



# Advances in understanding multilevel responses of seagrasses to hypersalinity

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## ABSTRACT

Human- and nature-induced hypersaline conditions in coastal systems can lead to profound alterations of the structure and vitality of seagrass meadows and their socio-ecological benefits. In the last two decades, recent research efforts (>50 publications) have contributed significantly to unravel the physiological basis underlying the seagrass–hypersalinity interactions, although most (~70%) are limited to few species (e.g. *Posidonia oceanica*, *Zostera marina*, *Thalassia testudinum*, *Cymodocea nodosa*). Variables related to photosynthesis and carbon metabolism are among the most prevalent in the literature, although other key metabolic processes such as plant water relations and responses at molecular (i.e. gene expression) and ultrastructure level are attracting attention. This review emphasises all these latest insights, offering an integrative perspective on the interplay among biological responses across different functional levels (from molecular to clonal structure), and their interaction with biotic/abiotic factors including those related to climate change. Other issues such as the role of salinity in driving the evolutionary trajectory of seagrasses, their acclimation mechanisms to withstand salinity increases or even the adaptive properties of populations that have historically lived under hypersaline conditions are also included. The pivotal role of the costs and limits of phenotypic plasticity in the successful acclimation of marine plants to hypersalinity is also discussed. Finally, some lines of research are proposed to fill the remaining knowledge gaps.

## 1. Introduction

Seagrasses are a relatively small polyphyletic group of clonal plants belonging to the order Alismatales (60–70 recognised species within four families, namely Posidoniaceae, Zosteraceae, Hydrocharitaceae and Cymodoceaceae) that have colonised the sea on at least three different occasions along their evolutionary history (Les et al., 1997; Janssen and Bremer, 2004). Seagrasses inhabit most coastal areas around the world, where they structure one of the most valuable ecosystems on Earth due to the ecological functions and socio-economic services they provide (Cullen-Unsworth et al., 2014). In the colonisation of the marine realm, seagrasses have faced critical challenges to complete their life cycles totally submerged in hypersaline waters, with high hydrodynamic forces, highly attenuated irradiance levels, reduced CO<sub>2</sub> availability and the presence of sulphide-rich anoxic sediments (Larkum et al., 2006). This challenging adaptation has resulted in the loss of several characters present in their terrestrial counterparts (e.g.

absence of stomata), but also the gain of others typical of macroalgae (e.g. cell wall composition and carbon concentration mechanisms; Lee et al., 2016; Olsen et al., 2016; Chen et al., 2021).

Seagrasses have also evolved particular properties at the physiological (e.g. osmotic capacities; Tyerman, 1989), ultrastructural (e.g. absence of a real cuticle and development of plasmalemma-mitochondrial transport system; Iyer and Barnabas, 1993) and morphological (e.g. protection of meristem tissues by petiolar sheaths; Tyerman, 1989) levels that allow them to tolerate seawater and to prosper in a wide range of salinity, including oceanic waters, estuaries and hypersaline coastal lagoons. These properties vary inter- and intraspecifically (Walker and McComb, 1990; van Katwijk et al., 1999; Touchette, 2007; Sandoval-Gil et al., 2014), determining the natural distribution of species and populations, as well as their tolerance to natural or human-induced salinity changes. Therefore, salinity is recognised as one of the major factors that have forced the evolutionary trajectory of seagrasses, conditioning their distribution, ecology and

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biology (Kuo and den Hartog, 2000).

