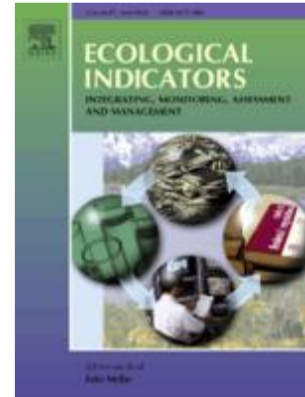


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**Metabolomics and traditional indicators unveil stress of a seagrass (*Cymodocea nodosa*) meadow at intermediate distance from a fish farm**

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**Abstract**

Seasonal variation of structural, physiological and growth indicators and the metabolome of the seagrass *Cymodocea nodosa*, as well as biogeochemical conditions of underlying sediment were studied in two meadows growing at increasing distance downstream from a fish farm in the Aegean Sea in order to assess seagrass performance under stress. Horizontal rhizome production decreased significantly with proximity to the fish farm (0.67 and 1.57 g DW m<sup>-2</sup> d<sup>-1</sup>)

<sup>1</sup> close and far from the fish-farm, respectively). This coincided with observed effects on ecophysiological indicators, such as rhizome nitrogen, leaf carbon and leaf  $\delta^{13}\text{C}$ , which were elevated with proximity to the fish-farm. Seasonality was shown by some indicators being elevated in either in the warm (C of all tissues and leaf  $\delta^{34}\text{S}$ ) or the cold period (N of all tissues). Growth promoting metabolites (sucrose, fructose, myo-inositol, heptacosane, tetracosane, stigmasterol, catechin and alpha-tocopherol) were lower close to the zone, whereas metabolites involved with stress-response (alanine, serine, proline, putrescine, ornithine, 3,4-dihydroxybenzoic acid and cinnamic acid) were higher. We found that growth-promoting metabolites were positively correlated with horizontal rhizome production, whereas the metabolites related to stress were negatively correlated. Metabolomic fingerprinting of seagrass provides opportunities for early detection of environmental degradation in marine ecological studies.

Key-words: Mediterranean; early warning indicators; coastal management; eutrophication; omics; seagrass

## Introduction

Seagrasses are indicators of environmental deterioration, as meadow declines often point to disturbances that affect the entire ecosystem (Orth et al., 2006). ‘Critical slowing down’ or mortality events show that meadows have reached a tipping point, after which there is low capacity for recovery (El-Hacen et al., 2018). Environmental stress plays the biggest part in plant growth and development (Steinberg, 2011) and therefore stress needs to be identified far earlier to avoid permanent loss of seagrasses. Many different indicators are used to evaluate seagrass health in Europe, with little overlap between regions, creating difficulty in making continent-wide assessments (Marbà et al., 2013). Traditional ecological indicators involve nutrient availability in water and sediment (Fourqurean et al., 1992; van Katwijk et al., 2011), structural variables like shoot density and biomass (Agawin et al., 1996; Ibarra-Obando et al., 2004), physiological variables such as elemental and isotopic composition of seagrass tissue (Christianen et al., 2012; M Pérez et al., 1991), and growth such as rhizome production (Duarte et al., 1994), all of which have been identified as good indicators of seagrass stress (Roca et al., 2016). The response time of traditional indicators is varied, with physiological indicators generally responding far more rapidly (leaf  $\delta^{15}\text{N}$  = ~8 weeks, leaf N = ~2-15 weeks), than structural (shoot density = ~5-25 weeks, above ground biomass = ~10-50 weeks) or growth indicators (leaf growth = ~2-20 weeks) (Roca et al., 2016).

Omics tools promise earlier and more targeted detection of stress (Malandrakis et al., 2017a), as they have the potential to reveal how seagrasses respond to abiotic stress, and how tolerance is obtained (Exadactylos, 2015), which could contribute to seagrass management and conservation (Macreadie et al., 2014). Since the turn of the century, and the genome sequencing of the model plant *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000), these tools have helped gain a deeper understanding into the molecular function of plants. Among omics, the metabolome provides the best indication of the physical composition of the biological

system in question (Horgan et al., 2009). Metabolomics involves detecting and measuring small molecules (<10 kDa), called metabolites, produced by cells during their lifetime (Fiehn, 2002). Due to their position as the end product of gene transcription, changes in metabolites are amplified compared to changes in the transcriptome or proteome (Urbanczyk-Wochniak et al., 2003). Hereby allowing earlier detection of changes in complex biological systems (Peng et al., 2015), making it an accurate tool to determine abiotic stress in plants (Jorge et al., 2016). For instance, plants biosynthesize specialized metabolites, such as the amino acids alanine and proline, in order to cope with environmental stress when stress response genes are expressed (Nakabayashi & Saito, 2015). Low levels of non-structural carbohydrates like soluble sugars can also be precise indicators of environmental stress in plants (Jiang et al., 2013). The observable traits of plants (e.g. biomass, growth rate, etc.) can be greatly influenced by resistance and stress response metabolites (Bino et al., 2004). Combining metabolomics with traditional indicators provides advantages for ecological monitoring. While traditional indicators deliver information on physiological state, environmental quality, and structural growth (Roca et al., 2016), the metabolome compliments these by presenting the chemical response of the plant (Nakabayashi & Saito, 2015), providing a holistic impression of the biological system. Metabolites change in response to environmental stimuli (Malandrakis et al., 2017a), and have cascading effects on indicators such as growth rate (Meyer et al., 2007), therefore exploring these relationships provides information on how changes in the metabolome are projected into changes in the measurable traits of a plant. Nevertheless, understanding of the molecular biology of seagrasses is very limited compared to what is known about terrestrial plants (Davey et al., 2016) and research into seagrass metabolomics is in its infancy (Kumar et al., 2016). Until now, there are only a few studies addressing the response of seagrass metabolites to environmental changes, which suggested that metabolomics was a useful tool in the early detection of *Zostera marina* stress previously

unidentifiable (Hasler-Sheetal et al., 2016, 2015). Once more information is gathered concerning stress-response, seagrasses can become even better bioindicators of marine ecosystem health (Malandrakis et al., 2017a), in particular by integrating them into traditional ecological monitoring programs (Kletou et al., 2018).

The seagrass *Cymodocea nodosa* forms meadows throughout the Mediterranean Sea and into the Atlantic. It is abundant in the Aegean Sea, where it grows in coastal waters up to 40 m depth (Borum & Greve, 2004). It is the most common species in shallow protected to semi-exposed sub-tidal environments (Orlando-Bonaca et al., 2016). *C. nodosa* is regarded as the most adaptable Mediterranean seagrass (Boudouresque et al., 2009), being more tolerant of nutrient enrichment (Delgado et al., 1997), heat fluctuations (Malandrakis et al., 2017b; Tutar et al., 2017), salinity stress (Piro et al., 2015), and general environmental stress (Garrido et al., 2013). *C. nodosa* is a suitable bioindicator species to assess anthropogenic stress in the marine environment as tissue-based metrics such as nitrogen content in the leaves can be used to distinguish meadows under stress (Papathanasiou et al., 2016).

