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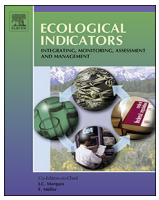

Original Articles

Metabolomic indicators for low-light stress in seagrass

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ABSTRACT

Monitoring of seagrass meadows is essential to inform adaptive management and address widespread declines in seagrass ecosystems. Effective monitoring techniques require sensitive indicators that are capable of detecting sub-lethal stressors and differentiating stress responses from background environmental variation. Here we explore untargeted metabolomics as a means to measure multi-parameter molecular responses of seagrass to low-light stress. We subjected *Zostera muelleri* to reduced light scenarios (< 10% natural light) in a six-week field experiment. Biomass loss was quantified over time and leaf samples were analysed by Nuclear Magnetic Resonance (NMR) spectroscopy to explore the effect of low-light exposure on metabolic activity. We identified several potential bioindicators of low-light stress: a reduction of soluble sugars and their derivatives, glucose, fructose, sucrose and myo-inositol, N-methylnicotinamide, organic acids and various phenolic compounds, and an increase in some amino acids. These signals were evident even amongst a noisy background of environmental variation and are consistent with inhibition of photosynthesis. Metabolite profiles showed a more consistent response to low-light stress than to biomass loss. These results suggest that metabolomics measurements may be useful bio-indicators of low-light stress in seagrass and that molecular indicators could inform on management of seagrass ecosystems.

1. Introduction

Seagrass ecosystems play a critical role in mitigating against climate change and provide essential habitat to fisheries, marine megafauna and protected species (Mtwana Nordlund et al., 2016; Sievers et al., 2019), however they are facing a widespread decline due to human impacts (Waycott et al., 2009). Turbidity has been identified as one of the most significant threats to seagrass, since the associated reduction in light inhibits growth (Collier et al., 2016). Extensive seagrass meadows are often found in estuaries and bays where ports and cities co-occur, so they are frequently exposed to poor water quality from capital dredging works and turbid river plumes associated with urban and agricultural development (Saunders et al., 2017). Prolonged periods of reduced light can result in complete loss of a meadow (Lavery et al., 2009). Monitoring of seagrass meadows, including their responses to human stressors, is required to inform on management actions to avert loss (Griffiths et al., 2020).

Recognising when meadows are stressed, or more susceptible to stress, can be difficult using traditional approaches for routine monitoring. Growth-condition metrics such as species composition, above ground biomass and spatial coverage are commonly used to indicate seagrass health (Collier et al., 2016; Moore et al., 2000) although a range of other metrics are also widely used (Chartrand et al., 2016; Petus et al., 2014; Vonk et al., 2015). Condition metrics detect change in seagrass biomass over time, however at times their efficacy is limited by substantial variation within sites and among seasons (Hossain et al., 2010). Further, they offer little insight into sub-lethal stresses that may be occurring at a physiological and molecular level, and which may precede morphological responses between routine sampling events. The development of sensitive alternative methodologies capable of revealing environmental perturbations at the molecular level are becoming essential for monitoring ecosystem health (Rotini et al., 2013), including for seagrass ecosystems (Macreadie et al., 2014). However, among the many methods previously used to measure light stress in seagrass, several of those reflecting physiological processes, such as respiration rate and concentrations of nitrogen, phosphorous and carbon (among others), do not respond consistently to light reduction and as such are not recommended as bioindicators (McMahon et al., 2013). Endpoints that integrate multi-parameter molecular responses to stress show great potential for addressing this shortcoming (Kumar et al., 2016).

Metabolomics has become a significant contributor to the

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identification of stress in plants (Shulaev et al., 2008). Metabolomics can identify by-products of stress metabolism and molecules that are part of the acclimation response of plants (Hong et al., 2016), so measurements of metabolites could indicate stress in seagrass meadows (Hasler-Sheetal et al., 2015). Physiological responses to stress may include elevated levels of amino acids or a reduction in sugars important in the signaling of cells under stress (Kumar et al., 2016). Liquid- and Gas-Chromatography paired with Mass Spectrometry (LC/GC–MS) and Nuclear Magnetic Resonance (NMR) spectroscopy are powerful tools for metabolomics analysis, each offering different strengths and weaknesses (Emwas, 2015; Pan and Raftery, 2007). NMR offers a particularly rapid and repeatable platform for untargeted metabolomics, including the ability to identify and quantify minor differences in a wide range of biologically relevant amino acids, carbohydrates, nucleotides and other metabolites (Melvin et al., 2017; Zou et al., 2014). The method has been used to obtain comprehensive metabolite profiles for the Northern Hemisphere seagrass species, *Zostera marina* (Hasler-Sheetal et al., 2015; Mochida et al., 2019). The untargeted nature of metabolomics is what makes it particularly relevant to ecological studies, because it reflects all interactions between and within species, and with their environment, rather than targeting specific biochemical pathways. This approach is referred to as eco-metabolomics and provides mechanistic evidence for biochemical processes that are relevant at ecological scales by detecting changes in metabolite concentrations (Peters et al., 2018). The results of early research has revealed considerable differences in profiles of metabolites between samples collected from an aquatic environment with variable levels of natural environmental stress (i.e., low-light, high temperature and anoxic conditions (Kumar et al., 2016; Zidorn, 2016). Thus, untargeted metabolomics is ideal to further test the adaptation strategies of seagrass to low-light stress. If stress responses can be identified in metabolomic measurements, then they may also have potential to be used as an early warning indicator of seagrass stress.

Here we utilised a common monitoring technique to measure the loss of biomass from light stressed *Z. muelleri,* a Southern Hemisphere seagrass. The morphology of stressed seagrass was compared to its metabolic state which was measured using NMR-based metabolomics. We subjected seagrass to a continuous period of low-light *in situ*. Loss of above-ground biomass was quantified over time and metabolites were extracted from leaf samples after 6 weeks and analysed by NMR. Two important questions were asked: 1) how does biomass loss in stressed seagrass compare to metabolic activity? And 2) are differences in metabolite profiles between seagrass subjected to low-light stress and those exposed to natural light conditions detectable? The overall aim of this paper is to explore the use of non-targeted metabolomics for characterising low-light stress on seagrass.

2. Materials and methods

2.1. Experimental set-up in the field

We subjected shallow subtidal (< 3 m) beds of the seagrass *Z.muelleri* to reduced light conditions using shading structures for a continual six week period during the summer of 2018/2019 in southern Moreton Bay, Queensland. Shading structures (1 m^2) were erected over five replicate patches of dense *Z.muelleri* (~3 m apart) along the subtidal fringe of a large meadow. Shading material was a mesh canopy that reduced light penetration to subtidal seagrass well below natural levels, tethered at each corner to a plastic stake. We installed vertical isolation borders 10 cm inside the edges of the treatment plots to a depth of 25 cm in the sediment to sever the roots and prevent the transfer of nutrients and carbohydrates from unshaded and shaded areas via rhizomes (Chartrand et al. 2016). Unshaded control plots that received natural light intensities were also prepared both with and without vertical isolation borders (two replicates of each). Light loggers (4 × Odyssey photosynthetically active radiation (PAR), and 5 × HOBO models) were placed in control and shaded plots to measure light levels throughout the period. Odyssey loggers were installed with automatic wipers. The shade cloth and HOBO light loggers were also manually cleaned every 2 days throughout the experiment to ensure relative differences in light penetration between treatments remained consistent, and to minimise sedimentation and biofouling. We quantified biomass inside a 25 cm^2 sub-quadrat installed within each 80 cm^2 plot. Shoot density (every shoot) and leaf-length (from five randomly selected leaves) were counted and measured, respectively, inside each sub-quadrat every 2 weeks throughout the experiment. A regression model was developed to quantify total above ground biomass (Biomass) based on shoot count (sc) and average leaf length (avll, Eq. (1)). This model was based on data collected from 6 seagrass cores (25 cm × 25 cm squares) from randomised locations adjacent to experimental plots prior to installation of the shade structures.

$$Biomass = (0.213 * sc) + (0.148 * avll) - 3.233 \quad (1)$$

The standard error of the biomass estimate was ± 1.36 g, or ~9% of the average starting biomass in each plot.

2.2. Sampling for metabolomics

We randomly collected between 7 and 20 single leaves of approximately 5 cm in length from each 80 cm shaded (treatment, n = 5) and unshaded (controls, n = 4) plot after a continual shading period of 39 days. Each leaf was manually cleaned of epiphytes whilst submerged and immediately placed inside plastic vials and snap frozen in a liquid nitrogen dry shipper (Taylor Wharton™). We took care to remove the 2nd or 3rd youngest leaf closest to the sheath, avoiding the youngest leaf to ensure samples were standardised, since leaf age can influence metabolite composition and concentration (Agostini et al., 1998; Hasler-Sheetal et al., 2015). Samples were stored at −80 °C in the laboratory until subsequent extraction of metabolites.