With a thorough revision, Touchette (2007) examined the mechanisms and processes enabling marine angiosperms to thrive in seawater, and how these mechanisms respond under changing saline conditions. The author reviewed a total of 160 studies on the interaction between plants (including marine algae and terrestrial halophytes) and salinity, of which less than half (57) were related to seagrasses and only 18 provided information on physiological responses. This highlights the limited knowledge that existed at the time regarding the interactions between salinity and seagrasses, especially at the physiological level, despite the fact that this is one of the most intriguing aspects of seagrasses. Since Touchette's revision, an increasing number of studies

have been published on the ecophysiological effects of hypersalinity on seagrasses (see Fig. 4 and Table A1 in Appendix). Particularly, >50 publications have contributed significantly to unravel the physiological basis underlying the seagrass–hypersalinity interactions, although most (~70%) are limited to few species, such as the Mediterranean *Posidonia oceanica* and *Cymodocea nodosa*. Some of them have focussed on how seagrasses cope with natural hypersaline conditions (see Box 1), as those existing in coastal lagoons with high evaporation rates and limited water exchange, such as the Stagnone di Marsala in Italy (Tomasello et al., 2009), the Mar Menor in Spain (Fernández-Torquemada and Sánchez-Lizaso, 2011) or Shark Bay in Australia (Bell et al., 2019). However, most of the research effort conducted during the last two

#### BOX 1

##### Seagrass populations living under natural hypersaline conditions.

Despite the potential sensitivity of seagrasses to hypersalinity, particular seagrass populations have been well established under saline conditions that should exceed species-specific tolerance thresholds (Koch et al., 2007b; Tomasello et al., 2009; Marín-Guirao et al., 2017). Coastal lagoons are generally characterised by a shallow depth and relatively weak water exchange with the open sea, a situation that favours evaporative processes and hence, the existence of hypersaline waters. These conditions can be further fuelled by the diversion and withdrawal of freshwater inflows, which partially contributes to alter other environmental parameters – for example, temperature (Koch et al., 2007b).

The stenohaline species *P. oceanica* grows on shallow bottoms of the Stagnone di Marsala, a semi-enclosed coastal lagoon located in the western coast of Sicily, Italy (La Loggia et al., 2004; Tomasello et al., 2009). The hydrodynamic and geomorphological features of this system promote environmental gradients of salinity and temperature (up to 48 and 30 °C, respectively), which have been correlated with a particular formation of atoll-shaped reefs, a meadow morphotype also documented in other coastal lagoons and particular areas along the Turkish and Corsican coasts (Boudouresque et al., 1990; Pasqualini et al., 1998; Pergent et al., 2002). Tomasello et al. (2009) argued that *P. oceanica*–forming atolls are able to persist in such extreme conditions at the expense of constrained vegetative productivity. Moreover, and contrary to the frequent blooming of meadows from outside the lagoon, there are no flowering events in inner lagoon meadows, which favours their genetic isolation and likely the selection of genotypes adapted to this more extreme conditions. More recently, Spinelli (2018) revealed that the innermost plants are better osmoadapted to increased salinity than plants growing at lower salinities, as inferred from their lower  $\psi_w$  and  $\psi_\pi$ , and higher leaf proline content. In addition, and in contrast to plants from other environments, these innermost plants withstood experimental hypersaline exposure, being able to maintain unchanged photosynthetic performance and to modify their leaf proline content for osmotic adjustment.

The subtropical Florida Bay (South Florida, USA) and the Mediterranean Mar Menor (South-east Spain) are shallow semi-enclosed coastal lagoons with restricted seawater circulation where seagrasses develop (Koch et al., 2007a; Lirman and Cropper, 2003). In Florida Bay, the seagrasses *T. testudinum*, *Halodule wrightii* and *R. maritima* can experience salinity up to 50 due to the synergistic effects of evaporation and restricted freshwater inflow during the drought season. By using different mesocosm approaches, Koch et al. (2007a) concluded that all the three species are able to osmoacclimate and maintain elevated vegetative and photosynthetic productivity in a wide range of hypersaline conditions, although this ability may change depending on the interaction with other environmental factors (i.e. temperature and sulphide in sediments; Koch and Erskine, 2001; Koch et al., 2007b). The physiological and vegetative performances of *C. nodosa* from the Mar Menor, with a salinity of 42–47, show a seasonal pattern quite similar to that of nearby populations from the adjacent Mediterranean Sea (with a lower and more stable salinity of ~37; Sandoval-Gil, 2012), suggesting that lagoon populations are fully adapted to the hypersaline waters. This has been corroborated in manipulative laboratory experiments: the lagoon plants have a higher tolerance to increased salinity than the Mediterranean populations (Fernández-Torquemada and Sánchez-Lizaso, 2006, 2011). The physiological tolerance of *C. nodosa* from the lagoon can again be partially explained by osmoadaptive properties such as the capacity to reach lower leaf osmotic potentials ( $\psi_\pi$ ) and to accumulate higher amounts of compatible osmolytes with osmoprotective functions (e.g. proline and soluble sugars; Sandoval-Gil, 2012).