Limited work has been carried out on the impact of fish farming on *C. nodosa*, however, in one study *C. nodosa* showed degraded growth and mortality 100 m from a fish-farm (Delgado et al., 1997). There are no guidelines in place to protect *C. nodosa* from fish farming, unlike the other Mediterranean seagrass species *Posidonia oceanica* which has narrow protections, such as the 400 m safety buffer recommendation between fish-cages and seagrass beds (Holmer et al., 2008). When factoring in current velocities, the intermediate distances are also impacted by fish-farming (Tsagaraki et al., 2013). There are several studies showing that the greatest effects of fish-farming on the suspended sections of the water column in the Mediterranean are between 100-500 m from the fish cages depending on current velocity (Sarà et al., 2006; Sarà, 2007; Tsagaraki et al., 2013). Intermediate impacts on the seagrass *P. oceanica* at distances 35-

400 m from fish-cages have caused vertical growth declines of 20-40 % of the pre-disturbed values (Marbà et al., 2006).

In this paper we assess the performance of *C. nodosa* meadows at intermediate distance from a fish farm using traditional seagrass-based indicators and metabolomics. We do so by quantifying the seasonal variability in nutrient availability in water, pore water and sediment, along with structural (density and biomass), physiological (elemental and isotopic composition of carbon, nitrogen and sulfur) and growth indicators (rhizome production) and the metabolome of seagrasses growing at intermediate distance and away from a fish farm in the Aegean Sea (Greece). We also explore the relationships of *C. nodosa* metabolites of stress and growth with horizontal production, to examine if *C. nodosa* has obstructed performance. The combination of traditional ecological indicators and metabolomics provides more understanding of how environmental stressors affect seagrass stress response. This is the first work to combine traditional seagrass-based indicators and metabolomics to reveal stress of seagrass meadows.

# Materials and Methods

## Sampling Strategy

Sampling was carried out at Vourlias Bay (Peloponnese, Greece), which is an allocated aquaculture zone with intense fish-farming activity (9 farms). The site was located approximately 2 km east of the main bay, separated by a headland where there is a fish-farm with 28 cages ( $37^{\circ} 27' 08''$  N  $23^{\circ} 04' 20''$  E) (Fig. 1). Apart from this aquaculture facility, there were no other features (e.g. rivers, towns) that could cause a disturbance to the environment. Two sampling stations were selected which will henceforth be referred to as 'Station A' and 'Station B' at 440 m and 780 m distance from the fish-farm, respectively. The seagrass meadow at Station A was the closest we could find to the fish-farm. Phosphorous sedimentation in seagrass meadows of the Mediterranean, which has been proved a reliable indicator of fish-farm waste, has been shown to decrease exponentially with distance from the cages and the sedimentation rates were roughly 100% and 40% higher at 250 m and 500 m from the fish-farm, respectively, than at non-impacted sites (Holmer et al., 2008). Yet, seagrass meadows growing 800 m downstream from fish farms have shown no evidence of impact (Marbà et al., 2006). Based on the above, it could be expected that there is an effect at 440 m (Station A) and that this effect should be low at Station B, and therefore we can expect to detect differences between stations. The predominant coastal currents in the area move from the North-West, travelling through Vourlias Bay and towards our sample sites, passing the adjacent fish farm. Station A is situated in a small bay where the currents circulate, whereas the currents pass Station B and move down the coast to the South-East (data taken from model described in Tsagaraki et al., 2011).

The site was visited twice, during a warm (June 2017) and a cold (March 2018) period, when water temperature was  $29^{\circ}$  C and  $15^{\circ}$  C respectively. The water depth was 8 m at both stations.

At each station we randomly collected 3 replicates (100 mL each) of bottom water using acid-washed plastic syringes, just above the seagrass canopy. Pore water samples (60 mL each, 3 replicates per station) were also collected by acid-washed plastic syringe with a perforated sipper which extracted water from the sediment down to 10 cm depth.

Bottom and pore water samples were filtered using pre-combusted filters (Whatman GF/F). Bottom water (25 ml) and pore water (900  $\mu$ l) samples were fixed with 10 mL of 10 mM and 100  $\mu$ l of 100 mM zinc acetate, respectively, and kept frozen for the determination of isotopic composition  $^{34}\text{S}$  of sulfate. The remaining filtered water and pore water were kept frozen, and later analyzed for ammonium and phosphate. The filters were kept frozen for the determination of particulate organic carbon and particulate total nitrogen.

Sediment samples (4 replicates, i.d. 4.5 cm) were collected by divers using Plexiglas cores, which were randomly inserted in the sediment down to 10 cm depth. Subsamples were taken from three of the replicates, and promptly fixed using 1 M zinc acetate and kept frozen at -20  $^{\circ}\text{C}$ , to prevent degradation for the determination of the  $^{34}\text{S}$  sulfide isotopic ratio. The remaining sediment was also frozen and later used for analysis on total organic carbon (TOC), total inorganic carbon (IC), total nitrogen (TN),  $^{13}\text{C}$  and  $^{15}\text{N}$ . The fourth sediment sample replicate was frozen and subsequently analyzed for granulometry.

Samples for measuring *C. nodosa* density, biomass, elemental and isotopic content, and metabolomics were collected using an aluminium cylindrical corer of area 177  $\text{cm}^2$ . At each station, 5 replicates were randomly taken. When out of the water, these samples were immediately processed to avoid stress to the plants, in order to ensure an accurate metabolic profile. At each station we also randomly hand-collected an additional 6-10 long (40 cm) horizontal *C. nodosa* ramets for analysis of yearly horizontal rhizome production. Seagrass core samples were washed in seawater to remove sediment, and the number of shoots (apical and vertical) were counted for shoot density. The two youngest leaves, rhizomes and root

bundles from each of the 5 replicates were rinsed using milliQ water and scraped free from epiphytes before they were put in liquid nitrogen and later stored at -80 °C, to be analysed for metabolites and nutrient content. The remaining seagrass tissue was kept frozen.

#### Laboratory Analysis

Ammonium analysis of water samples was carried out using the indophenol blue method (Ivancic & Degobbis, 1984) and determination of phosphate was carried out as described by Strickland and Parsons (1972). Samples for measuring  $\delta^{34}\text{S}_{\text{sulfate}}$  were treated by being boiled in acid before sulphate precipitation with  $\text{BaCl}_2$  resulting in  $\text{BaSO}_4$ . Particulate organic carbon (POC) and particulate organic nitrogen (PON) were measured from the frozen filters. The filtrate was analysed using an elemental analyser according to the Hedges & Stern (1984) procedure.

Sediment was dried at 60 °C for 48 hours to attain density, water content and porosity measurements. The dried sediment was pulverised before being measured for elemental and isotopic composition. Total carbon, nitrogen and  $^{15}\text{N}$  were analysed in sediment as it was. The sediment was then acidified for the determination of organic carbon (OC) and  $^{13}\text{C}$ . The inorganic carbon (IC) was estimated as the difference between total carbon and OC. The elemental and isotopic composition was expressed in % and  $\delta$  unit notation (‰ deviations from the established international standards ratio), respectively.