2.3. Sample extraction and processing for NMR spectroscopy

Samples were extracted and processed according to previously established methods (Melvin et al., 2017). In summary, after freeze-drying the samples, a modified Bligh-Dyer extraction (Bligh and Dyer, 1959) was performed to separate polar metabolites from lipids and cellular debris. Extracted metabolites were dried and re-suspended in 200 µL phosphate buffer made with deuterium oxide (D_2O), which contained 0.05% sodium-3-(tri-methylsilyl)-2,2,3,3-tetra-deuteriopropionate (TSP) as an internal standard (^1H δ 0.00, ^{13}C δ 0.0). The dry weight of each leaf in each sample was measured.

Samples were loaded into 3 mm NMR tubes and analysed using an 800 MHz Bruker® Avance III HDX spectrometer equipped with Triple (TCI) Resonance 5 mm Cryoprobe with Z-gradient and automatic tuning and matching. The general methodology has been described elsewhere (Melvin et al., 2018a, 2018b). Briefly, spectra were acquired at 298 K with D_2O used for field locking. The zg30 pulse program was used for proton (^1H) spectra, with 128 scans, 1.0 s relaxation delay, 6.80 µs pulse width and spectral width of 16 kHz (^1H δ −3.02–16.02). Peaks were post processed with MestReNova v8.1.4 (Mestrelab Research S.L., Spain). Post-processing included manual phase-correction, ablative baseline adjustment and normalisation of the spectra to the TSP standard (^1H δ 0.00). Individual spectral features were then manually integrated and the data was exported and normalised to individual sample weight prior to statistical analysis (Melvin et al., 2018a, 2018b). An edited ^1H–^{13}C Heteronuclear Single Quantum Coherence (HSQC) spectra was also acquired for one representative sample with 210 scans, 128 experiments, 0.8 s relaxation delay, 6.80 µs pulse width and spectral widths of 12.8 kHz (^1H δ −3.23–12.82) and 33.1 kHz (^{13}C δ −9.40–155.2). Metabolites were tentatively assigned using Chenomx NMR suite 8.5 software (ChenomxInc., Edmonton, Canada), and assignments were further validated by comparison of HSQC

spectra with freely available reference spectra (e.g., Human Metabolome Database; HMDB).

2.4. Multivariate statistical analysis

Data were grouped by the following treatments for multivariate analysis: 1) shaded plots with vertical isolation borders (shaded, treatment), 2) unshaded plots with vertical isolation borders (unshaded and bordered, treatment) and, 3) unshaded plots without vertical isolation borders (unshaded, control). We normalised the data to account for sample mass, and then scaled the data using the pareto scale function ('RFmarkerDetector' package in the R program (Palla and Armano, 2016)). Pareto scaling is common in metabolomics studies, because it reduces the influence of extreme outliers (Emwas et al., 2018). We used Principle Component Analysis (PCA) ('pca3d' package in the R program) initially to explore correlations between treatment and control groups (Weiner, 2017). Linear regression was used to explore differences between groups based on mass of whole dried leaves.

We conducted multivariate regression with the Bayesian Ordination and Regression AnaLysis model ('boral' package in the R program) with explanatory (light effect and optimal peak in metabolite activity) and two latent variables (Hui, 2016). The boral model is suitable for metabolomics data because it models correlations between different metabolites measured across the same set of samples. We included as a fixed effect the two treatment groups and random effects as two latent variables. The latent variables model correlations between metabolites from unexplained sources of variation (Hui, 2016). The effect size of the treatment relative to the control was taken to be the median difference (+ − 95% highest posterior density intervals) in each metabolite's scaled value between control and treatment plots. We then quantified the signal-to-noise ratio in terms of each metabolite's ability to detect a change in low-light relative to background variation in that metabolite. The signal-to-noise ratio was quantified as the per cent of the variance attributed to the treatment effect relative to the sum of the treatment effect and the variance attributed to the latent variables.

We then tested whether changes in the metabolite concentrations where more consistent with light loss or biomass loss. To do this test, we compared the fit of two boral models: (1) a model with biomass percent loss at 39 days as a fixed effect against (2) a model with light intensity as a fixed effect. We did not include treatment type in either model, because this would be confounded with biomass/light. We compared these two models for their WAIC statistic (Vehtari et al., 2017), where the model with the lowest WAIC is most consistent with the differences in the metabolites. To aid interpretation of these patterns, we plotted metabolic activity, treatment (light) exposure and biomass loss for the metabolites with the greatest effect sizes. We also plotted an ordination from a principal components analysis of the weight normalised and pareto-transformed metabolomics data.

3. Results

3.1. Light measurements and biomass loss

The average total daily light penetration measured from the light loggers was 0.7 mol m^{-2} d^{-1}in shaded plots compared to 8.2 mol m^{-2} d^{-1} in unshaded plots over the experiment duration. The average PAR was 52.8 μmol m^{-2} min^{-1} at the time of collection for metabolomics in the shaded plots compared to 273.7 μmol m^{-2} min^{-1} in the unshaded plots (averaged over 2-hour collection period). The water temperature was 27 °C across all plots.

A loss of biomass was observed over time in seagrass exposed to light stress through manipulative shading. In unshaded control plots, biomass on average declined slightly (13%) over the 6-week period. Shaded plots lost on average 82% of biomass (Fig. 1). Biomass loss in shaded plots was consistent with an average reduction of leaf length of 3.2 cm in shaded plots compared to an average increase of 3.1 cm in the unshaded plots. Loss of biomass in shaded plots was supported by a reduced leaf condition: individual leaves that were randomly sampled and dried for the metabolomics measurements had a lower weight in the shaded plot (10.6 mg) than leaves from the unshaded plots (14.7 mg, $p < 0.001$, $r^2 = 0.21$).

3.2. Metabolite differences

A total of 84 peaks were integrated from the ^1H spectra, corresponding to 33 distinct metabolites and 10 unidentified features (Fig. 2). Numbers were assigned to identify individual peaks from the same metabolite (i.e. Fructose-1). Differences between the three treatment groups were clearly visible in a plot of the principal components (Fig. 3). Further analysis with the Bayesian hierarchical models found that shaded plots had a high probability of lower glucose and fructose, some phenolics (and spectral regions where phenolics overlapped with other chemical features), sucrose (and regions where sucrose overlaps with other features), malic acid, N-methylnicotinamide, asparagine, myo-inositol and cinnamic acid (Fig. 4a, Fig. A.1). Shaded plots had higher levels of trigonelline and several amino acids (gamma aminobutyric acid, proline, betaine and glutamine), however, increases of trigonelline and the amino acids appear to be related to the effect of severing rhizomes from vertical isolation borders rather than shade stress (Fig. 4b). Glutamine was an exception and remained elevated in the shaded plots.

The combined variance explained by shading for all metabolites was 3.5% (signal to noise ratio), indicating that there was considerable leaf to leaf variation among plots. However, variation explained by shading for individual metabolites was higher; as much as 93% for N-methylnicotinamide, between 16 and 30% for glucose, 10% for fructose and several phenolic compounds and the remainder of metabolites generally < 10% (Fig. A.1). Overall high residual variation (Fig. A.2) suggested there was considerable variation in metabolites within and between plots for reasons other than the effect of shading.

Changes in light intensity were more consistent with changes in the metabolite profile than changes in biomass, according to the WAIC statistic (WAIC = 6448 for the light model versus 7006 for the biomass model). The assertion that shading, rather than biomass loss, was the predominant cause of metabolic variation was supported by visual interpretation of metabolite changes. For instance, peaks for glucose and fructose were elevated in unshaded plots, even when those unshaded plots had similar amounts of biomass loss as shaded plots (Fig. 5).

4. Discussion

Here we demonstrated that light-stress triggers distinguishable changes in metabolite profiles of the seagrass *Z. muelleri*, indicating that metabolomics may be a useful and sensitive tool for identifying stressed meadows. We also observed a loss of biomass from reduced light, consistent with findings from other studies (Collier et al., 2016; Silva et al., 2013). Several metabolites changed in a way that is consistent with plant responses to light stress (Hasler-Sheetal et al., 2016). Considerable background variation between and within treatment plots was also observed, with low variance explained by the treatment relative to residual variance (Table 1), which is not unexpected for complex field ecosystems. However, some of the responses to low-light were identified as being a result of isolating the treatment plots, which prevents plants from receiving nutrients via rhizomes in adjacent unshaded areas. For example, amino acids such as trigonelline and gamma-aminobutyric acid showed no difference between the effect of isolation (Fig. 4b) in comparison to the effect of shade and isolation (Fig. 4a). Other metabolites that showed a strong response to reduced light became lost in the noise of background variation, as was the case for fructose. We suggest that N-methylnicotinamide, glucose, fructose and malic acid have the most potential to be useful indicators of low-light stress in *Z.muelleri*, because these metabolites had the strongest effect to

