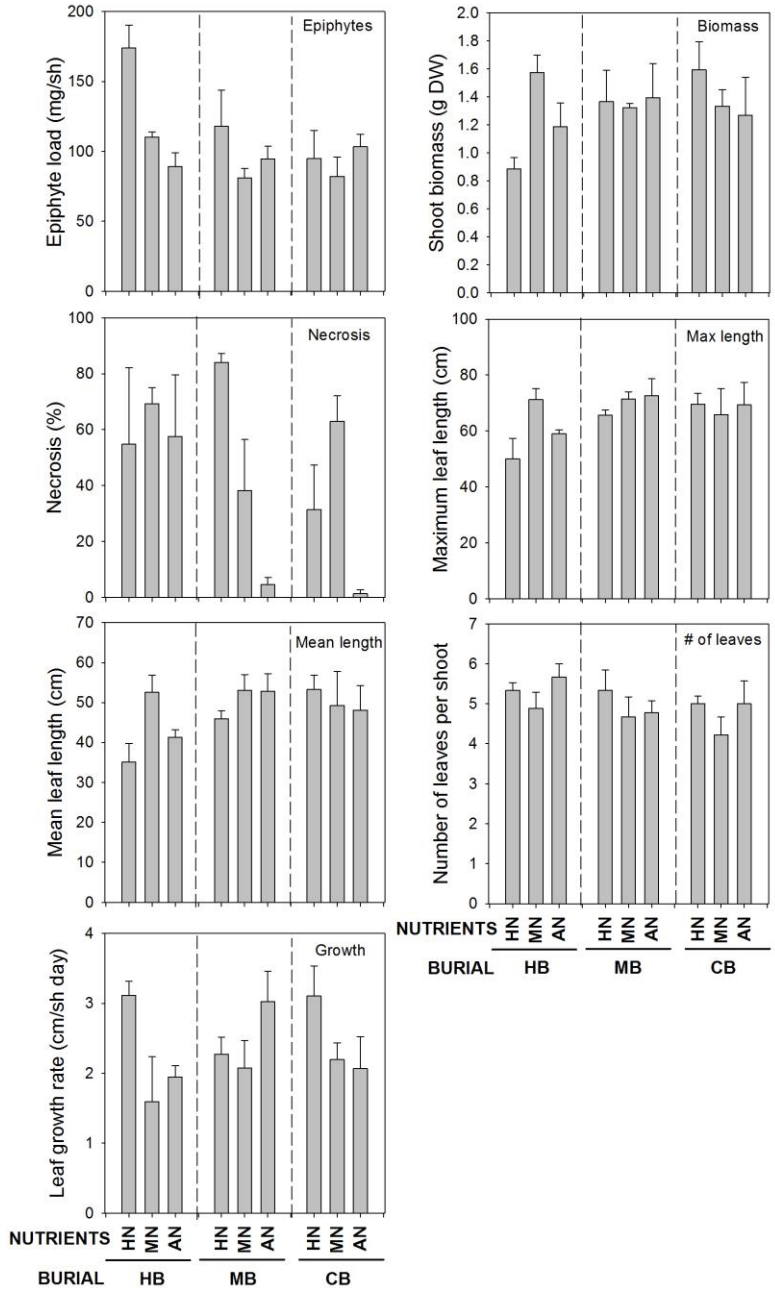

Epiphytes
Epiphyte load (mg/sh)
Biomass
Shoot biomass (g DW)
Necrosis
Necrosis (%)
Max length
Maximum leaf length (cm)
Mean length
Mean leaf length (cm)
# of leaves
Number of leaves per shoot
Growth
Leaf growth rate (cm/sh day)
NUTRIENTS HN MN AN HN MN AN HN MN AN
BURIAL HB MB CB