For sediment  $^{34}\text{S}$  sulfide measurements we used the two-step distillation method (Fossing and Jorgensen, 1989) to obtain the chromium reducible sulfur (CRS) and acid volatile sulfur (AVS) fractions. A 5-10 g fraction of homogenised sediment was combined with 10 mL 50% ethanol in a reaction flask, and degassed with  $\text{N}_2$  for 10 minutes. The AVS fraction was attained by distilling the mixture for 30 minutes at room temperature with 8 mL 12M HCl. The CRS fraction required reduction using 1M  $\text{Cr}_2^{+}$  in 0.5M HCl, and then boiling and distilling for 30 minutes. The sulfide extract was collected as  $\text{Ag}_2\text{S}$  precipitate in traps and  $\delta^{34}\text{S}$  was analysed

as described below. Granulometry assessment of the sediment was carried out by removing all grains larger than 63  $\mu\text{m}$  (sand) by wet sieving. The remaining sediment was then categorised using a Sedigraph (Micromeritics 5100) into < 63  $\mu\text{m}$  (silt) and > 0.1  $\mu\text{m}$  (clay).

The young seagrass tissue was freeze-dried to be analyzed for total sulfur (TS) and the  $\delta^{34}\text{S}$  isotopic fraction and their metabolic profile. The remaining (leaves, rhizomes and roots) tissue, which had not been quenched in liquid nitrogen, was dried at 60 °C for 48 hours and homogenized with the young tissue to determine dry weight. Young and remaining tissue were ground to a powder to be used for analysis of elemental and isotopic composition on total carbon (TC), nitrogen (TN),  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$  using elemental analyser combustion continuous flow isotope ratio mass spectroscopy. The  $\delta^{34}\text{S}$  measurement required the ground up seagrass tissue to be weighed into tin capsules along with vanadium pentoxide.

Metabolites were extracted, analysed and annotated as described in Hasler-Sheetal et al 2016. In brief, we extracted metabolites from freeze-dried seagrass tissue and analysed by GC-qtof-MS after derivatisation. The data was inspected, annotated and analysed using MassHunter and Mass Profiler Professional (Agilent Technologies, Santa Clara, USA).

Yearly horizontal production rates were determined through the measurement of the horizontal internodal lengths (the length between leaf scars) on the horizontal ramets (Duarte et al., 1994), in order to determine the leaf plastochrome interval (PI). All ramets were dried at 60 °C for 48 hours. The measurements were performed on a Zeiss model Discovery.V12 stereo microscope with a Jenoptik ProgRes® C14 plus camera addition and the aid of ProgRes® CapturePro image capture software.

## Calculations

Horizontal rhizome production ( $\text{g DW m}^{-2} \text{y}^{-1}$ ) was calculated from the measured rhizome internodal lengths. First a 30% running average was applied to the data, in order to account for

short-term variability (Duarte et al., 1994; Mishra et al., 2018), and then the distances between consecutive minima recorded, this estimates the annual horizontal growth. The sequence of internodal length along the horizontal rhizome was measured according to Duarte et al. (1994), which allowed an estimation of annual horizontal rhizome elongation rate per apex. The following formula was then applied to the data:

$$\text{Horizontal rhizome production} = \frac{\text{annual production} \times \text{horizontal weight} \times \text{apex density}}{\text{horizontal length}}$$

Where annual production ( $\text{cm y}^{-1}$ ) is each annual cycle measured from the consecutive minima, horizontal weight (g) is the total dry horizontal rhizome weight of the ramet, apex density ( $\text{m}^{-2}$ ) is the number of apical shoots per metre, and horizontal length (cm) is the total length of the ramet. We display here the mean production measurements of the two sampling campaigns.

Biomass ( $\text{g DW m}^{-2}$ ) was estimated as the dry weight of the seagrass tissue; leaves, rhizomes or roots, divided by the sampled area. Shoot density ( $\text{shoots m}^{-2}$ ) was estimated as the number of shoots divided by the sampled area. Biomass and density measurements of the three seagrass tissues were obtained by multiplying the measured value by the surface area of the aluminium cylindrical corer.

The percentage of sulfur in seagrass leaves, rhizomes and roots taken up from sediment sulfide ( $F_{\text{sulfide}}$ ) was calculated using the equation:

$$F_{\text{sulfide}} = \left( \frac{\delta^{34}\text{S}_{\text{tissue}} - \delta^{34}\text{S}_{\text{sulfate}}}{\delta^{34}\text{S}_{\text{sulfide}} - \delta^{34}\text{S}_{\text{sulfate}}} \right) \times 100$$

In this formula  $\delta^{34}\text{S}_{\text{tissue}}$  is the measured  $\delta^{34}\text{S}$  in the leaf, rhizome or root,  $\delta^{34}\text{S}_{\text{sulfate}}$  in the water, and  $\delta^{34}\text{S}_{\text{sulfide}}$  content of the sediment (Holmer & Kendrick, 2013).

Statistical analyses

Before statistical testing, we carried out normality and homogeneity checks in SPSS (IBM Corp., 2017) which included using Cochran's Q test. Two-way Analysis of Variance (ANOVA) in SPSS was used to test for differences between stations and seasons. Principal component analyses (PCA) were carried out using the R 'stats' package (R Core Team, 2013) and MetaboAnalyst 4.0 (Chong et al., 2018) in order to identify differences between stations or seasons. Missing values in the metabolomics dataset were calculated using a K-nearest neighbour algorithm, variables with over 50% missing values were discarded. Data was normalised by median, log transformed, and auto-scaled (mean-centred and divided by the standard deviation of each variable). The most influential metabolites in separating the seasons and stations along the principal components (PC) were identified by inspecting the loadings for each PC. Heatmaps were used to visualise how stress and growth-related metabolites differed between stations. Linear regression analysis was carried out on the  $F_{\text{sulfide}}$  data in order to identify sulfide intrusion and also in order to detect possible relationships between metabolites and growth. Correlations were carried out using Spearman's rho to assess whether stress and growth associated metabolites showed positive or negative trends with horizontal rhizome production. The statistical significance of these correlations were tested using one-tailed and two-tailed t-tests. As it was possible to predict the trend direction in advance, using one-tailed t-testing was appropriate. As two-tailed t-tests protect against type-I errors and cognitive biases, we also report it.

## Results

Pore water ammonium and sediment CRS pools and TN were elevated by 78%, 73% and 170%, respectively, at Station A compared to Station B (Table 1). The other measured biogeochemical variables (bottom and pore water  $\text{PO}_4$  and  $\delta^{34}\text{S}$ , bottom water  $\text{NH}_4$ , POC and PN, and sediment OC, IC,  $\delta^{15}\text{N}$  and  $\delta^{34}\text{S}$  sulfide pools) were not affected by station, except for sediment  $\delta^{13}\text{C}$  and AVS pools, but this depended on season.  $\delta^{13}\text{C}$  signal was highest during the warm period, and AVS pools had an interaction effect between station and season, where the biggest variation between stations was during the cold period. The other values reached significantly higher values during either the warm (pore water  $\text{PO}_4$ , sediment OC, TN,  $\delta^{13}\text{C}$ ) or the cold period (bottom water  $\text{PO}_4$ , sediment IC and  $\delta^{15}\text{N}$ , Table 1). The sediment grain size distribution was similar at the two sites (Station A = 91% sand, 9% silt and clay; Station B = 93% sand, 7% silt and clay).