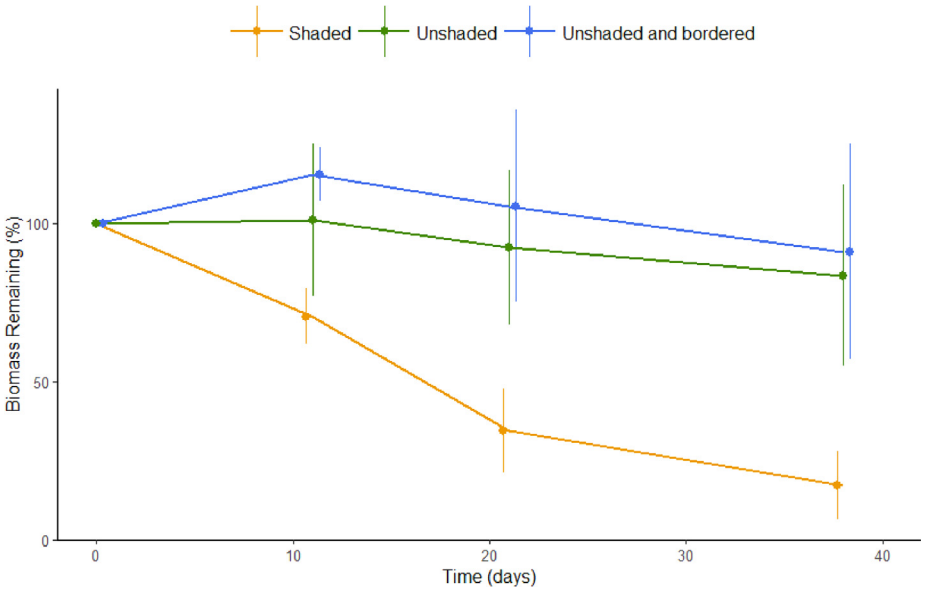

Fig. 1. Seagrass biomass change during the shading experiment (mean, SE), as change from initial biomass 38 days after shade structures were erected. Treatment groups are shaded plots with vertical isolation borders (Shaded), unshaded plots with vertical isolation borders (unshaded and bordered) and unshaded plots without any treatment (Unshaded). Metabolomics samples were collected 39 days after shade structures were erected.

shading relative to background variation.

4.1. Metabolome analysis

Carbohydrates were predicted to show the greatest response to low-light. Low-light reduces carbohydrates in seagrass (sucrose, fructose and glucose) due to lower rates of photosynthesis (Hasler-Sheetal et al., 2016; Kumar et al., 2017a). We found a reduction in all soluble carbohydrates in shaded seagrass (Table 1). The reduced abundance of carbohydrates is consistent with the morphological changes we observed of slower growth, smaller shoots, and higher mortality. The sensitivity of soluble sugars to reduced light was supported by the elevated concentrations of glucose, fructose and sucrose in the unshaded plots despite a loss of biomass in this treatment group (Fig. 5 and A.3, Table 1).

Glucose had the strongest response to shading (−10) and clearest (30% variance explained by shading) response to low-light and, as such, we suggest it is the best carbohydrate indicator of light stress (Table 1). It is unclear why levels of glucose showed a stronger response to low-light than levels of sucrose. Previous metabolomics studies with *Z. marina* have identified sucrose as having the largest magnitude of response to low-light (Mochida et al., 2019). In healthy plants, glucose levels are lower than other carbohydrates (Fig. 5), particularly in leaves compared to other parts of the plant, because glucose is readily converted into starch (Silva et al., 2013). Fructose showed a strong signal in response to low-light but was more readily influenced by other unknown environmental factors. The production of fructose is highly sensitive to multiple environmental stresses (Mochida et al., 2019) so the fructose response to shading may be masked by its response to other stressors.

N-methylnicotinamide showed the clearest response to low-light, with the treatment effect explaining 93% of the variance in this metabolite, and therefore it may be a good indicator of low-light stress (Table 1). N-methylnicotinamide is a co-enzyme in plant growth and may play a vital role in pathways controlling adaptation to environmental stresses, such as through the redox shuffle and retaining nicotinamide adenine dinucleotide phosphate (NADP) and NADPH homeostasis (Chai et al., 2005). The major NADPH generating source in darkness is the oxidative pentose phosphate pathway (OPPP) coupled with the central carbon metabolism in chloroplasts. This system maintains the redox potential necessary to protect the plant against oxidative stress (Kruger and Von Schaewen, 2003). Thus, it is possible that the reduction of N-methylnicotinamide from low-light is related to the physiological mechanisms aimed at preventing oxidative stress, although it is unclear why stressed plants have less of this compound. The OPPP pathway also utilises glucose and fructose rather than sucrose, which may explain the greater reduction in glucose and fructose compared to sucrose (Kruger and Von Schaewen, 2003).

Malic acid shows potential as an indicator of light stress. The reduced capacity of seagrass to fix carbon in low-light is a likely explanation for the reduced abundance of malic acid observed in light stressed plants. Current research suggests that seagrass are C3-C4 intermediate plants because some species have been identified to have CO_2 concentrating mechanisms (Larkum et al., 2017), but seagrasses lack many of the physiological characteristics consistent with C4 plants (Kim et al., 2018). The observed reduction in malic acid in plants exposed to low-light stress supports the theory that seagrass share some of the biochemical features characteristic of C4 plants. The reduction of malic acid and enzymes responsible for its synthesis in stressed seagrass has been shown elsewhere (unpublished data in Larkum et al., 2017; Moreno-Marín et al., 2018).

Some metabolites responded differently to previous studies in response to low-light stress, including phenolics and myo-inositol. For example, recent studies have shown that total phenols increased when subjected to low-light stress (up to 75% reduction in light) in *Z. marina* and *Cymodocea nodosa* (Silva et al., 2013), whereas we observed a decline in total phenols. This decline could be an artefact of the duration of stress exposure required before phenolic concentrations decline, because Silva et al. (2013) sampled after 3 weeks compared with approximately 6 weeks in this study. This hypothesis is supported by an observed reduction in secondary metabolites proportional to the degree of light reduction observed over longer timeframes (Toniolo et al., 2018). Alternatively, it could also be related to the sampling season, because reduced concentrations of phenolic compounds in seagrass have been observed in summer, in comparison to winter samples (Agostini et al., 1998). Similarly, an increase of myo-inositol in *Z. marina* was shown when subjected to darkness (Mochida et al., 2019), in contrast to the results of this study. More research is needed to better understand the significance of these responses and whether there are species specific differences.

4.2. Reliable indicators of light stress

Robust indicators should have a consistent response to stressors that can be distinguished from background variation (McMahon et al., 2013; Roca et al., 2016). In this study, individual metabolites responded more