The influence of the Mar Menor hypersaline waters is not limited to the species growing within the lagoon, as parts of the adjacent Mediterranean populations of *P. oceanica* are exposed to the exchange of water between the lagoon and the open ocean. Specifically, Marín-Guirao et al. (2017) compared a wide set of biological variables between plants growing under the influence of fluctuating hypersaline lagoon waters, and those from the same population growing far from this influence. They found that plants exposed to hypersaline waters had developed physiological strategies to tolerate the fluctuating hypersaline stress, including the ability to prevent the accumulation of toxic ions in plant cells, enhanced photosynthesis to compensate for increased respiration and augmented accumulation of organic osmolytes (sugars and proline) to achieve osmotic stability. These strategies allow plants to survive in this long-term unfavourable environment and to maintain the meadow structure in terms of plant density and cover, but with a considerable reduction in size. A reduced plant size has been proposed as a stress-coping mechanism based on more efficient utilisation of internal resources (see section 3.7), and it is also consistent with the existence of undersized *P. oceanica* shoots in other potentially unfavourable environments such as the Stagnone di Marsala.

Other studies have provided additional evidence of seagrasses growing under hypersaline conditions in coastal lagoons and estuarine systems. For example, the salinity levels in Biscayne Bay (up to 45; Florida, USA) correlate strongly with the abundance and distribution of seagrasses (Lirman and Cropper, 2003), which in turn may determine the structure of associated faunal communities, including fishes and crustaceans (Santos et al., 2018). In Laguna Madre (Texas, USA), *H. wrightii* meadows appear to be highly resistant to hypersaline conditions, including extreme levels (50–70) that occur during prolonged and exceptional periods of drought causing mass mortality of the coexistent species *Syringodium filiforme* (Wilson and Dunton, 2018). Finally, *A. antarctica* is the most abundant seagrass in Shark Bay (Western Australia), where salinity ranges from 35 to 65 and salinity gradients may be a primary factor driving the productivity of this species (Walker and McComb, 1990). Furthermore, it has been argued that hypersalinity in this system is a key 'biotic filter' conditioning the abundance and adaptive mechanisms of other species (e.g. *P. australis*; Booth et al., 2022), and even affecting the success of ambitious projects to restore degraded meadows (Statton et al., 2012).

decades has been driven by the growing environmental concern associated with expansion of the desalination industry. Seawater desalination has emerged to alleviate the problem of water scarcity in coastal regions worldwide, such as in the Mediterranean Sea, the Middle East–Arabian Gulf, Chile, Australia, California and the Red Sea (Lattemann and Höpner, 2008; Cambridge et al., 2017; Sola et al., 2019). The discharge of hypersaline effluents or brines from desalination plants currently represents a major threat to seagrasses (Ruiz et al., 2009b; Cambridge et al., 2019), which has led to the need to develop new scientific knowledge to establish effective conservation and management strategies for seagrass-based ecosystems.