Horizontal rhizome production and root biomass were decreased two-fold at Station A compared to Station B (Table 2). Rhizome biomass had an interaction effect between station and season, with the biggest variation between seasons at Station A, twice as high in the warm period. The majority of the structural variables were enhanced during the warm period (shoot density by 83 %, leaf biomass by 218 %, total biomass by 113 % and above:below ground biomass ratio by 100, Table 2).

The majority of the measured elemental concentrations and isotopic values were significantly elevated at Station A compared to Station B: Leaf C by 1.5 %, rhizome N by 27 %, root N by 37 %, rhizome S by 9.2 %, root S by 30 %, leaf  $\delta^{13}\text{C}$  by 11 %, rhizome  $\delta^{13}\text{C}$  by 2.4 %, root  $\delta^{13}\text{C}$  by 1.3 %, leaf  $\delta^{34}\text{S}$  by 0.9 %, root  $\delta^{34}\text{S}$  by 2.8% (Table 2). Some variables (S of leaves and rhizomes,  $\delta^{15}\text{N}$  of all tissues, and rhizome  $\delta^{34}\text{S}$ ) also had a station effect, but this depended on the season (Table 2). Seasonality was shown by many of the variables being elevated either

in the warm (C of all tissues and leaf  $\delta^{34}\text{S}$ ) or the cold period (N of all tissues, Table 2).  $F_{\text{sulfide}}$  did not show significant differences between stations or sampling periods.  $F_{\text{sulfide}}$  linear regressions of leaves and rhizomes ( $R^2 = 0.58$ ,  $p < 0.01$ ) and of rhizomes and roots ( $R^2 = 0.39$ ,  $p < 0.05$ ) were positively related indicating sulfide intrusion in the plants from the roots.

PCA analysis on metabolite levels in *C. nodosa* leaves revealed clear separations between stations but not seasons (Fig. 2). Metabolites in rhizome tissue showed a seasonal difference, and root metabolites separated mainly by season. However, there was also separation between Station A and B during the winter.

Mass spectrometry detected 203 leaf metabolites. A portion of them were related to seagrass stress or growth and varied between stations (Fig. 3). Analysis of the *C. nodosa* metabolome was carried out on leaves and roots. Particular focus was given to the leaves as this is the most active metabolic tissue in plants which has shown the biggest changes in metabolite composition in response to abiotic stress (Obata et al., 2015). Growth and stress-related metabolites varied between stations (Fig. 3, Table 3). Growth-promoting metabolites including sugars and sterols were lower at Station A. The growth metabolite with the largest difference between stations was fructose (2.5 times lower at Station A). Several stress-indicating metabolites including some amino acids were higher at Station A, and two metabolites known to decrease during abiotic stress (2-oxoglutaric acid and stearic acid) were lower at Station A (Fig. 3). Remarkably putrescine was 112 times higher at Station A. Spearman correlations between horizontal rhizome production and growth-promoting metabolite levels in the leaves yielded positive correlations (Table 4), the strongest significant ( $p < 0.05$ ) correlation being with the sugar myo-inositol ( $r_s = 0.647$ ). Spearman correlations between horizontal rhizome production and stress-indicating metabolites yielded significant negative correlations except for 2-oxoglutaric acid, which showed a positive correlation. The strongest correlation was with putrescine ( $r_s = -0.624$ , Table 4). Linear regression analysis showed a significant negative

relationship between horizontal rhizome production and alanine, and a significant positive relationship between horizontal rhizome production and sucrose (Fig. 4).

Of the 20 most significant (ANOVA) metabolites in root tissue, a large proportion of amino acids were higher at Station A during cold period (Fig. 5). We found significant negative relationships between leaf glutamate and horizontal rhizome production ( $R^2 = -0.45$ , Fig. 6), and leaf  $\delta^{15}\text{N}$  ( $R^2 = -0.43$ ), this establishes a link between the traditional ecological indicators and metabolomics.

## Discussion

Traditional seagrass-based indicators and metabolomic analyses revealed that the performance of *C. nodosa* was altered under abiotic stress from aquaculture effluents. The traditional indicators demonstrate higher nutrient loading and lower growth at Station A. Significant elevation of CRS pools and TN in the sediment and pore water ammonium at Station A suggests that the benthos was impacted by effluents from the fish-farm, similar to high sediment TN measured in an organically enriched *C. nodosa* meadow in Crete [0.8 % DW (Apostolaki et al., 2018)]. A comparable two-fold increase in pore-water ammonium with proximity to fish-cages has been found previously in the Mediterranean (Apostolaki et al., 2011). Decrease in root biomass has also been observed elsewhere, due to shading (Martin et al., 2018), salinity increases (Pagès et al., 2010) and effects of fish farming (Delgado et al., 1999), revealing that *C. nodosa* was under stress. Similarly, lower rhizome production at Station A suggests impacts of fish-farm effluent with proximity to the source, comparable to a two-fold reduction in *P. oceanica* growth after the installation of fish-cages within 400 m (Marbà et al., 2006). Nutrient availability has been shown to stimulate (Perez et al., 1991), or impair growth (Cunha & Duarte, 2004; Perez et al., 1994) depending on the degree of nutrient enrichment. The fact that the majority of elemental concentrations and isotopic levels in the three seagrass tissues were

higher at Station A implies a higher degree of impact from the fish-farm effluent (Marta Pérez et al., 2008), impairing growth. Leaf C and leaf  $\delta^{13}\text{C}$  at Station B were similar to the values reported from the Western Mediterranean for *C. nodosa* (Fourqurean et al., 2007), however, at Station A they exceeded these values (leaf C in warm period only), suggesting increased uptake. High growth demands in the summer result in seagrasses N demand exceeding the supply (Pedersen & Borum, 1992). This could explain why we found that N in all tissues followed a seasonal pattern comparable with previous studies, where the higher values were in the cold period not the warm (Fourqurean et al., 1997). Sulfide intrusion was moderate and ranged lower than previously reported (Apostolaki et al., 2018). [20 – 45 %, 25 – 45 %, 20 – 50 %, for leaves, rhizomes and roots respectively (Apostolaki et al., 2018)].

In accordance with the results of traditional indicators, analysis of the metabolome showed that the meadow at Station A has impaired growth, as seen by the decrease in the sugars sucrose, fructose and myo-inositol at Station A. The storage of carbohydrates in seagrass balances the use of carbon by respiration and growth (Alcoverro et al., 2001). Meadows growing in disturbed areas have been shown to contain lower carbohydrate levels in plant tissue, and lower biomass and productivity (Ruiz & Romero, 2003). Volatile organic compounds such as heptacosane and tetracosane, which were lower at Station A, have been identified as plant growth promoters (Jishma et al., 2017; Tahir et al., 2017). Stigmasterol, catechin and  $\alpha$ -tocopherol (vitamin E) all also have roles in promoting and regulating plant growth (Dufourc, 2008; Rani et al., 2011; Semida et al., 2014). As the majority of these ‘growth metabolites’ correlated positively with horizontal rhizome production, this indicates they have a positive association.

