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Seasonal Variability in Non-Structural Carbohydrate Content of Warm-Adapted *Zostera noltei* and *Zostera marina* Populations

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Abstract: Non-structural carbohydrates (NSCs) are energetic compounds that can be accumulated in tissues and mobilized during periods of unfavorable conditions to maintain the biological functions of plants. The balance of these biochemical compounds is controlled by environmental factors such as temperature and irradiance. *Zostera noltei* and *Zostera marina* find one of their southern distribution limits in southern Spain, where relatively high seawater temperatures are reached during summer (23–24 °C). To better understand the effects of elevated temperatures on the concentration of NSCs, we conducted a seasonal study at Cadiz Bay, representing warm-adapted populations of these species. Our results showed a bimodal pattern in both species, with the highest NSC content observed in December and June, followed by a depletion in March and August. In addition, the NSC content observed in the leaves of *Z. noltei* ($71.26 \pm 30.77 \text{ mg g}^{-1}$ dry weight) was higher than in the rhizomes and roots ($52.14 \pm 38.86 \text{ mg g}^{-1}$ DW). The observed patterns suggest that these species accumulated NSCs to cope with periods of unfavorable environmental conditions. We also suggest that the limited concentration of NSCs in *Z. noltei* rhizomes and roots indicates that this population may be suffering physiological stress.

Keywords: seagrasses; energetic reserves; sucrose; starch; plant performance; productivity; temperate species



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1. Introduction

Seagrasses are flowering plants that thrive in shallow and sheltered marine regions, commonly found in intertidal and subtidal zones [1]. These ecosystems serve as vital ecological components, providing essential services such as reductions in wave energy, nursery habitats for aquatic life, or carbon sinks, thus contributing to mitigating climate change [2]. Despite their significance, seagrasses face several threats globally, experiencing an annual habitat loss of 7% due to climate change, pollution, and coastal development [3,4].

In temperate regions, *Zostera noltei* Hornemann 1832 and *Z. marina* Linnaeus 1753 are two of the most predominant seagrass species. *Zostera noltei* is a small seagrass often found in intertidal zones distributed from 19.5° to 62° N (<https://www.gbif.org>; accessed on 11 February 2024) [5]. *Zostera marina* is a medium-sized seagrass that primarily forms subtidal meadows. This species exhibits an extensive distribution range, encompassing latitudes between 27° and 70° N (<https://www.gbif.org>; accessed on 23 October 2023) [5]. Both species exhibit high plasticity in terms of vegetative growth, life cycle, and biochemical modulation, allowing them to adapt to a wide range of environmental conditions [6].

Environmental factors such as temperature or irradiance are key factors driving the seasonal changes of temperate seagrass species, as they regulate plant physiology, metabolism and biochemical composition [7]. Among the biochemical compounds that seagrasses are capable of synthesizing, non-structural carbohydrates (NSCs) are crucial energetic compounds. NSCs are mainly synthesized in the form of sucrose (soluble) and starch (non-soluble), with each compound following different metabolic pathways for its synthesis, and sucrose is the primary NSC synthesized in most seagrass species [8,9]. When conditions for photosynthesis and plant development are favorable, seagrasses tend to accumulate NSCs in both belowground (rhizomes and roots; BG) and aboveground (leaves; AG) tissues, whereas they usually accumulate greater amounts in the rhizomes [8]. However, under adverse environmental conditions, seagrasses can use their energetic reserves to sustain metabolic and physiological demands [10,11]. Seagrasses exhibit species-specific responses to environmental factors, likely influencing their capacity for NSC production and accumulation, reflecting biochemical adaptations and acclimations that shape their ability and resilience to cope with varying climate scenarios [12].

In Cadiz Bay (southern Spain), *Zostera noltei* is one of the most common seagrass species, forming monospecific and mixed meadows commonly in association with *Cymodocea nodosa* (Ucria) Ascherson 1870 and *Caulerpa prolifera* (Forsskål) J.V. Lamouroux 1809 [13]. However, in recent years, there has been a significant regression in the distribution of this species (authors observation). In contrast, the *Z. marina* population from Cadiz Bay represents one of its southernmost geographical distribution limits, and its presence is limited to small patches or sparse shoots in mixed seagrass meadows. It has been reported that its distribution was common along both the Atlantic and Mediterranean coasts of southern Spain during the 20th century, but over the last 3–4 decades, its distribution has regressed significantly due to coastal development and water pollution [14]. Recent studies have suggested that seagrass populations in Cadiz Bay may experience thermal stress during summer when seawater temperatures exceed 23–24 °C [15,16]. Most of the studies on NSCs in seagrasses at Cadiz Bay have focused on experimental effects of light limitation or ammonium toxicity in *Z. noltei* (e.g., [10,17–19]). Only Brun et al. (2003) conducted a study of the seasonal cycle of *Z. noltei* in this region when this species was largely abundant in the area, but no studies on NSCs in *Z. marina* have been conducted to date [20].

With these considerations in mind, it is critical to understand the role that NSCs play in the adaptation and acclimation of seagrasses to shifting environmental conditions. Therefore, the objectives of this study were as follows: (i) To investigate seasonal variability in the concentration of NSCs (sucrose and starch) in two temperate *Zostera* species distributed within their southern distribution limits; (ii) to identify differences in NSC content between AG and BG tissues and potential correlations with plant performance (i.e., relative growth rate (RGR)) and productivity seagrass traits (i.e., leaf area production (LAP) and leaf formation (LF)); and lastly, (iii) to identify species-specific differences in the seasonal patterns of NSC content among *Z. noltei* and *Z. marina*.