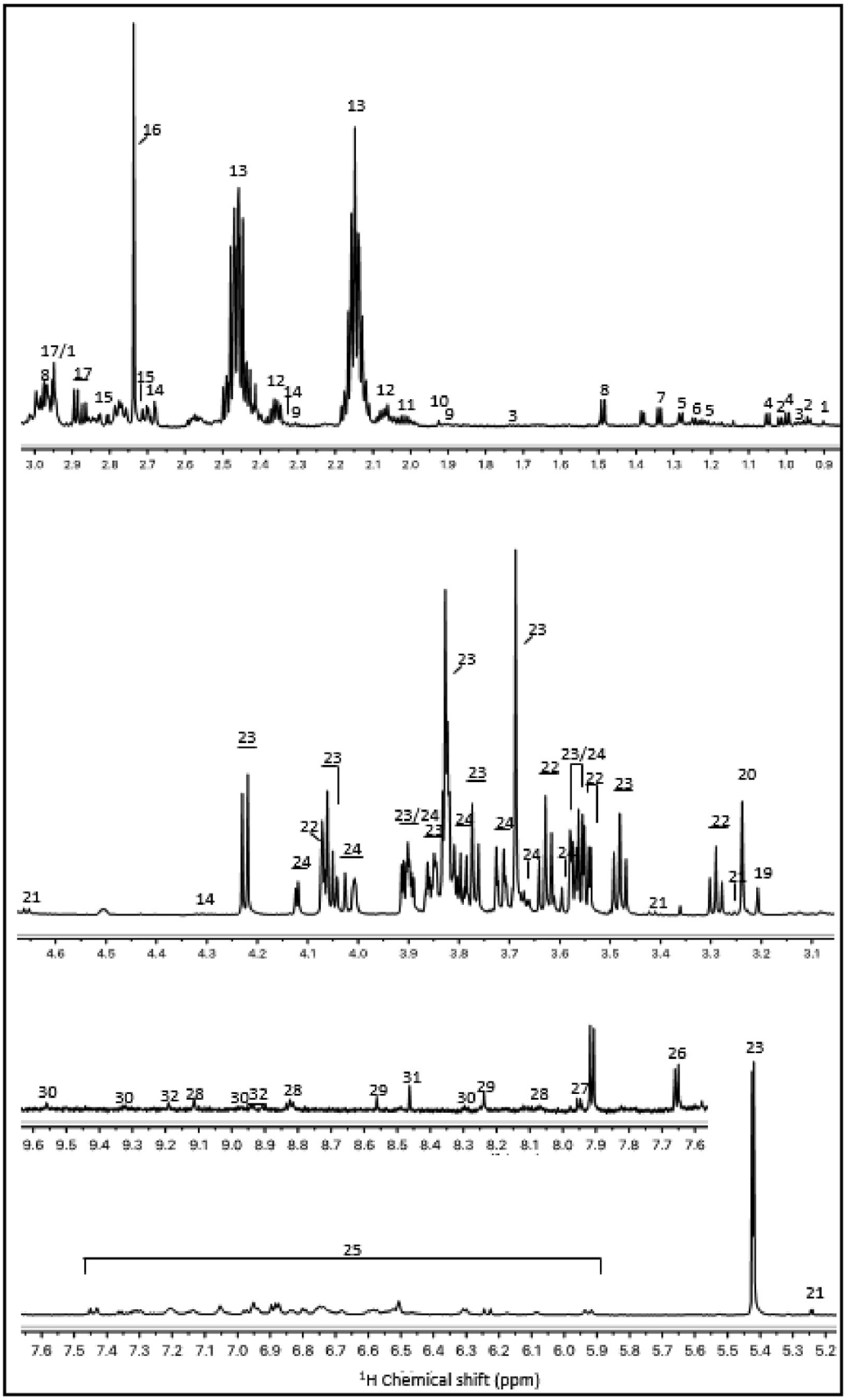

Fig. 2. Representative ^{1}H Nuclear Magnetic Resonance (NMR) spectra of *Zostera muelleri* leaf from the study site in Southern Moreton Bay, Queensland. 1 Pantothenate, 2 isoleucine, 3 leucine, 4 valine, 5 fucose, 6 allothreonine, 7 lactate, 8 alanine, 9 gamma-aminobutyric acid, 10 acetate, 11 proline, 12 glutamate, 13 glutamine, 14 malate, 15 aspartate, 16 sarcosine, 17 asparagine, 18 oxoglutarate, 19 choline, 20 betaine, 21 glucose, 22 myo-Inositol, 23 sucrose, 24 fructose, 25 phenolics, 26 cinnamic acid, 27 UDP-galactose, 28 trigonelline, 29 nucleotides (ADP/ATP), 30 Nicotinamide ribotide, 31 Formate, 32 1-methylnicotinamide.

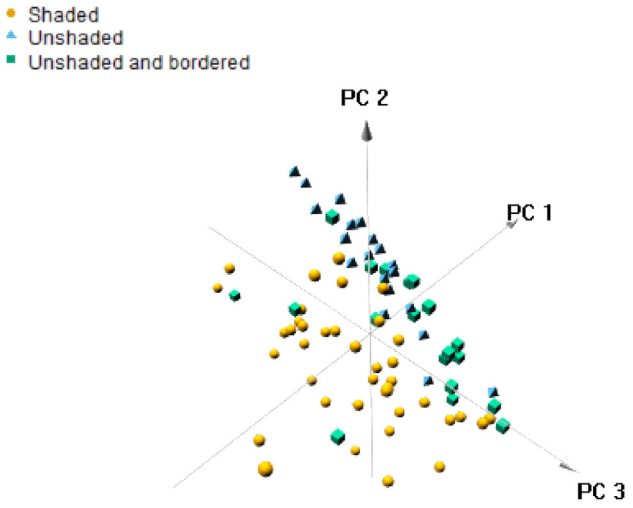

Fig. 3. PCA plot of metabolites showing separation between shaded and unshaded plots.

consistently to shading than to biomass loss (as indicated by the WAIC, also Fig. 5 & A.3). Biomass loss rates varied across control and treatment plots, with some control plots also losing considerable biomass. This biomass loss may be explained by this study occurring when *Z. muelleri* is approaching the summer period of natural senescence. This suggests that condition metrics such as biomass would be more suitable as a later warning indicator of functional change in contrast to early detection methods like metabolomics that has the potential to measure stress responses within a plant before external symptoms manifest.

Metabolomics could support existing indicators of low-light stress by providing biologically meaningful responses to light deprivation (de Kock et al., 2020). Metabolite responses to low-light in this study were consistent with inhibition of photosynthesis through a disruption to carbon fixation and energy metabolism (Table 1). Thus it served as a biological measure of sufficient light. Indicators of low-light stress allow managers to obtain an accurate indication of local conditions, which is particularly relevant for monitoring impacts in dredge operations and around ports, to facilitate prompt adaptive management (Chartrand et al., 2012; Griffiths et al., 2020; Statton et al., 2018). Light dependant thresholds and photosynthetic indicators, such as saturating irradiance for photosynthesis (E_K) and maximum electron transport rate (ETR_{max}) (Collier et al., 2009; Dattolo et al., 2014) are comonly used to monitor light conditions. However, biomass and below-ground productivity has been shown to be affected by light quality as well as light quantity in some seagrass species (Strydom et al., 2018).

A high level of metabolite specificity was achieved in the study, given the high magnetic strength of the NMR (800 MHz) and cryoprobe, and through confirmation of metabolite identification using the HSQC pulse experiment. Metabolomics using NMR spectroscopy also fills other criteria for bioindicator adequacy such as ease of collection, quick processing of samples and ease of interpretation of responses (McMahon et al., 2013). In addition, only small volumes of the sample are required for processing which reduces the impact of destructive techniques involved with biomass collections such as trampling (Rotini et al., 2013). Further, the method we have described here has the capacity to cover both polar and non-polar metabolites (Rivas-Ubach et al., 2013). Thus, it is able to identify metabolites involved in primary metabolism such as sugars, amino acids and small organic acids as well as secondary metabolites that can play a key role in an organism's response to environmental change such as phenolics. Further, this study utilises established protocols making data processing and metabolite identification for *Z. muelleri* more streamlined (Melvin et al., 2017, 2018).

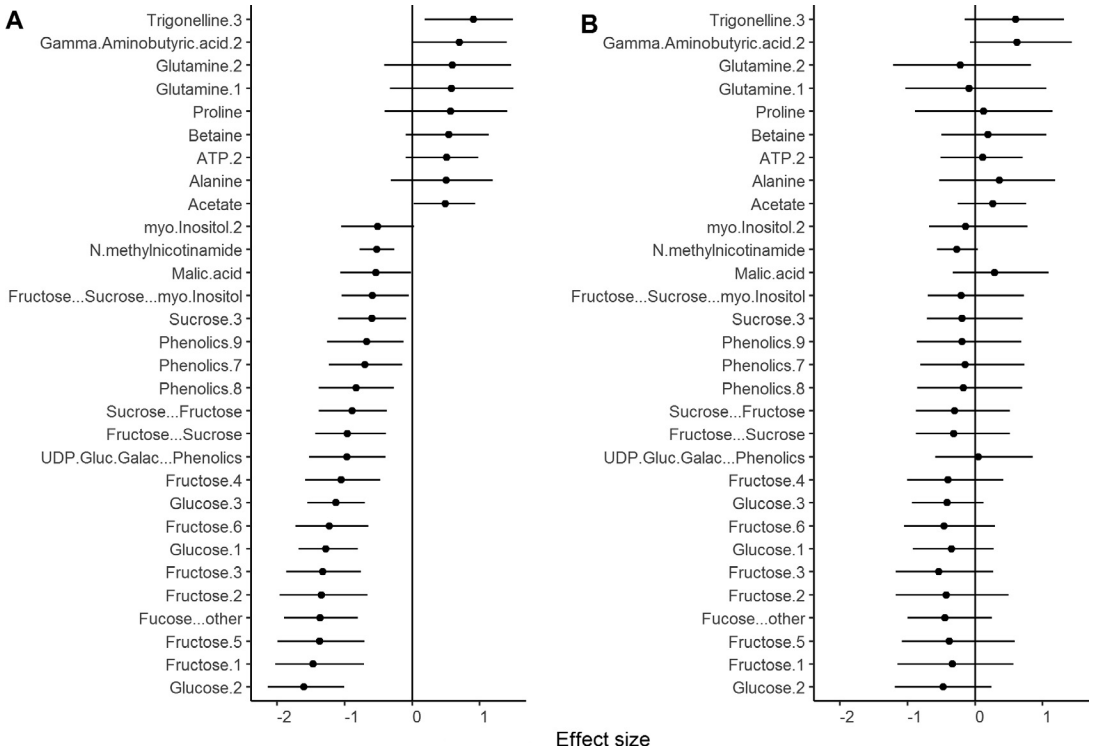

Fig. 4. Differences in the median effect sizes (black dots) with errors (95% highest posterior density intervals) for the highest 30 metabolic peaks of *Z. muelleri* showing the greatest response to shade stress. Plot A (left) shows differences between shaded (treatment) and unshaded (control) plots while Plot B (right) shows differences between unshaded (control) plots and unshaded plots with vertical isolation borders (treatment). A value above or below zero indicates an increase or decrease, respectively, in concentration of the treatment in comparison to the control. Bars indicate there is 95% probability of falling between the upper and lower limits.