We found that metabolites known to accumulate under plant stress were high in seagrass at Station A, indicating reduced performance. During abiotic stress in plants, specific metabolites increase due to their signaling or protective roles. The amino acid metabolism is reconfigured

under stress, which leads to the accumulation of amino acids such as alanine, by way of the alanine and GABA shunts (Diab & Limami, 2016; Jorge et al., 2016; Rocha et al., 2010). Alanine has been found increased in seagrass under sulfide stress (Hasler-Sheetal et al., 2016). Polyamines regulate plant growth, development and stress responses (Shu et al., 2015). The polyamine putrescine is well known to accumulate in plants under stress (Alet et al., 2011; Cuevas et al., 2008), which corresponds well to our measurements of an increase of two orders of magnitude at Station A. Stress can also lead to depletion of some metabolites, as we have observed with 2-oxoglutaric acid and stearic acid at Station A. Stearic acid, and other fatty acids, have been shown to deplete under temperature stress in terrestrial plants (Liu & Huang, 2004). The organic acid 2-oxoglutaric acid has been shown to deplete in the leaves of water stressed soybean plants (Silvente et al., 2012). Despite their unique environment, we could expect seagrasses to have similar stress responses as terrestrial plants as stress response mechanisms are largely conserved throughout the plant kingdom (Akpınar et al., 2012). Ongoing abiotic stress is expected to lead to reduced plant performance, and therefore less growth, which is in accordance with the negative correlations found between ‘stress metabolites’ and horizontal rhizome production.

The increase of amino acids in the roots at Station A during the cold period, coinciding with higher pore water ammonium levels, could be explained by increased ammonium assimilation. Rapid entry of ammonium into the cells causes many changes to seagrass physiology including impaired ATP production and photosynthetic electron transport (Alexandre et al., 2018; Villazán et al., 2013). To minimize these effects, seagrasses rapidly assimilate ammonium and convert it into amino acids, which depletes carbon skeletons (Brun et al., 2002, 2008).

The GOGAT cycle, where nitrate or ammonium are ultimately integrated into amino acids, is recognised as the most important route of nitrogen assimilation in higher plants (Robinson et al., 1991), of which glutamate is a central metabolite. Glutamate can induce the expression of

stress related genes as part of plant stress response (Kan et al., 2017). It has been suggested that regulation of genes in this pathway could be used as an indicator for exposure to ammonium pulses in seagrass (Pernice et al., 2016). Passive ammonium transport is not regulated (Touchette & Burkholder, 2000), therefore uptake of ammonium by the plant continues. The significant negative relationships between leaf glutamate and leaf  $\delta^{15}\text{N}$ , and horizontal rhizome production indicate the usefulness of glutamate as an indicator for plant stress.

Combining metabolomics and traditional indicators provide opportunities to speed up monitoring. Leaf  $\delta^{15}\text{N}$  is currently among the most rapid purpose-specific indicators for nutrient stress in small seagrasses, and has a response time of around eight weeks, considerably faster than shoot density (Roca et al., 2016). Metabolomics could allow even earlier detection because metabolites are altered from the moment the plant stress response is activated (Nakabayashi & Saito, 2015). Integrating metabolomics and traditional indicators provides a comprehensive picture that is benefitted by evidence of metabolism response. The pattern of ‘stress’ and ‘growth’ metabolites presented in this study are supported by the observations by the traditional tissue nutrient and biogeochemical indicators; that abiotic stress is increased with proximity to the fish-farm, and therefore *C. nodosa* performance is impaired although it is widely considered a resilient species (Boudouresque et al., 2009). In future research, the metabolism of *C. nodosa* should be studied in more depth. This study would have benefited from an increased number of sample stations. Yet the results of this study indicate that metabolomic fingerprinting can serve as a reliable early warning indicator and help provide a more thorough picture of plant performance, in particular ‘stress’ or ‘growth’ associated metabolites should be considered when selecting new indicators for seagrass health assessment.

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**Declarations of interest:** none

## Figure Legends

Fig. 1. Map of sampling area in the Argolikos Gulf with stations A and B indicated. Fish farm cages are indicated by circles.

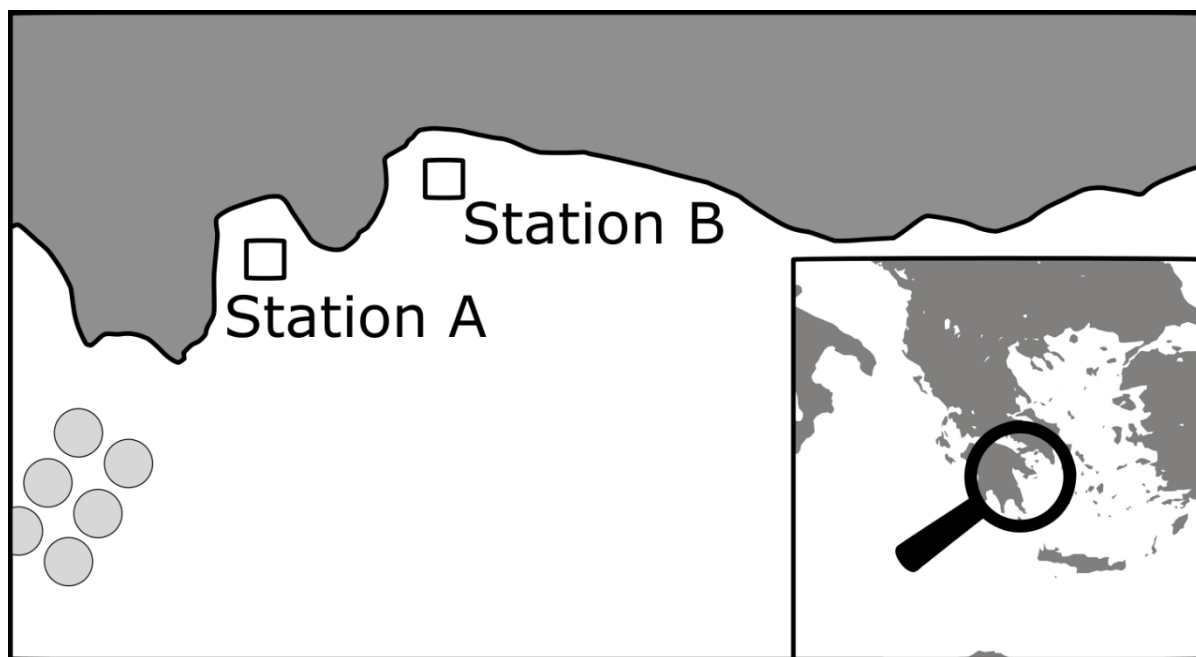
Fig. 2. Scores plots of PCA analyses of metabolite relative concentrations in *C. nodosa* leaves at the two sites, in two seasons.

Fig. 3. Heatmaps of metabolites in leaf tissue showing: (Left) plant growth associated metabolites, and (Right) Stress related metabolites in plants.