2. Materials and Methods

2.1. Habitat Description and Sample Collection

This study was conducted in two *Zostera noltei* and *Z. marina* populations located in an intertidal area of the inner bay of Cadiz Bay (36°29'23" N, 6°15'48" W; Figure 1). The population of *Z. noltei* formed a monospecific meadow, covering an estimated area of 150,000 m², while the population of *Z. marina* was a monospecific patch of an estimated area of 9 m² (for more detailed information, refer to Azcárate-García et al. (2022) [16]). Seagrass surveys were conducted monthly from October 2017 to August 2018. For the *Z. noltei* population, a permanent 30 m long transect was established during the studied period, while for the *Z. marina* patch, it was not possible to place a permanent transect due to its reduced size. For biochemical analyses, between 6 and 10 *Z. noltei* shoots were randomly collected along the permanent transect during each survey. For *Z. marina*, the second- and third-oldest leaves of four randomly selected shoots were collected, except for

December and March, when the entire shoot was collected. We decided not to collect the entire shoot to minimize damage to the patch. Collected samples were placed in zip-lock bags filled with marine seawater, stored in a cooler-box, and transported to the University of Cadiz (15 min drive from the sampling site). Once in the lab, shoots and leaves were cleaned of epiphytes and sediment with filtered seawater. Healthy aboveground (leaves; AG) and belowground (rhizomes and roots; BG) tissues without signs of necrosis were then separated and frozen at -80°C for 48 h. Afterward, they were freeze-dried for 48 h and stored at -80°C until biochemical analyses were conducted.

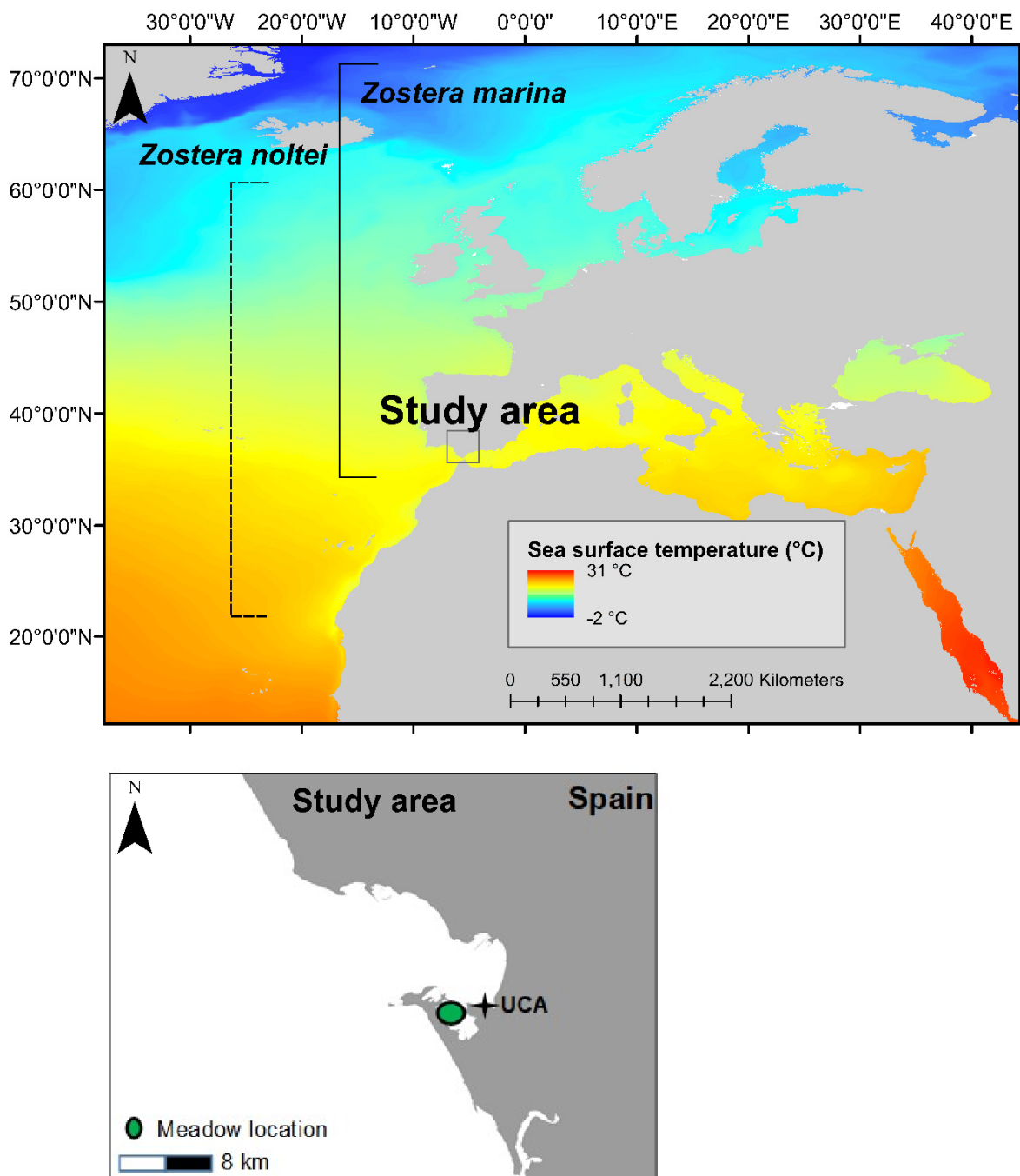


Figure 1. Map of satellite derived-data of mean sea surface temperature ($^{\circ}\text{C}$; <https://www.bio-oracle.org/>; accessed on 7 May 2024) indicating the latitudinal distribution range of *Zostera noltei* and *Z. marina* and map of Cadiz Bay indicating the sampling site (green circle; $36^{\circ}29'23''\text{N}$, $6^{\circ}15'48''\text{W}$) and the University of Cadiz (black cross; UCA).