Fig 4

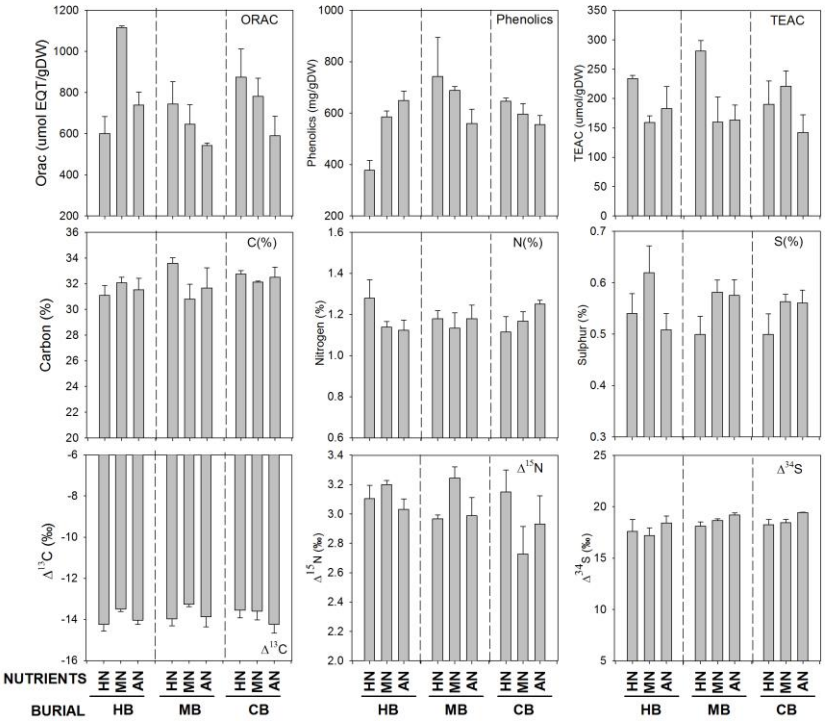

ORAC
Orac (umol EQT/gDW)
Phenolics
Phenolics (mg/gDW)
TEAC
TEAC (umol/gDW)
C(%)
Carbon (%)
N(%)
Nitrogen (%)
S(%)
Sulphur (%)
Δ13C (‰)
Δ13C
Δ15N
Δ15N (‰)
Δ34S
Δ34S (‰)
NUTRIENTS
HN MN AN
BURIAL
HB
MB
CB