Recent experimental approaches conducted in the field (Gacia et al., 2007; Ruiz et al., 2009b; Garrote-Moreno et al., 2014, 2015a; Capó et al., 2020) and under controlled laboratory conditions (Sandoval-Gil et al., 2014a; Oscar et al., 2018; Cambridge et al., 2019) have provided notable insights into the physiological basis behind the tolerance and resilience of seagrasses to increased salinity. Seagrass response variables have been studied across different functional levels, from the molecular, biochemical and physiological levels to the morphological and population levels. Additionally, notable progress has been made particularly on plant traits that had never or had been studied poorly in seagrasses. These include, among others, the osmoacclimative responses (Cambridge et al., 2017, 2019), changes in leaf optics (Durako and Howarth, 2017), photoprotection (Marín-Guirao et al., 2013b) and whole-shoot photochemistry (fluorescence imaging; Alfonso-Abellán et al., 2010). It is also worth noting some recent results derived from the use of novel ‘omics’ and molecular profiling techniques (i.e. metabolomics, transcriptomics and proteomics) that have revealed some critical mechanisms used by marine plants to withstand salinity changes (Davey et al., 2016; Malandrakis et al., 2017a). The present review provides an overview of these latest advances in knowledge on the mechanisms of salt tolerance in seagrasses, and offers an integrative perspective on acclimation and stress responses that are essential to understand the costs and benefits of seagrass phenotypic plasticity under hypersaline stress (Box 1).

## 2. Hypersalinity as a stress factor for seagrasses

The activation of rapid and reversible acclimation mechanisms allows marine plants to tolerate temporary fluctuations in environmental variables they naturally experience in their native environments,

including changes in salinity (Fig. 1). Typically, plants can maintain their optimum growth and physiological capacities under such conditions, or even improve them. This latter occurs when environmental changes act as ‘eustress’ forces that positively influence plant performance through the hardening of their metabolism and/or enhancing their stress tolerance (Lichtenthaler, 1996; Latzel et al., 2016; Nguyen et al., 2020; Jueterbock et al., 2021). Conversely, exposure to more intense or longer-lasting environmental changes can cause negative effects on the plants, which eventually result in a deviation from their optimum physiological status and development (Fig. 1). This kind of ‘real’ stress is known as ‘dis-stress’ and, consequently, promotes stress responses and acclimation to the new environmental conditions (Fig. 1). Under dis-stress, biological damage and resilience depend on internal (e.g. efficiency and integration of acclimation capabilities) and external (e.g. duration and/or intensity of the stress) factors, which ultimately determine the success of stressed plants to achieve a new optimum status (i.e. a phenotype that fits to the new conditions) and to recover after stress cessation (Lichtenthaler, 1996).

Regarding salinity, daily evaporation processes in the intertidal zone or the imbalance between water exchange and evaporation in (semi-) enclosed coastal systems are natural processes leading to saline fluctuations in seagrass ecosystems (Koch et al., 2007a; Tomasello et al., 2009; Sandoval-Gil et al., 2014b). Global climate change is intensifying these processes with evident consequences for coastal salinity levels (Durack et al., 2012). The current warming climate is driving changes in the global water cycle, while sea level rise is progressively increasing salinity intrusion in estuarine environments, producing increments in mean salinity levels and the peaks of salinity pulses. Thus, salinity changes boosted by ongoing climate change have the potential to affect the future survival and distribution of seagrasses (Short and Neckles, 1999). Human intervention modifying hydrological characteristics at the basin scale can also result in saline stress on seagrass meadows, as documented in the Greater Everglades system in Florida Bay (Koch et al., 2007b) and in Shark Bay, Western Australia (Walker et al., 1988). Nevertheless, the greatest source of saline stress in seagrasses is currently represented by dumping brines into coastal waters from reverse osmosis (RO) desalination plants (Sánchez-Lizaso et al., 2008; Ruiz et al., 2009b; Palomar and Losada, 2010; Sola et al., 2019). Desalination effluents form high-density hypersaline plumes that spread over large areas of the sea bottom, the extent of which depends on the brine characteristics (e.g. volume and salinity level), bottom bathymetry,