2.2. Environmental Data

Daily data of sea surface temperature ($^{\circ}\text{C}$; SST; $36^{\circ}29'33.5''$ N, $6^{\circ}15'36.6''$ W) and irradiance (Wh m^{-2} ; $36^{\circ}29'23''$ N, $6^{\circ}15'48''$ W) in the sampling site from October 2017 to August 2018 were obtained from SeaTemperature.info (<https://seatemperature.info>; accessed on 2 May 2021) and the Copernicus Atmosphere Monitoring Service (CAMS; <http://www.soda-pro.com/web-services/radiation/cams-radiation-service>; accessed on 2 May 2021), respectively.

2.3. Biochemical Analysis

To determine the content of non-structural carbohydrates (sucrose and starch; NSCs) in both AG and BG tissues, the methodology described by Brun et al. (2003) was followed [10]. Sucrose was extracted five consecutive times in 96% ethanol (EtOH) at 80°C for 15 min, followed by the evaporation of the extractions under a constant stream of air at 40°C . Dry extracts were then dissolved in distilled water. Starch was extracted from the remanent pellet in 0.1 N sodium hydroxide (NaOH) for 24 h. Both sets of extractions were analyzed spectrophotometrically using a resorcinol assay for sucrose and an anthrone assay for starch at 486 and 640 nm, respectively. Pure sucrose was used as standard to construct calibration curves. Data were expressed in mg g^{-1} dry weight (DW). For each sample, three pseudo-replicates were analyzed. The mean analytical error obtained from the pseudo-replicates measurements was $\pm 1.03 \text{ mg g}^{-1}$ DW. Total NSCs in AG and BG tissues were calculated as the sum of sucrose and starch.

2.4. Relative Growth Rate

The relative growth rate ($\text{cm}^2 \text{ cm}^{-2} \text{ day}^{-1}$; RGR) was obtained by the ratio of the leaf area production ($\text{cm}^2 \text{ day}^{-1}$; LAP) and the total leaf area (cm^2 ; LA) of 10–15 randomly marked shoots [21]. The shoots were marked following the “punching” method described by Short and Duarte (2001) [22]. This ratio represents the rate of foliar growth per day in relation to the total leaf area of the plant and may be used as indicative of plant's performance. Data of LAP, LA and leaf formation (n° of new leaves day^{-1} ; LF) were extracted from Azcárate-García et al. (2022) [16].

2.5. Statistical Analysis

For statistical analysis, normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test) of the data were tested. As data did not meet the criteria for normality and/or homogeneity, a non-parametric Kruskal–Wallis test and multiple post hoc comparison (Dunn test) were applied. To explore seasonal changes in the total NSC content and in RGR (objective i), a series of one-way Kruskal–Wallis tests with Month (Mo) as a single factor were applied, separately, to NSCs accumulated in the AG and BG tissues and RGR for both species (*Z. marina* BG tissues were excluded from this test, as we only collected data from two months). To evaluate differences in the total NSC content between tissues in *Z. noltei* (objective ii), a one-way Kruskal–Wallis test with Tissue (Ti) as a single factor was applied including AG and BG biomass as factor levels. To study differences in the accumulation among sucrose and starch in AG and BG tissues, a series of one-way Kruskal–Wallis tests with Compound (Co) as a single factor, including sucrose and starch as factor levels, were applied to the AG and BG tissues, separately for both species, except for *Z. marina* BG tissues. Additionally, a correlation matrix based on Pearson's correlation coefficient was conducted for each species to investigate potential correlations among NSCs (sucrose, starch and total), RGR, LAP, LF and environmental variables (SST and irradiance). Finally, to visually compare species-specific seasonal dynamics in NSCs (objective iii), a principal component analysis (PCA) was conducted based on sucrose, starch and total NSC content in AG tissues, RGR, LAP and LF. Before conducting PCA analyses, data were normalized. Statistical analyses were conducted using the statistical software Rstudio 4.1.2 and PRIMER and PERMANOVA 6.

3. Results

The sea surface temperature (SST) in Cadiz Bay during the sampling period (Figure 2) registered maximum values in July (22.2 ± 0.5 °C) and August (23.0 ± 0.5 °C) and minimum values in January (15.0 ± 0.5 °C) and February (14.2 ± 0.2 °C). Additionally, in situ measurements at the sampling site during a heatwave in August 2018, at a depth of approximately 2 m, revealed temperatures ranging from 29.0 to 30.0 °C. The irradiance in the sampling site showed maximum values in June (7301.5 ± 1044.1 Wh m⁻²) and July (7929.9 ± 448.8 Wh m⁻²) and minimum values in December (2566.4 ± 614.4 Wh m⁻²) and January (2617.9 ± 835.8 Wh m⁻²).