Fig 5

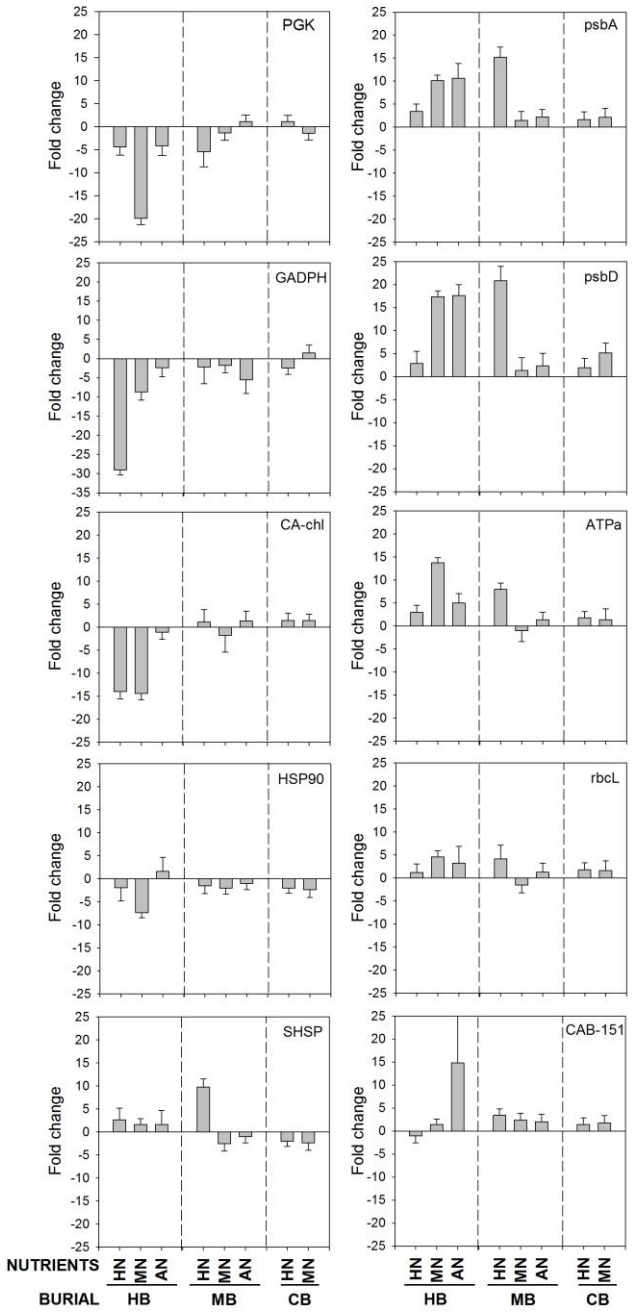

PGK
psbA
GADPH
psbD
CA-chl
ATPa
HSP90
rbcL
SHSP
CAB-151
Fold change
NUTRIENTS
HN MN AN HN MN AN HN MN HN MN AN HN MN AN HN MN
BURIAL
HB MB CB HB MB CB

Fig 6

# Seagrass collapse due to synergistic stressors is not anticipated by phenological changes

Giulia Ceccherelli, Silvia Oliva, Stefania Pinna, Luigi Piazzi, Gabriele Procaccini, Lazaro Marin-Guirao, Emanuela Dattolo, Roberto Gallia, Gabriella La Manna, Paola Gennaro, Monya M. Costa, Isabel Barrote, João Silva, Fabio Bulleri

**Supplementary material**

Table S1. List of genes analysed. Sequence of primers and stability curves are reported in the reference in which the genes have been selected for the first time. T: target genes; R: reference genes.

| Gene name | Symbol | References | Type |
|---|---|---|---|
| Photosystem II protein D1 | PSbA | Dattolo *et al*. 2014 | T |
| Photosystem II protein D2 | PSbD | Dattolo *et al*. 2014 | T |
| ATP synthase subunit alpha | ATPa | Marín-Guirao *et al.* 2016 | T |
| Chlorophyll a-b binding protein 151 | CAB-151 | Dattolo *et al*. 2014 | T |
| Phosphoglycerate kinase | PGK | Dattolo *et al*. 2017 | T |
| Carbonic anhydrase, chloroplastic | CA-chl | Dattolo *et al*. 2017 | T |
| Glyceraldehyde-3-phosphate dehydrogenase | GADPH | Serra *et al.* 2012 | T |
| Rubisco large subunit | rbcL | Marín-Guirao *et al*. 2016 | T |
| Heat Shock Protein 90 | HSP90 | Lauritano *et al*. 2015 | T |
| Small Heat Shock Protein | SHSP | Lauritano *et al.* 2015 | T |
| 18S ribosomal RNA | 18S | Serra *et al*. 2012 | R |
| Elongation factor 1 alpha | EF1A | Serra *et al.* 2012 | R |
| Ribosomal protein L23 | L23 | Serra *et al*. 2012 | R |