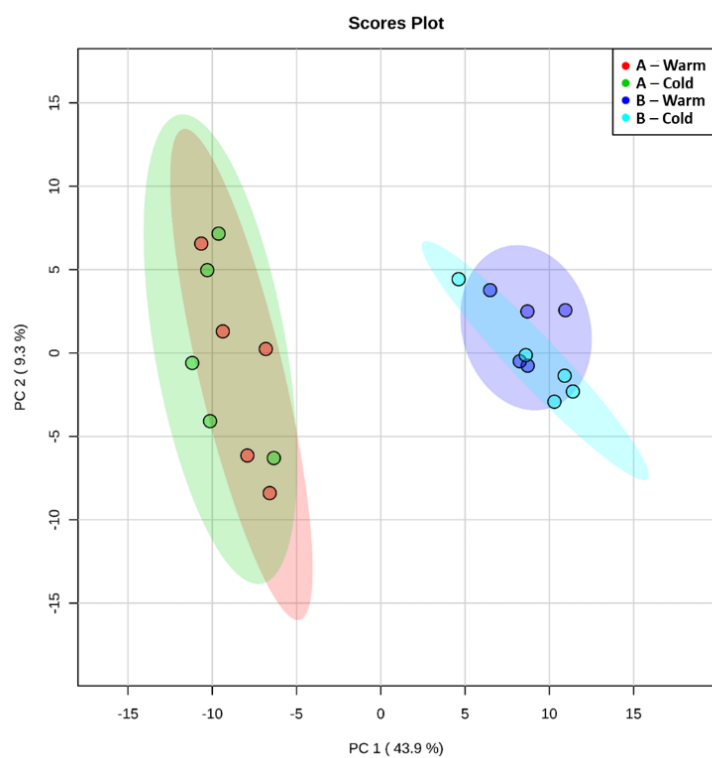
Fig. 4. Linear regressions between horizontal rhizome production and (Top) Sucrose and (Bottom) L-alanine.

Fig. 5. Heatmap of the 20 most significantly different root metabolites between station or sample site (2-way ANOVA).

Fig. 6. Linear regressions of (Top) Horizontal rhizome production and leaf glutamate, (Middle) Pore water ammonium and leaf  $\delta^{15}\text{N}$  and (Bottom) Leaf glutamate and leaf  $\delta^{15}\text{N}$ .

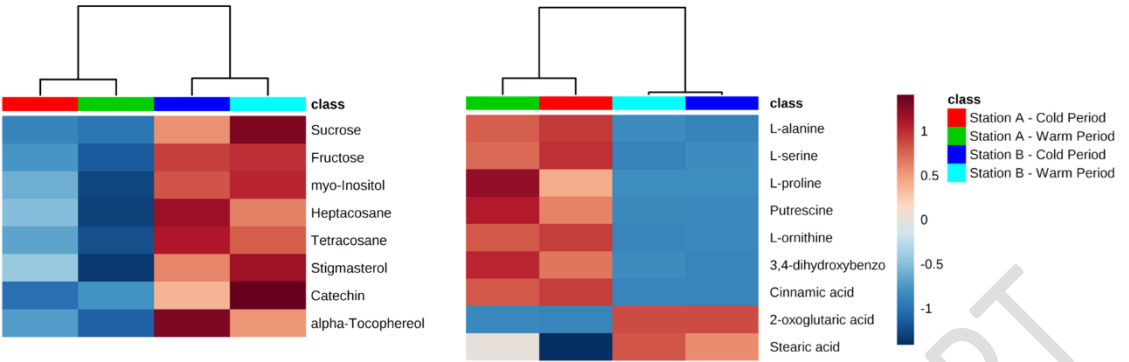


**Figure 1.**



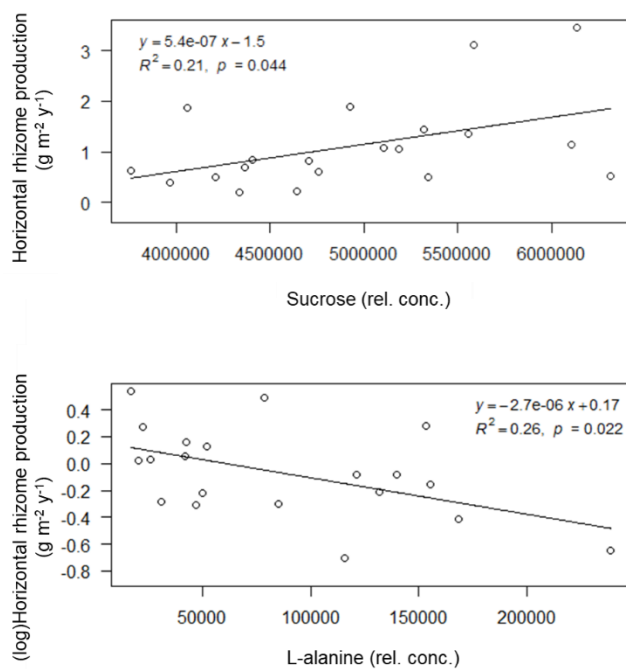
**Figure 2.**

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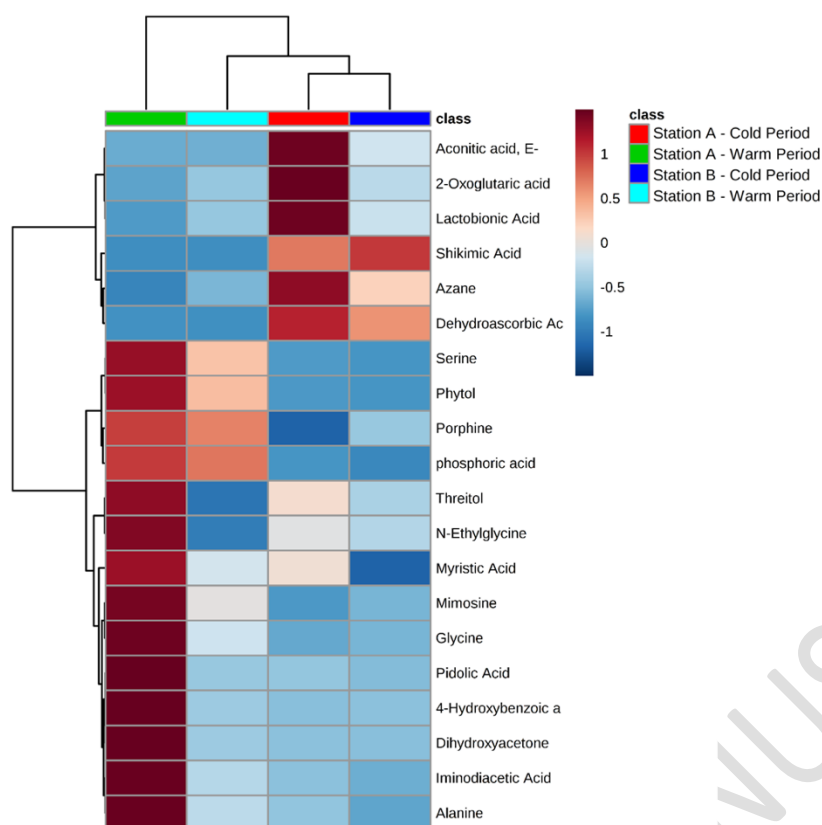