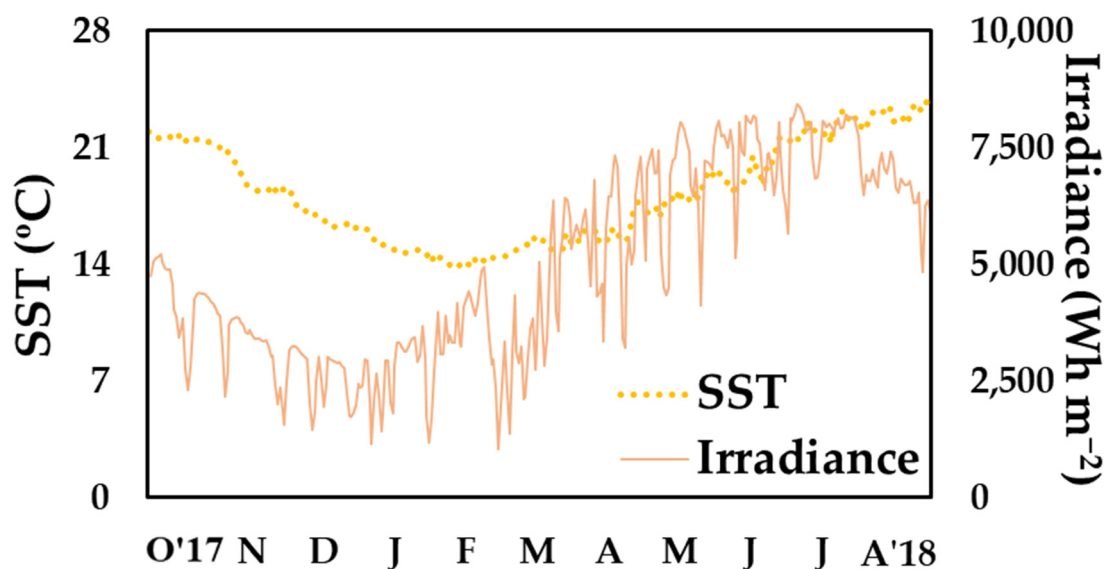


Figure 2. Daily sea surface temperature (°C; SST) and irradiance (Wh m⁻²) in Cadiz Bay from October 2017 to August 2018.

Significant differences in the seasonal accumulation of total non-structural carbohydrates (sucrose and starch; NSCs) in both seagrass species were reported (Kruskal–Wallis, $p < 0.05$; Table S1). In *Z. noltei*, two maximum annual peaks in the NSC content were observed. The first peak was noticed in December (Figure 3; Table S2), displaying a total NSC content of 85.20 ± 7.81 mg g⁻¹ dry weight (DW) in aboveground (leaves; AG) and 53.36 ± 19.05 mg g⁻¹ DW in belowground (rhizomes and roots, BG) tissues. The second and highest annual peak was observed in June, showing a total NSC content of 112.29 ± 43.41 and 92.24 ± 53.46 mg g⁻¹ DW in AG and BG tissues, respectively. Both annual peaks were followed by a significant drop in the total concentration of NSCs, reported in March (AG: 25.23 ± 5.23 ; BG: 32.41 ± 11.85 mg g⁻¹ DW) and August (AG: 89.89 ± 23.74 ; BG: 76.68 ± 17.52 mg g⁻¹ DW). *Zostera marina* displayed a similar annual pattern, showing two peaks in the concentration of NSCs in AG tissues. The first peak was observed in December (161.20 ± 22.06 mg g⁻¹ DW) and the second peak in June (124.04 ± 11.58 mg g⁻¹ DW). Both peaks were also followed by a depletion in the total NSC content of AG tissues, observed in March (89.69 ± 23.32 mg g⁻¹ DW) and August (83.55 ± 12.81 mg g⁻¹ DW). Additionally, significant differences in the concentration of sucrose and starch were also observed in both species (Kruskal–Wallis, $p < 0.05$). *Zostera noltei* showed higher concentration of sucrose in AG (49.57 ± 27.79 mg g⁻¹ DW) and BG tissues (21.69 ± 6.68 mg g⁻¹ DW) than starch (AG: 36.41 ± 31.72 ; BG: 15.73 ± 9.26 mg g⁻¹ DW). *Zostera marina* also had a higher concentration of sucrose than starch both in AG (sucrose: 93.17 ± 37.66 ; starch: 12.67 ± 7.28 mg g⁻¹ DW) and BG tissues (sucrose: 124.38 ± 29.47 ; starch: 40.66 ± 15.24 mg g⁻¹ DW).

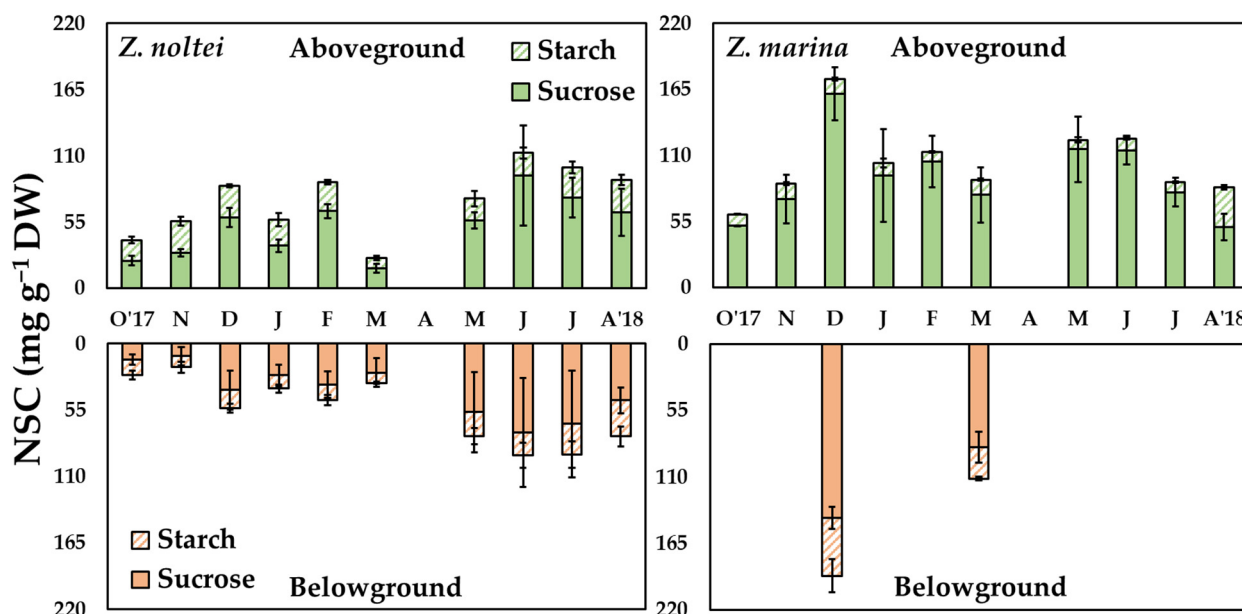


Figure 3. Non-structural carbohydrate (sucrose and starch; mg g^{-1} DW; NSC) content in aboveground (leaves) and belowground (rhizomes and roots) tissues of *Zostera noltei* and *Z. marina* at Cadiz Bay from October 2017 to August 2018.