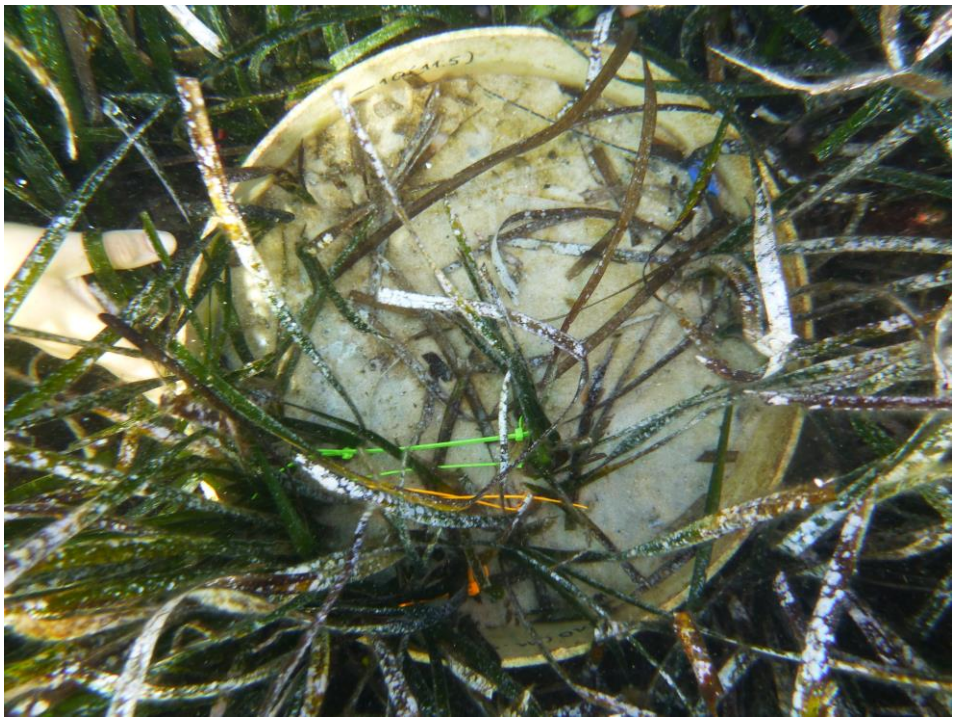


**Fig. S1** PVC cylinder bordering an HBHN experimental unit. The coloured cable ties indicate shoots punched for leaf growth rate estimates.

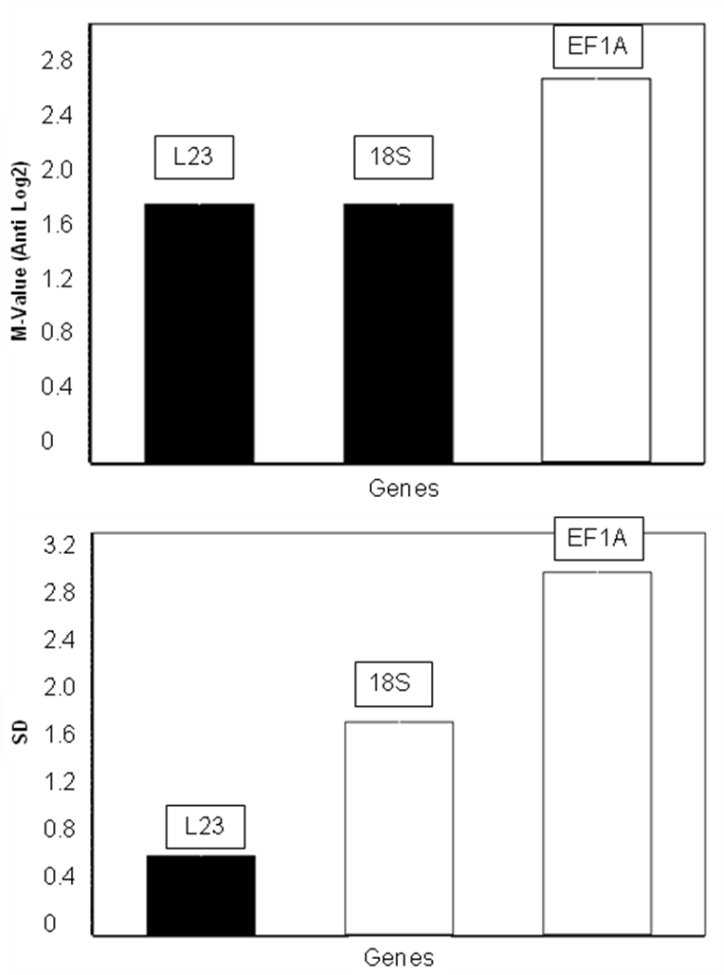
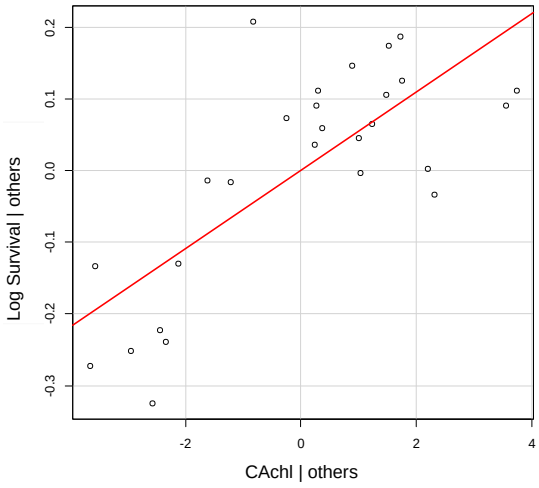