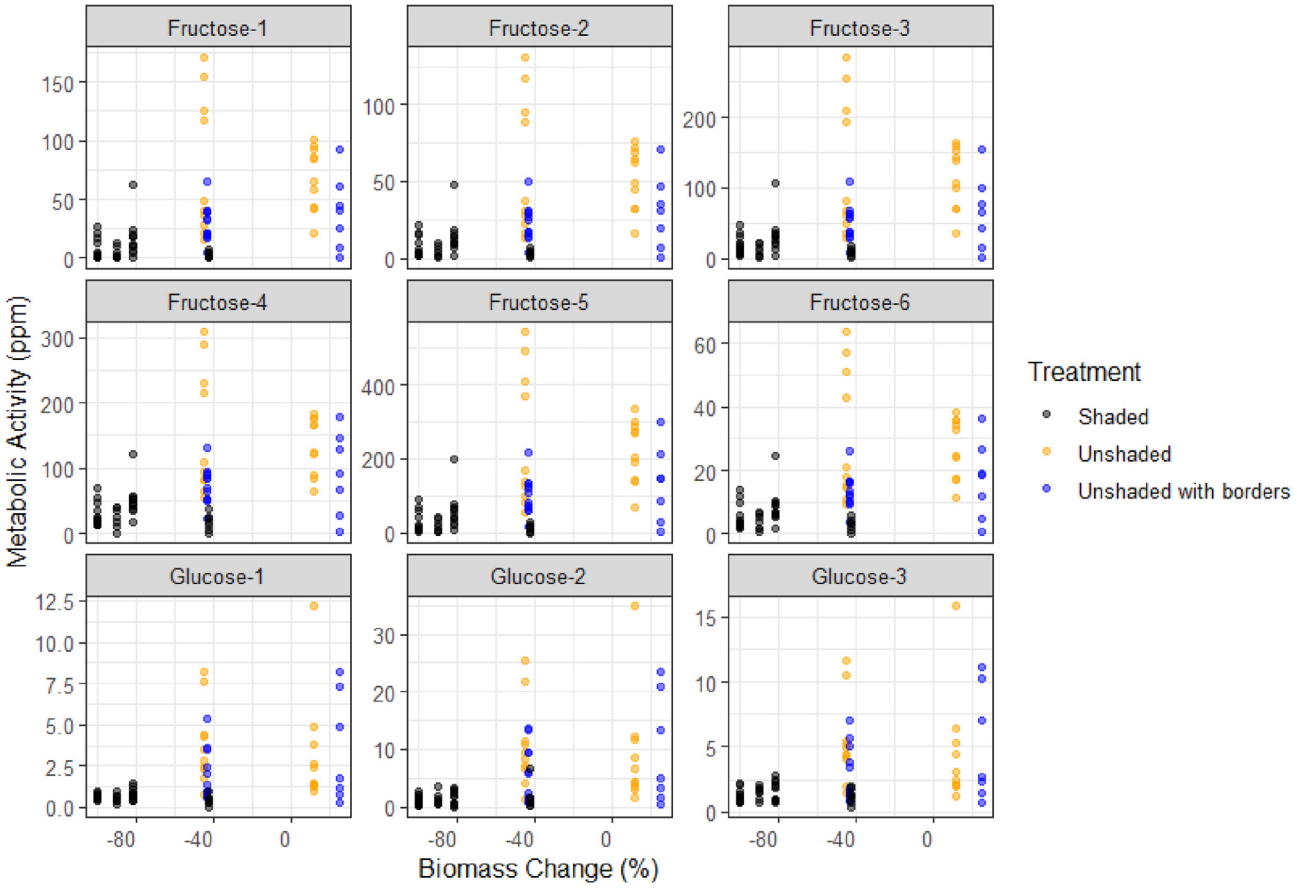

Fig. 5. Individual glucose and fructose metabolites showing biomass change and metabolic activity (spectra intensity measured as ^{1}H chemical shift in ppm) for each plot within each treatment group. Biomass change is the percent change over the 39 day experimental period from initial biomass measurements. Treatment groups include shaded plots with vertical isolation borders (Shaded), unshaded plots with vertical isolation borders (Unshaded with borders) and unshaded plots without any treatment (Unshaded). Numbers are assigned to metabolites to identify individual peaks in activity. Plot of individual sucrose metabolites is shown in Fig. A.3.

4.3. Limitations and future research directions

There are several caveats that limit interpretation of the results and the limitations of this study warrant further investigation to understand the other causes of variation in *Z. muelleri* metabolites. First, we were unable to identify ten of the individual metabolite features from their peaks in activity because reference spectra were unavailable. Therefore, we may have missed part of the plant's stress response. Further work identifying metabolites and developing standards for this species are needed.

Second, the various phenolic components were not readily distinguishable, which limits interpretation of the functional role of phenolics in the response to light stress. This relates to our use of D_2O to optimise comparison of primary metabolites against reference spectra, which was a trade-off since D_2O is not an ideal solvent for plant phenolics. Further work characterising individual phenolics may help to associate discrete stressors with specific biochemical responses.

Third, our parameters were limited to biomass and light intensity, however other ecological parameters would have helped to associate changes in metabolites with changes in ecosystem function (Jesch et al., 2018). For example, metabolite profiles have been shown to be affected by sediment structure (Holmer and Hasler-Sheetal, 2014), temperature (Kaldy, 2014), leaf herbivory (Arnold et al., 2008), prevalence of competitors (Dumay et al., 2004) and taxonomic relationships (Gillan et al., 1984). These abiotic effects can vary over small spatial scales. For example, different metabolic profiles have been shown for the same plant over small temporal and spatial scales for *Arabidopsis* and *Silene* plants (Dötterl et al., 2012; Stitt et al., 2007). Future research of light stress on the metabolome would therefore benefit from laboratory or mesocosm studies to limit the amount of environmental variation confounding interpretation of results (e.g. Bertelli and Unsworth, 2018). Fully or partially controlled environments would allow those metabolites involved specifically in the light stress response to be identified, so that field testing could focus on detecting change in those metabolites.

Fourth, samples for metabolite analyses could also be extracted at the onset of shade stress and at regular intervals throughout the shading experiment to understand temporal changes in the biochemical response to shading. This would help to understand the role of secondary metabolites (particularly phenolics) and to monitor sub-lethal responses to low-light stress. It is particularly important to identify metabolites that play a specific role in the plant's response to light deprivation. The metabolites identified in this study, such as glucose and fructose, have broad functions, and so may be more subject to environmental noise from other non-light stressors. Metabolomic sampling at the onset of shading may help to identify specific light-response metabolites. It is also needed to test how the method performs as an early-warning indicator. For instance, depletion of sugars in leaves may not be apparent early in the shade response, because the plant can translocate stored sugars from rhizomes to leaves (Mackey et al., 2007).

Fifth, it would be helpful to look at the effect of varying light levels. We initially attempted to create a gradient of light levels in the treatments, but were unsuccessful in retaining the variable light penetrations due to constant covering of the shade cloth with sediment and biofouling. Further, the study should be expanded to other seagrass species as tolerance to light has shown to vary between species (Silva

Table 1
Metabolites that responded most notably to low-light stress in *Z. muellieri* and their usefulness as a bioindicator.

Metabolite	Functional role	Response of metabolite to light reduction	Consistent with literature?	Effect size^	Variance explained by shading (%)	Useful as an indicator?	References
N-methylnicotinamide	A metabolite of niacin which synthesizes nicotinamide adenine dinucleotide (NAD) through a salvage pathway.	Possible response to reduced carbon fixation due to its role in maintaining redox potential to prevent oxidative stress.	Unknown	–5	93.3	Yes	Berglund et al., 2017; Chai et al., 2005; Matsui et al., 2007
Glucose	Signalling and regulatory molecule that controls growth and developmental programs, gene and protein expression, cell-cycle progression and primary and secondary metabolism in plants.	Response to suppressed photosynthesis, which suppresses glucose production	Yes	–10	29.9	Yes	Sheen, 2014
Fructose	An important signalling molecule in plant development and stress response	Response to suppressed photosynthesis, which suppresses fructose production	Yes	–10	9.3	Yes	Cho and Yoo, 2011
Malic acid	A dicarboxylic acid particularly important in C4 or CAM plants, which convert CO_2 into carboxylic acids and reduces the rate of photorespiration.	Response to lower rates of carbon fixation from the downregulation of enzyme (carbonic anhydrase) responsible for converting CO_2 to carboxyl acids in *Z.muelleri* exposed to 90% light reduction.	Yes	–5	7.0	Yes	Unpub. data in Larkum et al., 2017; Moreno-Marín et al., 2018; Ubierna et al., 2013
Phenolics	Are essential as defence mechanisms to protect against stress and constituent of secondary metabolites implicated in plant allelopathy in seagrass by either beneficially or adversely effecting neighbouring plants through the release of chemicals	Could be a secondary response from reduction in photosynthesis and limited carbohydrates available for production of energy demanding plant defence metabolites. Alternatively, it could be a growth promoting response that benefits the rest of the meadow 'neighbours' to release the individual plant from competitive growth when energy reserves are low.	Yes	–5	5.0	Maybe	Bryant et al., 1983; Caretto et al., 2015; Fajer et al., 1992; Li et al., 2010; Zapata et al., 1979
Asparagine	A major nitrogen storage amino acid that accumulates during periods of low rates of protein synthesis.	Response as a nitrogen carrier to remobilise and salvage nitrogen during light suppression and senescence.	Yes	+2	5.0	Maybe	Forde and Lea, 2007; Kim et al., 2018; Lea et al., 2007; Lin and Wu, 2004; Ubierna et al., 2013
Glutamine	An abundant free amino acid synthesised from ammonia and it is a major amino donor for synthesis of amino acids and other nitrogen-containing compounds	Response to insufficient energy available for its synthesis.	Mixed	+5	3.6	No	Hasler-Sheetal et al., 2016; Kumar et al., 2017b; Mochida et al., 2019; Moreno-Marín et al., 2018
Cinnamic acid	A monocarboxylic acid that plays a role in stress response.	Response to stress to prevent significant growth against bacterial and fungal species	Yes	+2	3.4	No	de Kock et al., 2020; Subhashini et al., 2013
Sucrose	The primary storage carbohydrate in seagrass which forms up to 90% of the total soluble carbohydrate pool.	Response to suppressed photosynthesis which suppresses sucrose production and remobilises sucrose from stored carbohydrates.	Yes	–2	2.4	No	Touchette and Burkholder, 2000
Myo-inositol	A sugar-like carbohydrate biosynthesised from glucose and it is central to the growth and development of plants and participates in the plant's stress response	Myo-inositol is rapidly metabolized, so this may be a response to reduced availability of carbohydrates for biosynthesis.	No	–5	2.3	No	Loewus and Murthy, 2000

^ Effect size range from −10 (strong negative) to +10 (strong positive)

et al., 2013).