Regarding differences among AG and BG tissues, *Zostera noltei* showed a significantly larger mean annual total NSC content in AG tissues of $71.26 \pm 30.77 \text{ mg g}^{-1}$ DW than in BG tissues, which had $52.14 \pm 38.86 \text{ mg g}^{-1}$ DW (Kruskal–Wallis, $p < 0.05$). This pattern was likely observed throughout the seasonal cycle, except in March, when there were no significant differences among the BG ($32.41 \pm 11.85 \text{ mg g}^{-1}$ DW) and AG ($25.23 \pm 5.23 \text{ mg g}^{-1}$ DW) tissues. In contrast, the mean annual total NSCs in the AG tissues of *Z. marina* was $105.85 \pm 35.94 \text{ mg g}^{-1}$ DW, while the mean total NSCs estimated in the BG tissues was $165.04 \pm 40.79 \text{ mg g}^{-1}$ DW.

Seasonal significant differences in the relative growth rate (RGR) of *Z. noltei* were observed (Kruskal–Wallis, $p < 0.05$), revealing two annual maximum peaks. The first peak was reported in November ($0.045 \pm 0.017 \text{ cm}^2 \text{ cm}^{-2} \text{ day}^{-1}$) and was followed by a decline in December ($0.026 \pm 0.011 \text{ cm}^2 \text{ cm}^{-2} \text{ day}^{-1}$), when the minimum value was registered (Figure 4; Table S3). Subsequently, an increasing trend was observed until August ($0.062 \pm 0.020 \text{ cm}^2 \text{ cm}^{-2} \text{ day}^{-1}$), when the second peak was registered. Seasonal significant differences in the RGR of *Z. marina* were also observed (Kruskal–Wallis, $p < 0.05$). This species showed a more stable tendency of RGR values and one annual peak in February ($0.029 \pm 0.008 \text{ cm}^2 \text{ cm}^{-2} \text{ day}^{-1}$). The minimum value in this species was also observed in December ($0.017 \pm 0.006 \text{ cm}^2 \text{ cm}^{-2} \text{ day}^{-1}$).

The principal component analysis (PCA; Figure 5) grouped both species separately, indicating species-specific differences in the NSC content, plant performance and biomass production traits among both species. The PCA results showed that PC1 (% of variation: 52%; λ : 3.12) and PC2 (% of variation: 28%; λ : 1.68) were both significant and explained 80% of the variance. Based on the PCA results, *Zostera noltei* tends to exhibit higher starch content in leaves relative to its total NSC content during the annual cycle, showing an increase in the sucrose content during summer (June, July and August) and late winter (February), when the RGR and leaf formation (LF) also increases. On the contrary, *Zostera marina* showed higher sucrose content relative to its total NSC content during most of the annual cycle, except in August, when the concentration of starch increased, and October, when a reduction in the NSC content was registered.

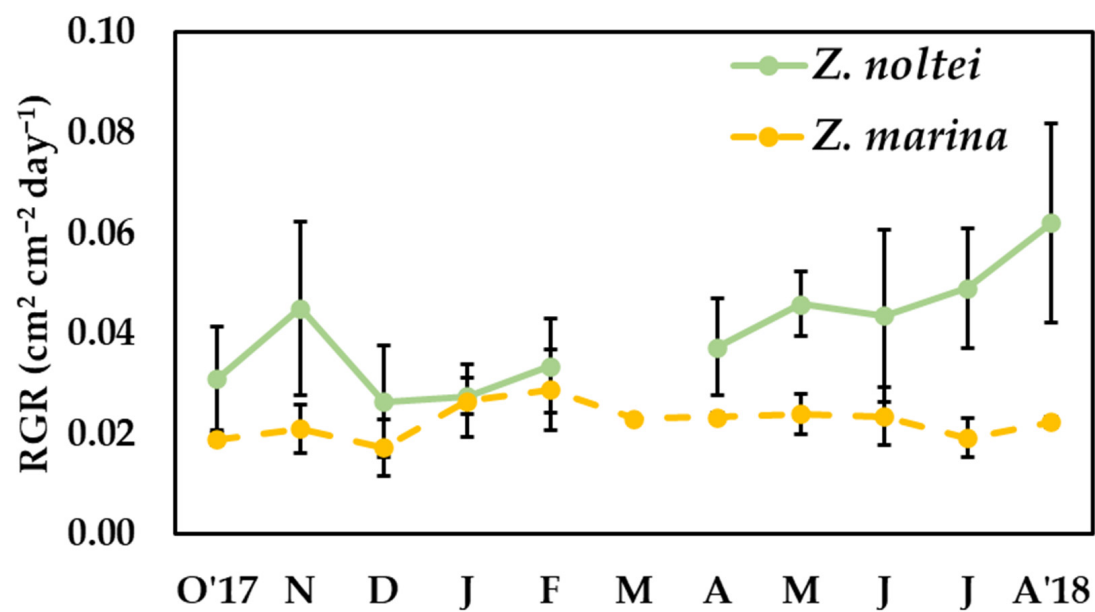


Figure 4. Relative growth rate ($\text{cm}^2 \text{cm}^{-2} \text{day}^{-1}$; RGR) of *Zostera noltei* and *Z. marina* at Cadiz Bay from October 2017 to August 2018.