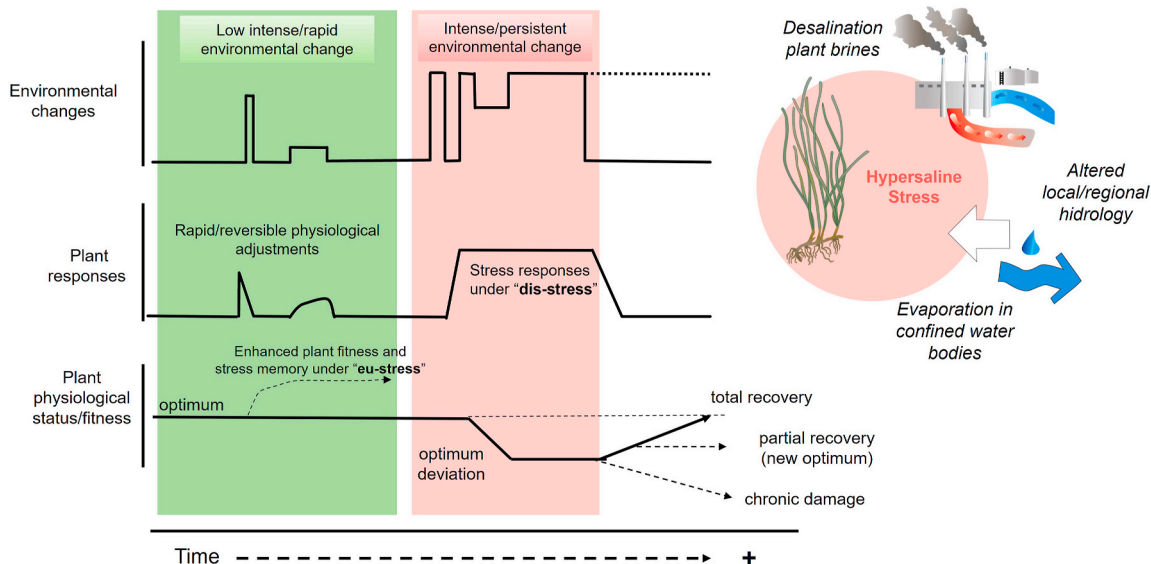


Fig. 1. A general model of the plant stress responses, based on stress concepts proposed by Lichtenthaler (1996). See the text for additional details. Some images have been modified from <http://ian.umces.edu/>.

hydrodynamism and the use of brine dilution devices such as venturi diffusers (Fernández-Torquemada et al., 2005a; Ruiz-Mateo, 2007; Lattemann and Höpner, 2008; Portillo et al., 2013). The overall impact of these brines on adjacent benthic vegetation (and associated biota) also depends on community properties such as species composition, their phenotypic plasticity and their salinity tolerance ranges (Morton et al., 1997; Del-Pilar-Ruso et al., 2007; Riera et al., 2012; Petersen et al., 2018).

Regarding seagrasses, different experimental approaches have been used to assess the effects of hypersaline waters, including: i) laboratory experiments under controlled conditions (microcosm and mesocosms; Pagès et al., 2010; Marín-Guirao et al., 2011; Oscar et al., 2018), ii) long-term monitoring of natural populations under the influence of desalination discharges (Gacia et al., 2007), iii) *in situ* experimental manipulation of salinity (Ruiz et al., 2009b), iv) exposure of seagrass transplants to desalination effluents (Garrote-Moreno et al., 2014) and v) studies of the biological attributes of seagrasses naturally growing under hypersaline conditions (Tomasello et al., 2009; Marín-Guirao et al., 2017). Preliminary studies conducted during the early years of the 21st century aimed at protecting the valuable *Posidonia oceanica*