Finally, while the relative change in the abundance of different metabolites were used to indicate the effect of low-light stress on seagrass, absolute values for the metabolites were not quantified. Future studies could therefore utilise other methods capable of quantifying values more specifically, such as LC-MS (Kim et al., 2015). This would be particularly important if discrete quantities of metabolites were a central goal of the study.

The method developed here could be complementary to support other research on stress responses in seagrass. For example, metabolomics coupled with other omics technologies, such as genomics (Bruno et al., 2010) or proteomics (Kumar et al., 2017a), can identify the functional role of metabolites in stress responses (Toniolo et al., 2018). Genomics in particular, could be useful to map how genotypes shape local scale variation to stresses (Salo et al., 2015). Metabolomics would also complement more traditional approaches such as biomass measurements. Multi-variable measurements will provide an integrated view of the functional status of seagrass as it responds to light stress. For example, metabolite analysis could support intermittent measurements of biomass loss from light stress experiments to determine if biochemical tipping points can be detected prior to loss in biomass. The complement of molecular interpretation combined with other physiological or morphometric analysis are put forward as strategies to evaluate the impact of human stressors on the ecosystem and to monitor environmental changes (Toniolo et al., 2018).

5. Conclusion

This study used experimental manipulations of seagrass meadows in the field to determine if metabolomic indicators could be identified for the response of *Z. muelleri* to prolonged low-light. Clear separation in metabolite profiles and strong signals from identifiable metabolites in comparison to traditional monitoring methods, suggest that metabolomics have potential as an indicator of low-light stress in seagrass. We suggest support for more research on metabolomics as a potential bioindicator for early impact assessment monitoring and to assess its contribution to the list of alternative indicators for low-light stress in seagrass meadows (McMahon et al., 2013).

CRediT authorship contribution statement

LL. Griffiths: Investigation, Methodology, Writing - original draft, Formal analysis. **SD. Melvin:** Formal analysis, Validation, Writing - review & editing. **RM. Connolly:** Conceptualization, Resources, Writing - review & editing. **RM. Pearson:** Methodology, Writing - review & editing. **CJ. Brown:** Supervision, Methodology, Formal analysis, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.106316.

References

Agostini, S., Desjobert, J.M., Pergent, G., 1998. Distribution of phenolic compounds in the seagrass Posidonia oceanica. Phytochemistry 48, 611–617. https://doi.org/10.1016/S0031-9422(97)01118-7.

Arnold, T.M., Tanner, C.E., Rothen, M., Bullington, J., 2008. Wound-induced accumulations of condensed tannins in turtlegrass, Thalassia testudinum. Aquat. Bot. 89, 27–33. https://doi.org/10.1016/j.aquabot.2008.02.001.

Berglund, T., Wallström, A., Nguyen, T. Van, Laurell, C., Ohlsson, A.B., 2017. Nicotinamide; antioxidative and DNA hypomethylation effects in plant cells. Plant Physiol. Biochem. 118, 551–560. https://doi.org/10.1016/j.plaphy.2017.07.023.

Bertelli, C.M., Unsworth, R.K.F., 2018. Light stress responses by the eelgrass, Zostera marina (L). Front. Environ. Sci. 6, 1–13. https://doi.org/10.3389/fenvs.2018.00039.

Bligh, E.G., Dyer, W.J., 1959. Canadian Journal of Biochemistry and Physiology. Can. J. Biochem. Physiol. 37.

Bruno, A., Bruno, L., Chiappetta, A., Giannino, D., Bitonti, M.B., 2010. PoCHL P expression pattern in Posidonia oceanica is related to critical light conditions. Mar. Ecol. Prog. Ser. 415, 61–71. https://doi.org/10.3354/meps08725.

Bryant, J.P., Chapin, F.S., Klein, D.R., 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivore. Oikos 40, 357–368.

Caretto, S., Linsalata, V., Colella, G., Mita, G., Lattanzio, V., 2015. Carbon fluxes between primary metabolism and phenolic pathway in plant tissues under stress. Int. J. Mol. Sci. 16, 26378–26394. https://doi.org/10.3390/ijms161125967.

Chai, M.F., Chen, Q.J., An, R., Chen, Y.M., Chen, J., Wang, X.C., 2005. NADK2, an Arabidopsis chloroplastic NAD kinase, plays a vital role in both chlorophyll synthesis and chloroplast protection. Plant Mol. Biol. 59, 553–564. https://doi.org/10.1007/s11103-005-6802-y.

Chartrand, K., Rasheed, M.A., Petrou, K., Ralph, P.J., 2012. Establishing tropical seagrass light requirements in a dynamic port environment, in: Proceedings of the 12th International Coral Reef Symposium 9-12 July 2012. Cairns.

Chartrand, K.M., Bryant, C.V., Carter, A.B., Ralph, P.J., Rasheed, M.A., 2016. Light thresholds to prevent dredging impacts on the great barrier reef seagrass, Zostera muelleri ssp. capricorni. Front. Mar. Sci. 3, 1–17. https://doi.org/10.3389/fmars.2016.00106.

Cho, Y.H., Yoo, S.D., 2011. Signaling role of fructose mediated by FINS1/FBP in Arabidopsis thaliana. PLoS Genet. 7, 1–10. https://doi.org/10.1371/journal.pgen.1001263.

Collier, C.J., Adams, M.P., Langlois, L., Waycott, M., O'Brien, K.R., Maxwell, P.S., McKenzie, L., 2016. Thresholds for morphological response to light reduction for four tropical seagrass species. Ecol. Indic. 67, 358–366. https://doi.org/10.1016/j.ecolind.2016.02.050.

Collier, C.J., Lavery, P.S., Ralph, P.J., Masini, R.J., 2009. Shade-induced response and recovery of the seagrass Posidonia sinuosa. J. Exp. Mar. Bio. Ecol. 370, 89–103. https://doi.org/10.1016/j.jembe.2008.12.003.

Dattolo, E., Ruocco, M., Brunet, C., Lorenti, M., Lauritano, C., D'Esposito, D., de Luca, P., Sanges, R., Mazzuca, S., Procaccini, G., 2014. Response of the seagrass Posidonia oceanica to different light environments: Insights from a combined molecular and photo-physiological study. Mar. Environ. Res. 101, 225–236. https://doi.org/10.1016/j.marenvres.2014.07.010.

de Kock, W., Hasler-Sheetal, H., Holmer, M., Tsapakis, M., Apostolaki, E.T., 2020. Metabolomics and traditional indicators unveil stress of a seagrass (Cymodocea nodosa) meadow at intermediate distance from a fish farm. Ecol. Indic. 109, 105765. https://doi.org/10.1016/j.ecolind.2019.105765.

Dötterl, S., Jahreiß, K., Jhumur, U.S., Jürgens, A., 2012. Temporal variation of flower scent in Silene otites (Caryophyllaceae): A species with a mixed pollination system. Bot. J. Linn. Soc. 169, 447–460. https://doi.org/10.1111/j.1095-8339.2012.01239.x.

Dumay, O., Costa, J., Desjobert, J.M., Pergent, G., 2004. Variations in the concentration of phenolic compounds in the seagrass Posidonia oceanica under conditions of competition. Phytochemistry 65, 3211–3220. https://doi.org/10.1016/j.phytochem.2004.09.003.

Emwas, A.H., 2015. The Strengths and Weaknesses of NMR Spectroscopy and Mass Spectrometry with Particular Focus on Metabolomics Research, in: Jacob T. Bjerrum (Ed.), Metabonomics. Humana Press, New York, NY, pp. 161–193. https://doi.org/https://doi.org/10.1007/978-1-4939-2377-9_13.

Emwas, A.H., Saccenti, E., Gao, X., McKay, R.T., dos Santos, V.A.P.M., Roy, R., Wishart, D.S., 2018. Recommended strategies for spectral processing and post-processing of 1D 1 H-NMR data of biofluids with a particular focus on urine. Metabolomics 14, 1–23. https://doi.org/10.1007/s11306-018-1321-4.