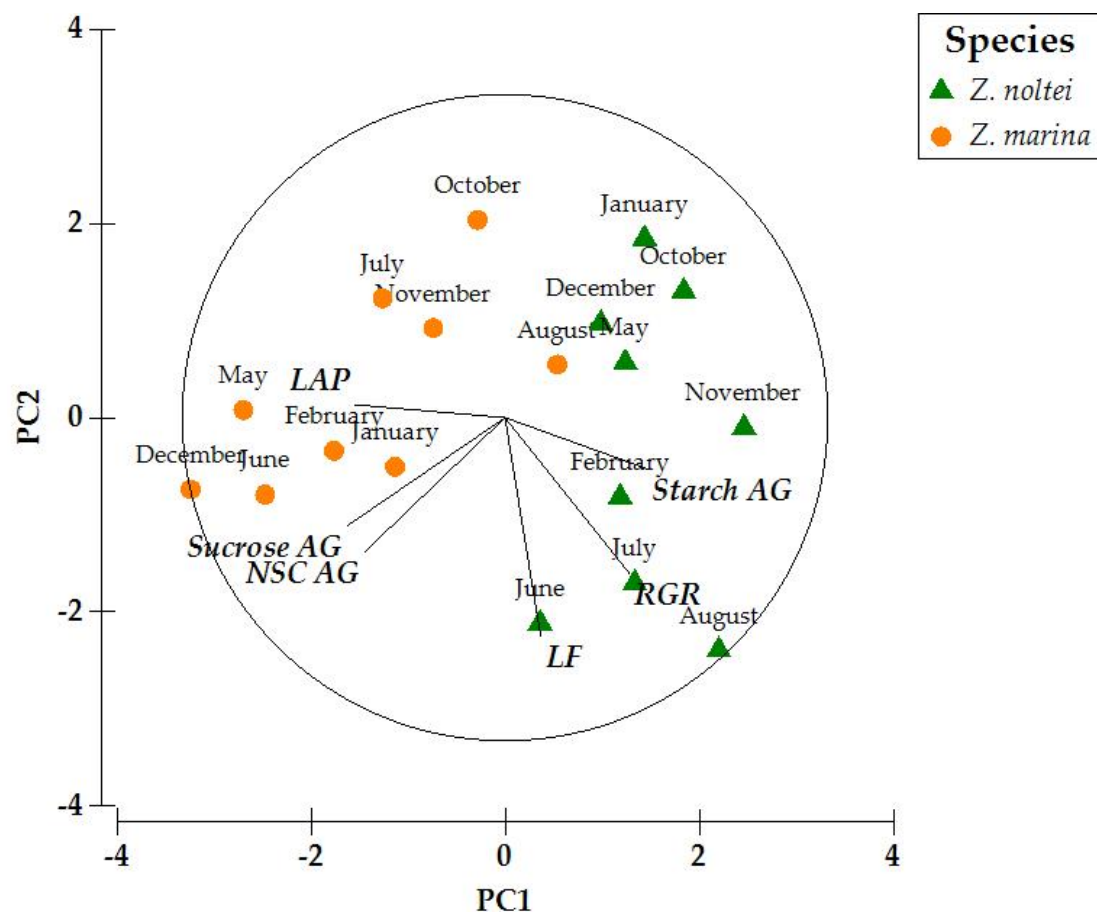


Figure 5. Principal component analysis (PCA) showing the resemblance of *Zostera noltei* (green triangles) and *Z. marina* (orange circles) populations from Cadiz Bay based on the sucrose, starch and total non-structural carbohydrates (NSCs) content in aboveground (leaves; AG) tissues, relative growth rate (RGR), leaf area production (LAP) and leaf formation (LF).

4. Discussion

This study represents a progress in understanding the seasonal variability of non-structural carbohydrates (NSCs) in populations of temperate seagrass species distributed within their southern distribution range and adapted to relatively high temperatures. Two main outcomes can be highlighted from this study: (i) both seagrass species exhibited a bimodal pattern in NSC content, displaying two annual peaks in early winter and summer, with drops observed in late summer and late winter, coinciding with the highest and lowest sea surface temperatures (SST) during the annual cycle, respectively. (ii) The concentration of NSCs in *Zostera noltei* was significantly higher in aboveground (leaves; AG) than in belowground (rhizomes and roots; BG) tissues, suggesting that this species may be suffering physiological stress likely associated with unfavorable environmental conditions.

4.1. Seasonal Variability in NSC Content

The seasonal pattern observed for NSCs in *Z. noltei* is likely linked to the plant's responses to environmental changes, as well as physiological demands [11,12]. Overall, the larger concentration of NSCs during an annual cycle in temperate seagrasses has been attributed to two main factors. The first one involves the conversion of excess energy generated during optimal photosynthetic conditions into NSCs when the produced energy exceeds the plant's demands [20,23]. The second one is related to the preconditioning of plants to cope with upcoming environmental conditions, which is directly dependent on the environmental conditions of the previous season [24–26]. In support, the studied *Z. noltei* population accumulated higher energetic compounds in December and June to cope with the lowest (14 °C) and highest (23–24 °C) annual temperatures reached in late winter and late summer, respectively. The observed increase in NSC content in early winter was also accompanied by a reduction in the relative growth rate (RGR), which may favor NSC accumulation. Then, the depletion in the NSC content during late winter and late summer may suggest the utilization of energetic reserves during unfavorable environmental conditions that exceed the physiological optimal conditions of the plants. In support, the population of *Z. noltei* in Cadiz Bay exhibited an increase in shoot density and coverage during the summer season, favoring shading among adjacent shoots, thereby reducing incident irradiance. At the same time, that they are being affected by high temperatures [10,16,27]. This, together with the observed depletions in body size and leaf production during late summer, suggested that they experienced some degree of thermal stress at the highest summer temperatures [16]. Similarly, in a *Z. noltei* population from the Adriatic Sea, also exposed to warm summer temperatures (25 °C), plants exhibited a decline in the concentration of NSCs with the highest annual temperatures [28]. Brun et al. (2003) reported that *Z. noltei* plants at Cadiz Bay accumulated more NSCs during autumn, exhibiting minimum NSC content during spring due to the light deprivation mainly associated with the exacerbated growth of *Ulva* species [20]. Contrary to southern populations, northern distributed *Z. noltei* populations, exposed to colder thermal regimens, exhibited a different annual NSC pattern. Northern populations displayed the highest annual NSC content in summer, aligning with the warmest temperatures of the year, which also favored plant production, with no depletion of NSCs observed. Subsequently, a depletion in NSC content during winter season is noticed due to the mobilization of NCS reserves for plant surviving since photosynthetic tissues are practically absent [25,29]. Interestingly, we found no correlation between plant performance and production and the concentration of NSCs in the studied plants (Figure S1). This suggests that growth patterns and the concentration of NSCs displayed diverse and uncoupled responses to seasonal environmental changes.