**Fig. S2** Reference genes. Assessment of the stability of three putative reference genes. More stable genes are the ones in black, according to the software geNorm (top panel) and NormFinder (bottom panel). Both L23 and 18S have been utilized as reference genes in the analysis.



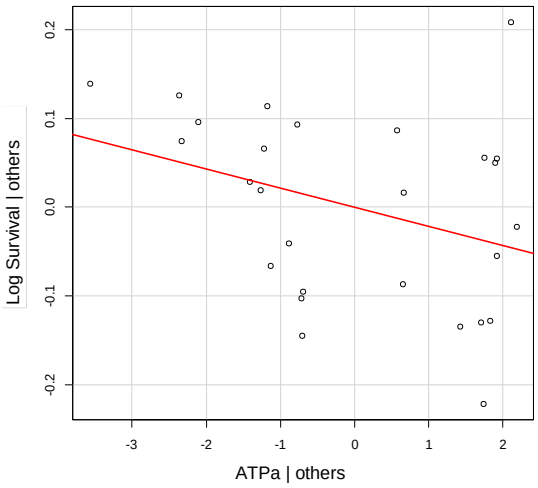




**Fig. S3** Partial regression plots for each of the variables retained by the best-fit multiple regression model at the level of gene expression.

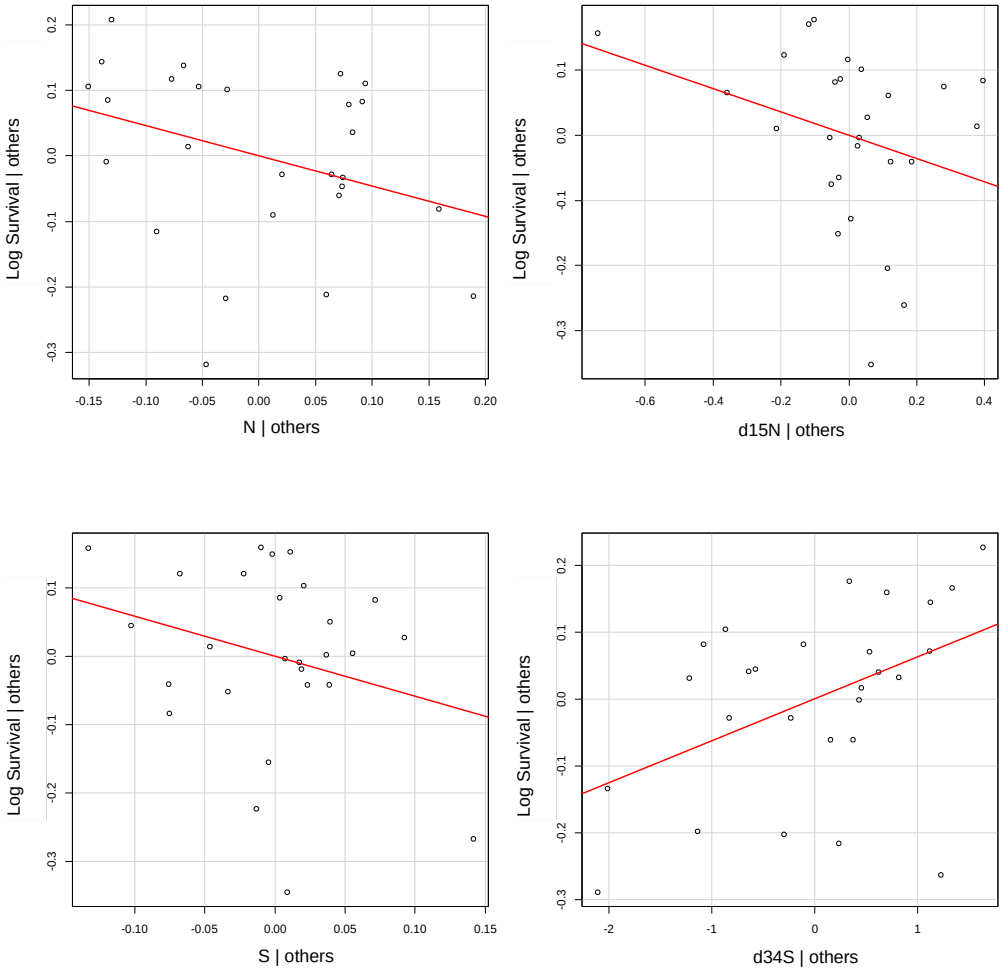



**Fig. S4** Partial regression plots for each of the variables retained by the best-fit multiple regression model at the level of isotopes.