Fajer, E.D., Bowers, M.D., Bazzaz, F.A., 1992. The effect of nutrients and enriched CO2 environments on production of carbon-based allelochemicals in Plantago: a test of the carbon/nutrient balance hypothesis. Am. Nat. 140, 707–723. https://doi.org/10.1086/285436.

Forde, B.G., Lea, P.J., 2007. Glutamate in plants: Metabolism, regulation, and signalling. J. Exp. Bot. 58, 2339–2358. https://doi.org/10.1093/jxb/erm121.

Gillan, F.T., Hogg, R.W., Drew, E.A., 1984. The sterol and fatty acid compositions of seven tropical seagrasses from North Queensland, Australia. Phytochemistry 23, 2817–2821. https://doi.org/10.1016/0031-9422(84)83021-6.

Griffiths, L.L., Connolly, R.M., Brown, C.J., 2020. Critical gaps in seagrass protection reveal the need to address multiple pressures and cumulative impacts. Ocean Coast.

Manag. 183. https://doi.org/10.1016/j.ocecoaman.2019.104946.
Hasler-Sheetal, H., Castorani, M.C.N., Glud, R.N., Canfield, D.E., Holmer, M., 2016. Metabolomics reveals cryptic interactive effects of species interactions and environmental stress on nitrogen and sulfur metabolism in seagrass. Environ. Sci. Technol. 50, 11602–11609. https://doi.org/10.1021/acs.est.6b04647.
Hasler-Sheetal, H., Fragner, L., Holmer, M., Weckwerth, W., 2015. Diurnal effects of anoxia on the metabolome of the seagrass Zostera marina. Metabolomics 11, 1208–1218. https://doi.org/10.1007/s11306-015-0776-9.
Holmer, M., Hasler-Sheetal, H., 2014. Sulfide intrusion in seagrasses assessed by stable sulfur isotopes-A synthesis of current results. Front. Mar. Sci. 1, 1–12. https://doi.org/10.3389/fmars.2014.00064.
Hong, J., Yang, L., Zhang, D., Shi, J., 2016. Plant metabolomics: An indispensable system biology tool for plant science. Int. J. Mol. Sci. 17. https://doi.org/10.3390/ijms17060767.
Hossain, M.K., Rogers, K., Saintilan, N., 2010. Variation in seagrass biomass estimates in low and high density settings: implications for the selection of sample size. Environ. Indic. 5, 17–27.
Hui, F.K.C., 2016. boral – bayesian ordination and regression analysis of multivariate abundance data in r. Methods Ecol. Evol. 7, 744–750. https://doi.org/10.1111/2041-210X.12514.
Jesch, A., Barry, K.E., Ravenek, J.M., Bachmann, D., Strecker, T., Weigelt, A., Buchmann, N., de Kroon, H., Gessler, A., Mommer, L., Roscher, C., Scherer-Lorenzen, M., 2018. Below-ground resource partitioning alone cannot explain the biodiversity–ecosystem function relationship: A field test using multiple tracers. J. Ecol. 106, 2002–2018. https://doi.org/10.1111/1365-2745.12947.
Kaldy, J.E., 2014. Effect of temperature and nutrient manipulations on eelgrass Zostera marina L. from the Pacific Northwest. USA. J. Exp. Mar. Bio. Ecol. 453, 108–115. https://doi.org/10.1016/j.jembe.2013.12.020.
Kim, D.-H., Achcar, F., Breitling, R., Burgess, K.E., Barrett, M.P., 2015. LC–MS-based absolute metabolite quantification: application to metabolic flux measurement in trypanosomes. Metabolomics 11, 1721–1732. https://doi.org/10.1007/s11306-015-0827-2.
Kim, M., Brodersen, K.E., Szabó, M., Larkum, A.W.D., Raven, J.A., Ralph, P.J., Pernice, M., 2018. Low oxygen affects photophysiology and the level of expression of two-carbon metabolism genes in the seagrass Zostera muelleri. Photosynth. Res. 136, 147–160. https://doi.org/10.1007/s11120-017-0452-1.
Kruger, N.J., Von Schaewen, A., 2003. The oxidative pentose phosphate pathway: Structure and organisation. Curr. Opin. Plant Biol. 6, 236–246. https://doi.org/10.1016/S1369-5266(03)00039-6.
Kumar, M., Kuzhiumparambil, U., Pernice, M., Jiang, Z., Ralph, P.J., 2016. Metabolomics: an emerging frontier of systems biology in marine macrophytes. Algal Res. 16, 76–92. https://doi.org/10.1016/j.algal.2016.02.033.
Kumar, M., Padula, M.P., Davey, P., Pernice, M., Jiang, Z., Sablok, G., Contreras-Porcia, L., Ralph, P.J., 2017a. Proteome analysis reveals extensive light stress-response re-programming in the seagrass Zostera muelleri (alismatales, zosteraceae) metabolism. Front. Plant Sci. 7, 1–19. https://doi.org/10.3389/fpls.2016.02023.
Kumar, R., Bohra, A., Pandey, A.K., Pandey, M.K., Kumar, A., 2017b. Metabolomics for plant improvement: Status and prospects. Front. Plant Sci. 8, 1–27. https://doi.org/10.3389/fpls.2017.01302.
Larkum, A.W.D., Davey, P.A., Kuo, J., Ralph, P.J., Raven, J.A., 2017. Carbon-concentrating mechanisms in seagrasses. J. Exp. Bot. 68, 3773–3784. https://doi.org/10.1093/jxb/erx206.
Lavery, P.S., McMahon, K., Mulligan, M., Tennyson, A., 2009. Interactive effects of timing, intensity and duration of experimental shading on Amphibolis griffithii. Mar. Ecol. Prog. Ser. 394, 21–33. https://doi.org/10.3354/meps08242.
Lea, P.J., Sodek, L., Parry, M.A.J., Shewry, P.R., Halford, N.G., 2007. Asparagine in plants. Ann. Appl. Biol. 150, 1–26. https://doi.org/10.1111/j.1744-7348.2006.00104.x.
Li, Z.H., Wang, Q., Ruan, X., Pan, C. De, Jiang, D.A., 2010. Phenolics and plant allelopathy. Molecules 15, 8933–8952. https://doi.org/10.3390/molecules15128933.
Lin, J.F., Wu, S.H., 2004. Molecular events in senescing Arabidopsis leaves. Plant J. 39, 612–628. https://doi.org/10.1111/j.1365-313X.2004.02160.x.
Loewus, F.A., Murthy, P.P.N., 2000. myo-Inositol metabolism in plants. Plant Sci. 150, 1–19. https://doi.org/10.1016/S0168-9452(99)00150-8.
Mackey, P, Collier, C.J., Lavery, P.S., 2007. Effects of experimental reduction of light availability on the seagrass Amphibolis griffithii. Mar. Ecol. Prog. Ser. 342, 117–126.
Macreadie, P.I., Schliep, M.T., Rasheed, M.A., Chartrand, K.M., Ralph, P.J., 2014. Molecular indicators of chronic seagrass stress: A new era in the management of seagrass ecosystems? Ecol. Indic. 38, 279–281. https://doi.org/10.1016/j.ecolind.2013.11.017.
Matsui, A., Yin, Y., Yamanaka, K., Iwasaki, M., Ashihara, H., 2007. Metabolic fate of nicotinamide in higher plants. Physiol. Plant. 131, 191–200. https://doi.org/10.1111/j.1399-3054.2007.00959.x.
McMahon, K., Collier, C., Lavery, P.S., 2013. Identifying robust bioindicators of light stress in seagrasses: a meta-analysis. Ecol. Indic. 30, 7–15. https://doi.org/10.1016/j.ecolind.2013.01.030.
Melvin, S.D., Habener, L.J., Leusch, F.D.L., Carroll, A.R., 2017. 1H NMR-based metabolomics reveals sub-lethal toxicity of a mixture of diabetic and lipid-regulating pharmaceuticals on amphibian larvae. Aquat. Toxicol. 184, 123–132. https://doi.org/10.1016/j.aquatox.2017.01.012.
Melvin, S.D., Jones, O.A.H., Carroll, A.R., Leusch, F.D.L., 2018a. 1H NMR-based metabolomics reveals interactive effects between the carrier solvent methanol and a pharmaceutical mixture in an amphibian developmental bioassay with Limnodynastes peronii. Chemosphere 199, 372–381. https://doi.org/10.1016/j.chemosphere.2018.02.063.
Melvin, S.D., Leusch, F.D.L., Carroll, A.R., 2018b. Metabolite profiles of striped marsh frog (Limnodynastes peronii) larvae exposed to the anti-androgenic fungicides vinclozolin and propiconazole are consistent with altered steroidogenesis and oxidative stress. Aquat. Toxicol. 199, 232–239. https://doi.org/10.1016/j.aquatox.2018.04.004.
Mochida, K., Hano, T., Onduka, T., Ito, K., Yoshida, G., 2019. Physiological responses of eelgrass (Zostera marina) to ambient stresses such as herbicide, insufficient light, and high water temperature. Aquat. Toxicol. 208, 20–28. https://doi.org/10.1016/j.aquatox.2018.12.018.
Moore, K.A., Wilcox, D.J., Orth, R.J., 2000. Analysis of the abundance of submersed aquatic vegetation communities in the Chesapeake Bay. Estuaries 23, 115–127. https://doi.org/10.2307/1353229.
Moreno-Marín, F., Brun, F.G., Pedersen, M.F., 2018. Additive response to multiple environmental stressors in the seagrass Zostera marina L. Limnol. Oceanogr. 63, 1528–1544. https://doi.org/10.1002/lno.10789.
Mtwana Nordlund, L., Koch, E.W., Barbier, E.B., Creed, J.C., 2016. Seagrass ecosystem services and their variability across genera and geographical regions. PLoS One 11, e0163091. https://doi.org/10.1371/journal.pone.0163091.
Palla, P., Armano, G., 2016. RFmarkerDetector: Multivariate Analysis of Metabolomics Data using Random Forests.
Pan, Z., Raftery, D., 2007. Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. Anal. Bioanal. Chem. 387, 525–527. https://doi.org/10.1007/s00216-006-0687-8.
Peters, K., Worrich, A., Weinhold, A., Alka, O., Balcke, G., Birkemeyer, C., Bruelheide, H., Calf, O.W., Dietz, S., Dührkop, K., Gaquerel, E., Heinig, U., Kücklich, M., Macel, M., Müller, C., Poeschl, Y., Pohnert, G., Ristok, C., Rodríguez, V.M., Ruttkies, C., Schuman, M., Schweiger, R., Shahaf, N., Steinbeck, C., Tortosa, M., Treutler, H., Ueberschaar, N., Velasco, P., Weiß, B.M., Widdig, A., Neumann, S., van Dam, N.M., 2018. Current challenges in plant Eco-Metabolomics. Int. J. Mol. Sci. 19, 1–38. https://doi.org/10.3390/ijms19051385.
Petus, C., Collier, C., Devlin, M., Rasheed, M., McKenna, S., 2014. Using MODIS data for understanding changes in seagrass meadow health: A case study in the Great Barrier Reef (Australia). Mar. Environ. Res. 98, 68–85. https://doi.org/10.1016/j.marenvres.2014.03.006.
Rivas-Ubach, A., Pérez-Trujillo, M., Sardans, J., Gargallo-Garriga, A., Parella, T., Peñuelas, J., 2013. Ecometabolomics: optimized NMR-based method. Methods Ecol. Evol. 4, 464–473. https://doi.org/10.1111/2041-210X.12028.
Roca, G., Alcoverro, T., Krause-Jensen, D., Balsby, T.J.S., Van Katwijk, M.M., Marbà, N., Santos, R., Arthur, R., Mascaró, O., Fernández-Torquemada, Y., Pérez, M., Duarte, C.M., Romero, J., 2016. Response of seagrass indicators to shifts in environmental stressors: a global review and management synthesis. Ecol. Indic. 63, 310–323. https://doi.org/10.1016/j.ecolind.2015.12.007.
Rotini, A., Belmonte, A., Barrote, I., Micheli, C., Peirano, A., Santos, R.O., Silva, J., Migliore, L., 2013. Effectiveness and consistency of a suite of descriptors for assessing the ecological status of seagrass meadows (Posidonia oceanica L. Delile). Estuar. Coast. Shelf Sci. 130, 252–259. https://doi.org/10.1016/j.ecss.2013.06.015.
Salo, T., Reusch, T.B.H., Boström, C., 2015. Genotype-specific responses to light stress in eelgrass Zostera marina, a marine foundation plant. Mar. Ecol. Prog. Ser. 519, 129–140. https://doi.org/10.3354/meps11083.
Saunders, M.I., Bode, M., Atkinson, S., Klein, C.J., Metaxas, A., Beher, J., Beger, M., Mills, M., Giakoumi, S., Tulloch, V., Possingham, H.P., 2017. Simple rules can guide whether land- or ocean-based conservation will best benefit marine ecosystems. PLoS Biol 15, e2001886. https://doi.org/10.1371/journal.pbio.2001886.
Sheen, J., 2014. Master Regulators in Plant Glucose Signaling Networks. J. Plant Biol. 57, 67–79. https://doi.org/10.1007/s12374-014-0902-7.
Shulaev, V., Cortes, D., Miller, G., Mittler, R., 2008. Metabolomics for plant stress response. Physiol. Plant. 132, 199–208. https://doi.org/10.1111/j.1399-3054.2007.01025.x.
Sievers, M., Brown, C.J., Tulloch, V.J.D., Pearson, R.M., Haig, J.A., Turschwell, M.P., Connolly, R.M., 2019. The role of vegetated coastal wetlands for marine megafauna conservation. Trends Ecol. Evol. 34, 807–817. https://doi.org/10.1016/j.tree.2019.04.004.
Silva, J., Barrote, I., Costa, M.M., Albano, S., Santos, R., 2013. Physiological responses of Zostera marina and Cymodocea nodosa to light-limitation stress. PLoS One 8. https://doi.org/10.1371/journal.pone.0081058.
Statton, J., McMahon, K., Lavery, P., Kendrick, G.A., 2018. Determining light stress responses for a tropical multi-species seagrass assemblage. Mar. Pollut. Bull. 128, 508–518. https://doi.org/10.1016/j.marpolbul.2018.01.060.
Stitt, M., Gibon, Y., Lunn, J.E., Piques, M., 2007. Multilevel genomics analysis of carbon signalling during low carbon availability: Coordinating the supply and utilisation of carbon in a fluctuating environment. Funct. Plant Biol. 34, 526–549. https://doi.org/10.1071/FP06249.
Strydom, S., McMahon, K.M., Kendrick, G.A., Statton, J., Lavery, P.S., 2018. Short-term responses of posidonia australis to changes in light quality. Front. Plant Sci. 8, 1–13. https://doi.org/10.3389/fpls.2017.02224.
Subhashini, P., Dilipan, E., Thangaradjou, T., Papenbrock, J., 2013. Bioactive natural products from marine angiosperms: abundance and functions. Nat. Products Bioprospect. 3, 129–136. https://doi.org/10.1007/s13659-013-0043-6.
Toniolo, C., Di Sotto, A., Di Giacomo, S., Ventura, D., Casoli, E., Belluscio, A., Nicoletti, M., Ardizzone, G., 2018. Seagrass Posidonia oceanica (L.) Delile as a marine biomarker: A metabolomic and toxicological analysis. Ecosphere 9. https://doi.org/10.1002/ecs2.2054.
Touchette, B.W., Burkholder, J.A.M., 2000. Overview of the physiological ecology of carbon metabolism in seagrasses. J. Exp. Mar. Bio. Ecol. 250, 169–205. https://doi.org/10.1016/S0022-0981(00)00196-9.
Ubierna, N., Sun, W., Kramer, D.M., Cousins, A.B., 2013. The efficiency of C4 photosynthesis under low light conditions in Zea mays, Miscanthus x giganteus and