The observed bimodal pattern of NSC accumulation in *Z. marina* suggests that this species also showed higher NSC content before periods of unfavorable environmental conditions for growth and biomass production. This was evidenced by an increase in the NSC content in early winter and early summer, which was preceded by a depletion in late winter and late summer. Consistent with this, a reduction in the biomass production

of this population was also observed in late summer [16]. Furthermore, a parallel study involving the same population reported significant necrosis in photosynthetic tissues and a marked reduction in unsaturation levels in seagrass leaves under the warmest environmental conditions reached in summer, indicative of thermal stress in marine plants [15]. Conversely, northern *Z. marina* populations exposed to likely cold temperatures displayed a different pattern in NSC accumulation in leaves compared to the studied warm adapted population. Northern populations reported a single peak in their NSC content during the warmest summer months when environmental conditions were more suitable to growth and biomass production. This peak was followed by a depletion in NSC content in seagrass leaves, reaching its lowest values in winter under darker and colder conditions—when environmental conditions were likely less favorable for seagrass development [12,30]. In support of this, *Z. marina* plants from Ireland exhibited a significant positive correlation between plant production and the concentration of NSCs [12]. However, no correlation was observed between plant performance and production and NSC content in the studied population from Cadiz Bay (Figure S2).

Common to both seagrass species, plants exhibited a higher concentration of sucrose in comparison with starch in both AG and BG tissues. This observation aligns with findings in temperate seagrass species, where sucrose was identified as the primary NSC [9,18]. This can be attributed to sucrose serving as a readily mobilizable energy source, facilitating faster responses and higher accumulation fluctuations in response to the metabolic and physiological demands of seagrasses [23]. In contrast, starch, while present, may play a more substantial role in longer-term energy storage [31]. Furthermore, our results revealed that during the highest SST reached in August, sucrose content was decreased, while starch increased. Similarly, previous studies observed that under periods of physiological stress, seagrasses may prioritize the utilization of their sucrose reserves over their starch reserves [23,32].

4.2. Differences in NSC Accumulation among Tissues

Overall, carbon reserves in seagrasses are primarily accumulated in rhizomes, as these structures have the capacity to store larger reserves compared to photosynthetic structures and ensure the existence of energetic reserves during periods of unfavorable environmental conditions and reduced or absent photosynthetic biomass [8,10]. However, in this study, we observed higher NSC content in AG than in BG tissues during the annual cycle, contrasting previous observations in *Z. noltei* populations at Cadiz Bay [20]. This unusual pattern has been previously observed and was associated with plants experiencing some degree of physiological stress, thereby reducing the accumulation of NSCs in rhizomes due to the mobilization of these energetic reserves to cope with unfavorable environmental conditions [10,17]. Cabaço and Santos (2007) observed that under experimentally reduced irradiance conditions, higher sucrose content was measured in leaves compared to rhizomes, suggesting that under conditions of physiological stress, NSCs may be mobilized to maintain plant metabolic demands [33] and thus not accumulated in rhizomes. Therefore, the observed pattern in this study also suggests that *Z. noltei* plants in Cadiz Bay could be exposed to some degree of environmental stress conditions, such as elevated temperatures. These conditions may affect the carbon balance of the plants by reducing the allocation of NSCs in rhizomes due to their rapid consumption.

4.3. Species-Specific Differences

Despite observing that *Z. noltei* and *Z. marina* reported a similar seasonal pattern of NSC accumulation, the principal component analysis (PCA) revealed species-specific differences. For instance, *Zostera noltei* showed more NSC content in early summer than in early winter, while *Z. marina* NSC accumulation during early winter was higher than in early summer. Additionally, *Zostera noltei* showed lower concentration of NSCs in AG tissues than *Z. marina*, probably due to the fact that *Z. noltei* is a fast-growing seagrass species with higher growth rates relative to its size and shorter leaf lifespan, thus resulting

in a faster utilization of NSCs for growth [18,20]. In this context, both species adopted different growth strategies. *Zostera noltei* displayed a strategy focused on maximizing growth rates, particularly during late summer months, while *Z. marina* exhibited a more stable growth pattern, prioritizing steady plant development. In addition, *Zostera noltei* showed a higher concentration of starch relative to total NSCs than *Z. marina*, which is in line with previous studies showing that *Z. noltei* can accumulate more starch, relative to its total NSC content, than other seagrass species [9].