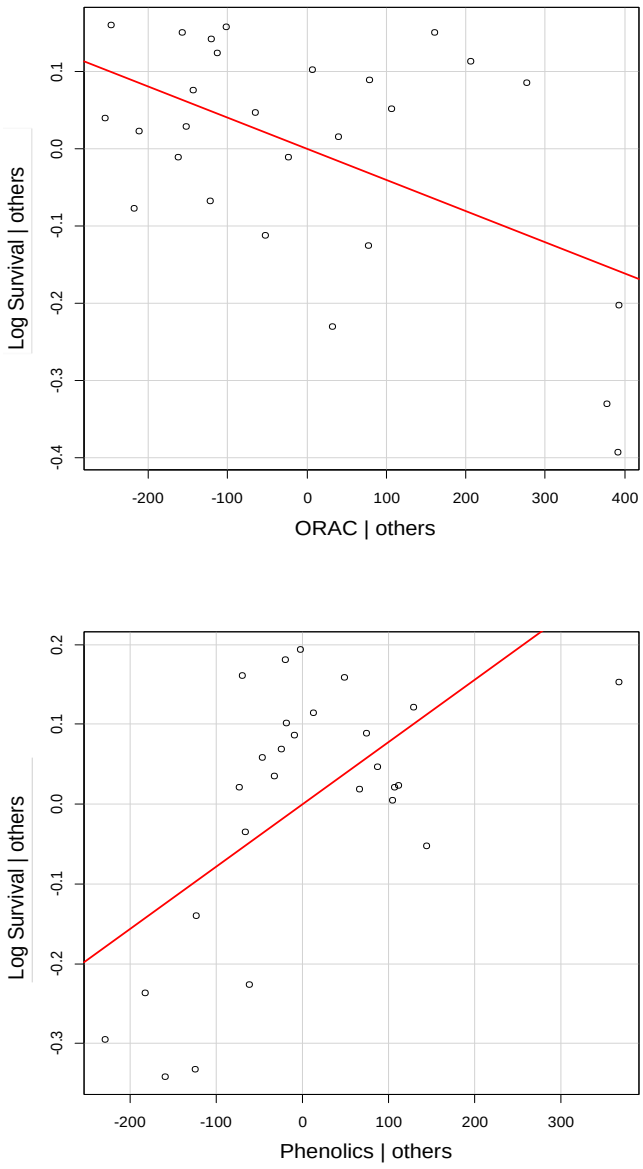



**Fig. S5** Partial regression plots for each of the variables retained by the best-fit multiple regression model at the level of antioxidant.