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781 **Figure 3.**

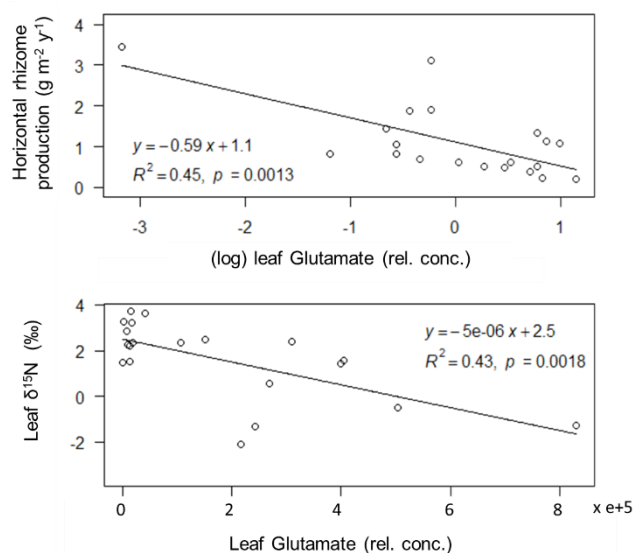


**Figure 4.**



**Figure 5.**

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791 **Figure 6.**

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**Table Legends**

Table 1. Ammonium, phosphate and  $\delta^{34}\text{S}$  sulfate of bottom water and pore water for each station. Organic (OC) and inorganic (IC) carbon and total nitrogen (TN) pools and  $\delta^{34}\text{S}$  sulfide of seagrass sediments. Significant differences ( $P < 0.05$ ) between stations are given by capital letters and between season by small letters, \* indicates station X season effect.

Table 2. Shoot density, leaf, rhizome and root biomass, above to below ground biomass ratio, total biomass and horizontal rhizome production, and average TC, TN, TS,  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ,  $\delta^{34}\text{S}$ ,  $F_{\text{sulfide}}$  AVS and  $F_{\text{sulfide}}$  CRS for leaves rhizomes and roots at the two stations during the warm and cold periods. Significant differences ( $P < 0.05$ ) between stations are given by capital letters (A) and between seasons by small letters (a), \* indicates station X season effect.

Table 3. Two-way ANOVA testing of stress-associated and growth-associated metabolites in seagrass leaves.

Table 4. Spearman correlations between horizontal rhizome production and metabolites associated with growth or stress in seagrass leaves in both sampling periods.

**Table 1.**

	Station A		Station B	
	Warm Period	Cold Period	Warm Period	Cold Period
<i>Bottom water</i>				
NH <sub>4</sub> (μM)	0.45 ± 0.10	0.99 ± 0.55	0.40 ± 0.09	0.54 ± 0.10
PO <sub>4</sub> (μM)	0.02 ± 0.01 a	0.07 ± 0.06 a	0.01 ± 0.006 a	0.05 ± 0.03 a
δ <sup>34</sup> S sulfate (‰)	20.71	20.69	20.28	20.70
POC (μg/L)	153.40 ± 5.37	301.29 ± 323.33	139.23 ± 67.07	100.29 ± 76.73
PN (μg/L)	50.10 ± 5.02	20.02 ± 16.19	16.18 ± 15.10	11.71 ± 10.34
<i>Pore water</i>				
NH <sub>4</sub> (μM)	2.74 ± 0.85 A	6.07 ± 2.13 A	2.48 ± 0.36 A	2.46 ± 1.08 A
PO <sub>4</sub> (μM)	0.73 ± 0.30 a	0.18 ± 0.04 a	0.47 ± 0.11 a	0.275 ± 0.03 a
δ <sup>34</sup> S sulfate (‰)	20.58 ± 0.06	20.53 ± 0.20	20.41 ± 0.14	19.91 ± 1.06
<i>Sediment</i>				
OC (% DW)	6.25 ± 0.42 a	3.51 ± 0.12 a	5.99 ± 0.76 a	3.36 ± 0.08 a
IC (% DW)	3.75 ± 0.19 a	6.78 ± 0.23 a	3.98 ± 0.86 a	6.36 ± 0.79 a
TN (% DW)	0.074 ± 0.01 Aa	0.061 ± 0.003 Aa	0.05 ± 0.006 Aa	0.05 ± 0.0009 Aa
δ <sup>13</sup> C (‰)	-0.85 ± 0.69 a*	-1.66 ± 0.58 a*	0.43 ± 0.23 a*	-1.84 ± 0.73 a*
δ <sup>15</sup> N (‰)	0.66 ± 0.35 a	1.49 ± 0.15 a	0.77 ± 0.19 a	1.81 ± 0.48 a
δ <sup>34</sup> S sulfide (AVS pools) (‰)	-19.92 ± 1.64	-17.82 ± 3.26	-20.11 ± 1.49	-18.27 ± 6.85
δ <sup>34</sup> S sulfide (CRS pools) (‰)	-26.81 ± 0.60	-25.26 ± 1.27	-27.46 ± 0.58	-27.67 ± 1.51
S AVS pools (μM)	155.75 ± 45.17 *	55.28 ± 41.90 *	110.98 ± 43.17 *	185.87 ± 50.57 *
S CRS pools (μM)	11686.35 ± 1051.82 A	8813.33 ± 5381.55 A	4465.4 ± 713.35 A	7413.33 ± 1250.34 A