4.4. Conservation Status of Seagrass Populations in Cadiz Bay

Over the last two decades the *Z. noltei* populations located in Cadiz Bay has undergone a significant decline (authors observation), including the meadow where this study was conducted (Figure 6). While the decline of other *Z. noltei* populations in southern Spain have been linked to eutrophication processes [17], the combination of various factors could have contributed to the habitat reduction of the studied population. These factors include the high pressure of shell fishing in the meadow, the discharge of wastewater into the area promoting eutrophication events, and increased temperatures, including recurring heat waves, associated with global warming [34–36]. However, the causes of the decline of the *Z. noltei* populations in Cadiz Bay remain unclear. Additionally, the recent appearance of the invasive algae species *Rugulopteryx okamurae* (E.Y. Dawson) I.K. Hwang, W.J. Lee and H.S. Kim, 2009 in the coast outside the bay (authors observation) may represent a potential risk to the seagrass populations inhabiting Cadiz Bay, whether it spreads into the inner bay. Noteworthy, the higher NSC content observed in the leaves compared to the rhizomes may serve as an early indicator of stress due to habitat degradation affecting this population. In this support, seagrass indicators based on NSC content are characterized by a rapid response to unfavorable conditions [37].

Regarding *Z. marina*, the populations in Cadiz Bay have remained stable since their first documentation in 2006, with distribution limited to a few patches. These patches sometimes form mixed meadows with *Cymodocea nodosa* and *Z. noltei* along the intertidal zone of the inner bay [13,16]. Floating shoots of *Z. marina* have been observed along the coast outside the bay (authors' observation), suggesting that its distribution in this region may be greater than expected. This highlights the necessity for additional habitat mapping to document potential novel *Z. marina* populations. Due to the degradation experienced by the seagrass populations in Cadiz Bay over the last 3–4 decades, increased conservation efforts focused on their restoration have been recently undertaken. For instance, an ongoing research project at the University of Cadiz has established cultures of temperate seagrass species (i.e., *Zostera marina*, *Zostera noltei*, and *C. nodosa*) in earthen ponds within Cadiz Bay. The aim is to generate a seed bank for restoration and blue carbon projects [38]. Additionally, this project has promoted the use of non-viable *Z. marina* seeds in gastronomy ("sea rice"), adding extra value to these cultures as a highly nutritious food source [38].

Lastly, this study provided new insights about the responses to environmental changes in temperate seagrass species, specifically focusing on the concentration of NSCs in warm-adapted populations located within their southern distribution limit. The reduction in NSCs during warmer months and the limited concentration in the BG tissues suggest that seagrass populations in Cadiz Bay may be potentially exposed to a certain degree of thermal physiological stress. These outcomes are particularly relevant because they focus on the last remaining *Z. marina* populations in southern Spain, generating critical knowledge for ongoing projects aiming to restore meadows of this species in the region. Additionally, *Zostera noltei* populations are experiencing critical habitat reduction. Therefore, the reported results are of high importance, as these observed patterns may become more common for temperate seagrass ecosystems in the coming years due to the consequences of global warming.



Figure 6. Photo series from 2009 to 2024 of the *Z. noltei* population where this study was conducted (36°29'23" N, 6°15'48" W).

5. Conclusions

This study provides new data on the seasonal patterns of non-structural carbohydrate (NSC) content in *Zostera noltei* and *Z. marina* populations located within their southern distribution range, representing warm-adapted populations of these species. Our results revealed that both seagrass species presented a bimodal pattern in the accumulation of NSCs during the annual cycle, exhibiting two peaks in NSC accumulation during early winter and early summer, followed by a decline after each peak. The observed seasonal patterns may suggest that plants exhibit a higher amount of NSCs before periods of un-

favorable environmental conditions. In addition, we observed that the seasonal cycle of NSCs and plant development and production were not coupled, suggesting that these variables presented different responses to seasonal changes in environmental conditions. Furthermore, the higher NSC content observed in the aboveground tissues of *Z. noltei* compared to belowground tissues may indicate that the rapid utilization of NSCs is likely due to physiological stress. Species-specific differences in the NSC accumulation of both species were also identified. Since the knowledge of the seasonal patterns of NSC accumulation in southern distributed populations of temperate seagrass species is still limited, and considering that these populations will be among the first to be affected by rising global temperatures, further studies should delve into assessing the effect of exceeding physiological optimal conditions on these species. Such data could play a crucial role in identifying habitat degradation resulting from climate change, thereby facilitating the implementation of conservation projects.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d16070391/s1>, Table S1: Results of Kruskal-Wallis tests. *Chi-square* values are shown together with degree of freedom (df), sum of ranks (SR) and significance levels (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$); Table S2: Results of non-structural carbohydrate (mg g⁻¹ dry weight (DW); NSC) content in aboveground (leaves; AG) and belowground (rhizomes and roots; BG) tissues of *Z. noltei* and *Z. marina* plants at Cadiz Bay. Data represents mean $\pm$ standard deviation. (-): no data available; Table S3: Results of relative growth rate (cm² cm⁻² day⁻¹; RGR), leaf area production (cm² day⁻¹; LAP), leaf formation (n° of new leaves day⁻¹; LF) and leaf area (cm²; LA) in *Z. noltei* and *Z. marina* at Cadiz Bay. Data of LAP and LF was extracted from Azcárate-García et al. (2022) [16]. Data represents mean $\pm$ standard deviation. (-): no data available; Figure S1: *Zostera noltei* correlation matrix based on Pearson's coefficient indicating positive (green) or negative (blue) correlations among sucrose, starch, total non-structural carbohydrates (NSCs) in aboveground (leaves; AG) and belowground (rhizomes and roots; BG) tissues, relative growth rate (RGR), leaf area production (LAP), leaf formation (LF), sea surface temperature (SST) and irradiance. Higher correlations are indicated by higher sizes; Figure S2: *Zostera marina* correlation matrix based on Pearson's coefficient indicating positive (green) or negative (blue) correlations among sucrose, starch, total non-structural carbohydrates (NSCs), relative growth rate (RGR), leaf area production (LAP), leaf formation (LF), sea surface temperature (SST) and irradiance. Higher correlations are indicated by higher sizes.

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