Flaveria bidentis Plant. Cell Environ. 36, 365–381. https://doi.org/10.1111/j.1365-3040.2012.02579.x.
Vehtari, A., Gelman, A., Gabry, J., 2017. Practical Bayesian model evaluation using leave-one-out cross-validation and WAIC. Stat. Comput. 27, 1413–1432. https://doi.org/10.1007/s11222-016-9696-4.
Vonk, J.A., Christianen, M.J.A., Stapel, J., O'Brien, K.R., 2015. What lies beneath: Why knowledge of belowground biomass dynamics is crucial to effective seagrass management. Ecol. Indic. 57, 259–267. https://doi.org/10.1016/j.ecolind.2015.05.008.
Waycott, M., Duarte, C.M., Carruthers, T.J.B., Orth, R.J., Dennison, W., Olyarnike, S., Calladine, A., Fourqurean, J.W., Heck, K.L.J., Hughes, A.R., Kendrick, G.A., Kenworthy, W.J., Short, F.T., Williams, S.L., 2009. Accelerating loss of seagrasses across the globe threatens coastal ecosystems. PNAS 106, 12377–12381.
Weiner, J., 2017. Package "pca3d": Three Dimensional PCA Plots. CRAN.
Zapata, O., Mcmillan, C., Texas, U.S.A., 1979. University of Oklahoma . The California studies have emphasized the inter- actions between higher plants ; the Oklahoma studies have clarified the effects on microorganisms of phenolic c o m p o u n d s produced b y higher plants and have related the resu 7.
Zidorn, C., 2016. Secondary metabolites of seagrasses (Alismatales and Potamogetonales; Alismatidae): Chemical diversity, bioactivity, and ecological function. Phytochemistry 124, 5–28. https://doi.org/10.1016/j.phytochem.2016.02.004.
Zou, H.X., Pang, Q.Y., Lin, L.D., Zhang, A.Q., Li, N., Lin, Y.Q., Li, L.M., Wu, Q.Q., Yan, X.F., 2014. Behavior of the edible seaweed Sargassum fusiforme to copper pollution: Short-term acclimation and long-term adaptation. PLoS One 9. https://doi.org/10.1371/journal.pone.0101960.