**Table 2.**

	Station A		Station B	
	Warm Period	Cold Period	Warm Period	Cold Period
Shoot density (shoots m <sup>-2</sup> )	2569.19 ± 185.97 a	1032.77 ± 297.21 a	1822.20 ± 967.34 a	1369.48 ± 374.09 a
Leaf biomass (g DW m <sup>-2</sup> )	37.50 ± 4.16 a	10.33 ± 2.74 a	36.75 ± 19.48 a	13.00 ± 1.89 a
Rhizome biomass (g DW m <sup>-2</sup> )	113.83 ± 46.38 *	46.22 ± 12.48 *	78.05 ± 22.60 *	93.85 ± 33.41 *
Root biomass (g DW m <sup>-2</sup> )	33.86 ± 8.70 A	25.00 ± 6.71 A	48.37 ± 26.20 A	69.31 ± 40.80 A
Above:Below-ground biomass	0.21 ± 0.05 a	0.13 ± 0.01 a	0.21 ± 0.06 a	0.08 ± 0.04 a
Total biomass (g DW m <sup>-2</sup> )	185.19 ± 46.00 a	56.55 ± 14.95 a	163.17 ± 62.17 a	106.84 ± 34.99 a
Horizontal rhizome production (g m <sup>-2</sup> yr <sup>-1</sup> )	0.67 ± 0.54 A		1.57 ± 0.78 A	
Leaf C (% DW)	41.07 ± 0.21 Aa	37.79 ± 1.64 Aa	39.84 ± 2.95 Aa	37.86 ± 0.56 Aa
Rhizome C (% DW)	38.50 ± 1.57 Aa	36.56 ± 1.51 Aa	39.92 ± 0.84 Aa	37.69 ± 0.86 Aa
Root C (% DW)	40.69 ± 0.70 a	37.26 ± 0.85 a	40.08 ± 0.83 a	37.51 ± 1.19 a
Leaf N (% DW)	1.81 ± 0.12 a	2.22 ± 0.45 a	1.73 ± 0.33 a	2.29 ± 0.11 a
Rhizome N (% DW)	0.69 ± 0.05 Aa	1.80 ± 0.25 Aa	0.52 ± 0.15 Aa	1.43 ± 0.15 Aa
Root N (% DW)	0.76 ± 0.10 Aa	1.09 ± 0.07 Aa	0.52 ± 0.15 Aa	0.83 ± 0.09 Aa
Leaf S (% DW)	0.39 ± 0.03 A*	0.31 ± 0.04 A*	0.34 ± 0.03 A*	0.39 ± 0.04 A*
Rhizome S (% DW)	0.45 ± 0.04 A*	0.38 ± 0.10 A*	0.35 ± 0.06 A*	0.41 ± 0.04 A*
Root S (% DW)	0.48 ± 0.08 A	0.42 ± 0.13 A	0.34 ± 0.04 A	0.35 ± 0.05 A
Leaf δ <sup>13</sup> C (‰)	-7.69 ± 0.25 A	-6.24 ± 3.86 A	-8.16 ± 0.22 A	-7.53 ± 0.16 A
Rhizome δ <sup>13</sup> C (‰)	-7.87 ± 0.44 A	-7.83 ± 0.26 A	-8.09 ± 0.08 A	-7.99 ± 0.28 A
Root δ <sup>13</sup> C (‰)	-8.22 ± 0.41 A	-8.34 ± 0.36 A	-8.36 ± 0.53 A	-8.41 ± 0.28 A
Leaf δ <sup>15</sup> N (‰)	2.05 ± 0.51 Aa*	-0.93 ± 0.99 Aa*	1.97 ± 0.43 Aa*	3.34 ± 0.35 Aa*
Rhizome δ <sup>15</sup> N (‰)	1.14 ± 0.64 a*	-0.31 ± 1.08 a*	-0.19 ± 1.11 a*	3.02 ± 0.23 a*
Root δ <sup>15</sup> N (‰)	1.92 ± 0.66 A*	0.13 ± 1.03 A*	0.96 ± 0.93 A*	3.51 ± 0.48 A*
Leaf δ <sup>34</sup> S (‰)	16.79 ± 0.28 Aa	15.33 ± 0.74 Aa	16.27 ± 0.64 Aa	15.55 ± 0.89 Aa
Rhizome δ <sup>34</sup> S (‰)	10.17 ± 1.76 A*	8.03 ± 0.49 A*	9.37 ± 1.79 A*	10.57 ± 1.53 A*
Root δ <sup>34</sup> S (‰)	10.55 ± 2.57 A	11.69 ± 1.50 A	11.99 ± 2.63 A	9.64 ± 2.79 A
Leaf F <sub>sulfide</sub> AVS (%)	9.51 ± 1.13	14.96 ± 0.70	9.77 ± 1.67	13.07 ± 1.82
Rhizome F <sub>sulfide</sub> AVS (%)	25.41 ± 5.20	32.85 ± 1.46	26.87 ± 3.77	28.10 ± 5.45
Root F <sub>sulfide</sub> AVS (%)	23.84 ± 9.60	27.10 ± 1.60	16.41 ± 1.97	30.80 ± 5.80
Leaf F <sub>sulfide</sub> CRS (%)	8.11 ± 0.63	11.43 ± 2.01	8.30 ± 1.73	10.37 ± 0.83
Rhizome F <sub>sulfide</sub> CRS (%)	21.63 ± 3.64	27.36 ± 0.05	22.80 ± 3.93	22.28 ± 3.69
Root F <sub>sulfide</sub> CRS (%)	20.18 ± 7.26	20.54 ± 3.53	13.89 ± 1.73	24.76 ± 6.58

825 **Table 3**

two-way ANOVA test						two-way ANOVA test					
Metabolite	Source of variability	df	MS	F	p-value	Metabolite	Source of variability	df	MS	F	p-value
<i>Stress Associated</i>						<i>Growth Associated</i>					
L-alanine	Station	1	7.546	10.824	**	Sucrose	Station	1	17.200	170.386	***
	Season	1	0.057	0.081	ns		Season	1	0.115	1.139	ns
	Station x Season	1	0.244	0.349	ns		Station x Season	1	0.070	0.692	ns
L-serine	Station	1	8.710	14.143	**	Fructose	Station	1	16.476	114.642	***
	Season	1	0.316	0.513	ns		Season	1	0.141	0.984	ns
	Station x Season	1	0.120	0.195	ns		Station x Season	1	0.083	0.576	ns
L-proline	Station	1	9.529	16.243	***	myo-Inositol	Station	1	15.399	69.854	***
	Season	1	0.039	0.067	ns		Season	1	0.073	0.331	ns
	Station x Season	1	0.045	0.077	ns		Station x Season	1	0.001	0.005	ns
Putrescine	Station	1	12.405	34.869	***	Heptacosane	Station	1	17.765	260.750	***
	Season	1	0.885	2.486	ns		Season	1	0.072	1.052	ns
	Station x Season	1	0.018	0.051	ns		Station x Season	1	0.074	1.082	ns
L-ornithine	Station	1	13.188	37.759	***	Tetracosane	Station	1	16.851	135.093	***
	Season	1	0.205	0.587	ns		Season	1	0.001	0.009	ns
	Station x Season	1	0.018	0.051	ns		Station x Season	1	0.152	1.219	ns
3,4-dihydroxybenzoic acid	Station	1	11.007	34.301	***	Stigmasterol	Station	1	18.081	315.980	***
	Season	1	1.472	4.588	*		Season	1	0.002	0.040	ns
	Station x Season	1	1.386	4.319	ns		Station x Season	1	0.001	0.013	ns
Cinnamic acid	Station	1	14.593	53.525	***	Catechin	Station	1	14.677	58.787	***
	Season	1	0.004	0.016	ns		Season	1	0.283	1.132	ns
	Station x Season	1	0.041	0.149	ns		Station x Season	1	0.046	0.185	ns
2-oxoglutaric acid	Station	1	16.179	91.875	***	alpha-Tocophereol	Station	1	14.357	50.831	***
	Season	1	0.002	0.010	ns		Season	1	0.027	0.096	ns
	Station x Season	1	0.002	0.010	ns		Station x Season	1	0.098	0.345	ns
Stearic acid	Station	1	15.699	107.759	***						
	Season	1	0.346	2.372	ns						
	Station x Season	1	0.624	4.286	ns						

ns not significant  
 \* p < 0.05.  
 \*\* p < 0.01.  
 \*\*\* p < 0.001.

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Metabolites correlated with horizontal rhizome production	N	Spearman's rho Correlation coefficient	Sig. (1-tailed)	Sig. (2-tailed)
<i>Growth</i>				
Sucrose	20	0.453	*	*
Fructose	20	0.577	**	**
myo-Inositol	20	0.647	**	**
Heptacosane	20	0.620	**	**
Tetracosane	20	0.368	ns	ns
Stigmasterol	20	0.562	**	**
Catechin	20	0.289	ns	ns
alpha-Tocophereol	20	0.525	**	*
<i>Stress</i>				
L-alanine	20	-0.460	*	*
L-serine	20	-0.439	*	ns
L-proline	20	-0.316	ns	ns
Putrescine	20	-0.624	**	**
L-ornithine	20	-0.412	*	ns
3,4-Dihydroxybenzoic acid	20	-0.430	*	ns
Cinnamic acid	20	-0.400	*	ns
2-Oxoglutaric acid	20	0.510	*	*
Stearic acid	20	0.071	ns	ns

ns not significant

\* p &lt; 0.05.

\*\* p &lt; 0.01.

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