meadows from the growing desalination industry in the Mediterranean region (Fernández-Torquemada and Sánchez-Lizaso, 2005; Gacia et al., 2007; Ruiz et al., 2009b). These pioneer studies have helped to establish provisional tolerance thresholds for this iconic species and have provided recommendations to help environmental managers to minimise the impact of brine discharges (Sánchez-Lizaso et al., 2008; Barrio et al., 2020). More recently, sophisticated mesocosm systems have been used to conduct fine-tuned and highly controlled experiments, which have allowed accurate exploration of salinity-induced physiological effects on seagrasses (Marín-Guirao et al., 2011; Cambridge et al., 2017; Oscar et al., 2018).

### 3. Hypersalinity and seagrass multilevel responses

The effects of increased salinity on seagrasses and how they respond depends largely on the nature of the (hyper)saline condition, including the level of salinity (Pagès et al., 2010; Marín-Guirao et al., 2011; Sandoval-Gil et al., 2012b), whether it increases slowly or rapidly (Koch et al., 2007a; Fernández-Torquemada and Sánchez-Lizaso, 2011), the duration of exposure (Murphy et al., 2003; Ruiz et al., 2009a;

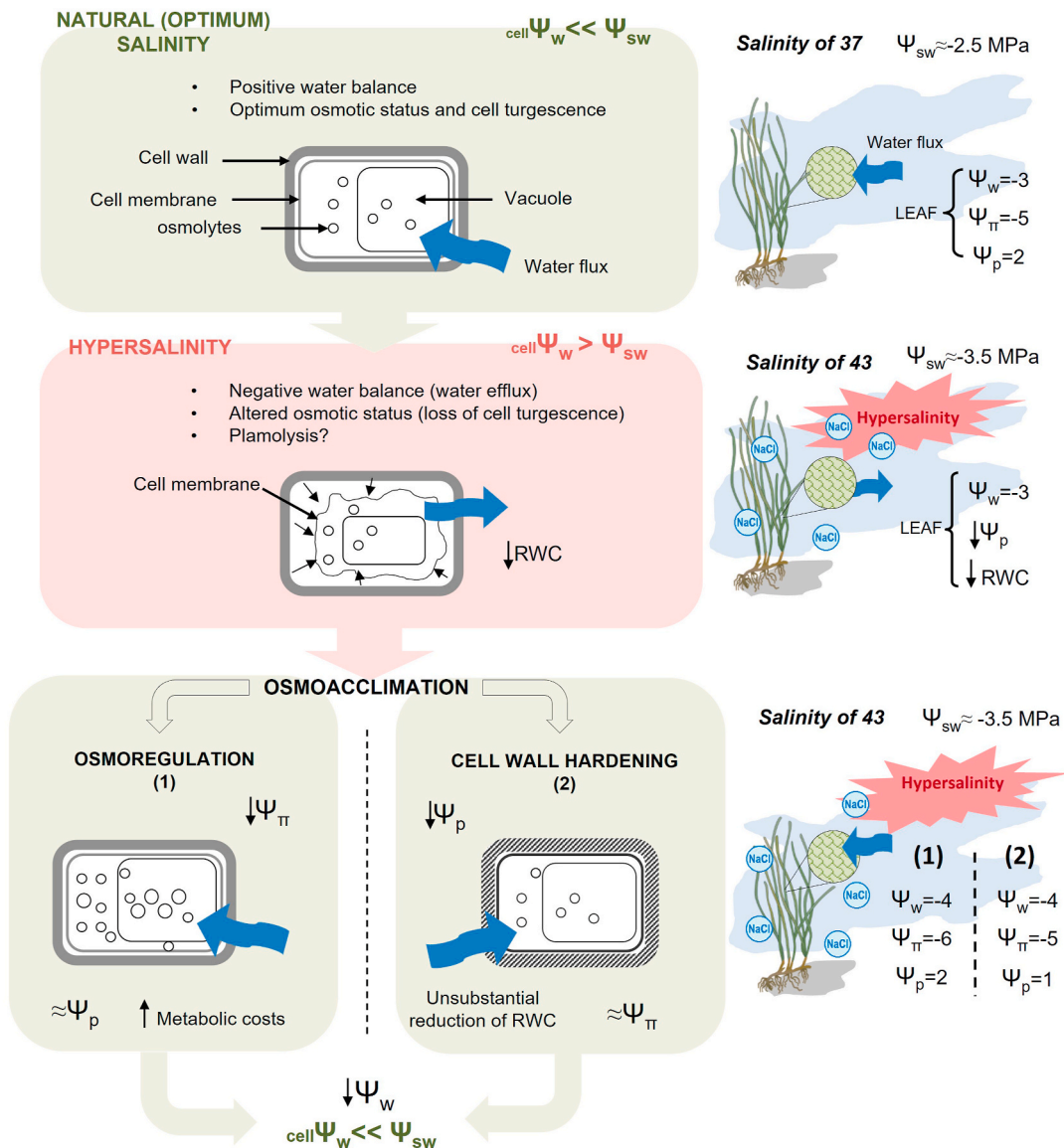


Fig. 2. Schematic representation of seagrass responses to hypersalinity integrated at three main levels of biological organisation – cell, shoot/ramet and meadow – including the interaction with some biotic and abiotic factors. Some images have been modified from <http://ian.umces.edu/> and <https://edu.glogster.com/>.



Marín-Guirao et al., 2013a) and its temporal pattern (i.e. press versus pulse) (Ruiz et al., 2009a; Garrote-Moreno et al., 2014; Marín-Guirao et al., 2017). Hypersalinity has the potential to affect seagrasses across their different levels of biological organisation, including the cellular, individual and population levels (Fig. 2). Increased salinity generates hyper-osmotic conditions with the potential to disrupt the water balance between plant tissues and the surrounding seawater, eventually leading to cell dehydration and dysfunction. Increased salt concentrations