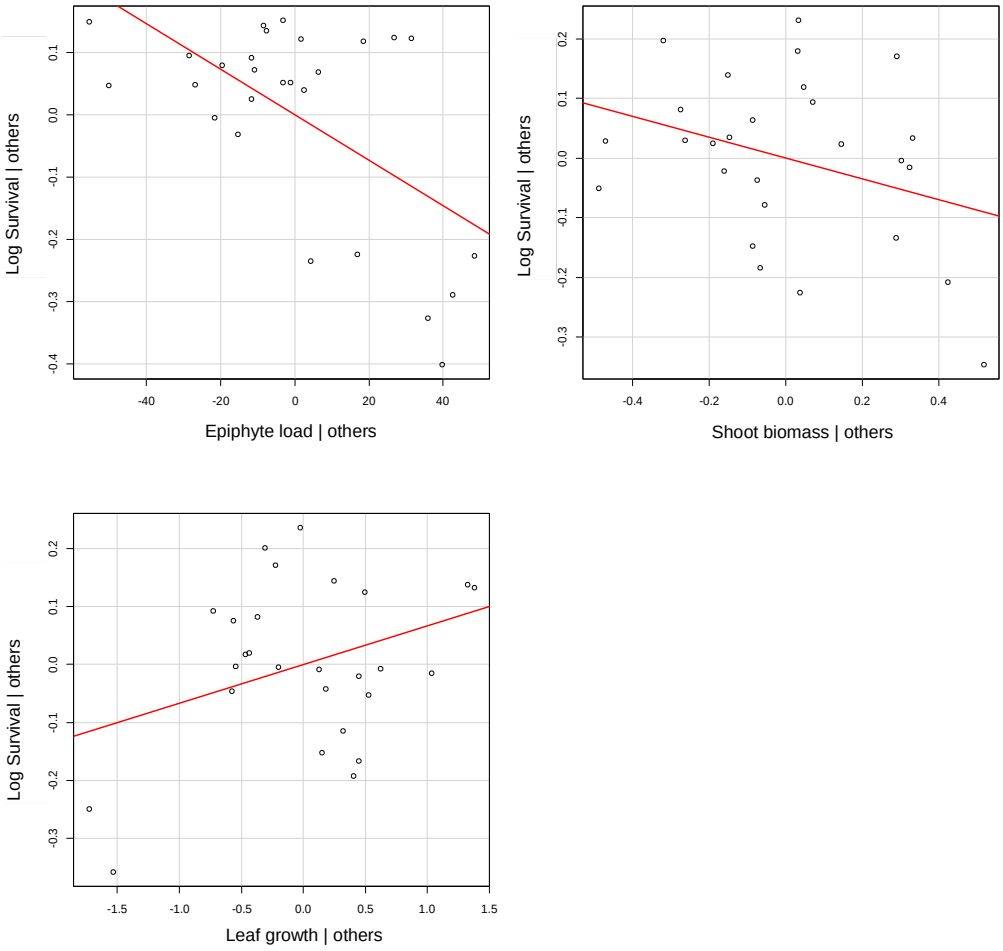



**Fig. S6** Partial regression plots for each of the variables retained by the best-fit multiple regression model at the level of plant morphology/growth**.**