also create hyper-ionic conditions that can lead to ionic imbalances in plant tissues, and thus to alterations in cell enzyme pathways and metabolic toxicity (Tyerman, 1989; Bisson and Kirst, 1995). Both conditions are closely related to the terms 'limitation' and 'disruptive stress' that Davison and Pearson (1996) described in seaweeds, on the basis of the restricted water acquisition and the ion-induced physiological damage promoted by hypersalinity.

Stressful saline conditions also affect organisms associated with seagrasses, their biotic interactions and, thus, the entire biocenosis and ecosystem. Finally, the effects of hypersalinity on seagrasses may be altered when hypersaline stress interacts with other abiotic stressors that often occur in coastal waters (see section 4).

### 3.1. Molecular responses

The application of relatively novel high-throughput 'omics' technologies (e.g. genomics, proteomics, metabolomics, transcriptomics and ionomics) in combination with eco-physiological approaches have contributed to address knowledge gaps on seagrass responses, resilience and acclimation to abiotic stress factors, including salinity, light and temperature (Franssen et al., 2011; Davey et al., 2016; Malandrakis et al., 2017a; Marín-Guirao et al., 2017b; Davey et al., 2018; Kongrueang, 2018; Sablok et al., 2018; Booth et al., 2022). Although the seagrass molecular ecology field has emerged, knowledge of the molecular basis of seagrass tolerance to (hyper-)salinity is still very limited compared with other stress factors (e.g. heat, light and eutrophication).

By combining semiquantitative proteomics with physiological approaches, it has been observed that *Cymodocea nodosa* subjected to hypersalinity accumulated the cytochrome b559 alpha subunit, a protein involved in photosystem II (PSII) repair and biogenesis (Piro et al., 2015). This finding reflects an improvement in photosystem assembly and repair that may underlie the absence of photochemical alterations in the species subjected to increased salinity. That study also showed a marked downregulation of the carbon-fixing enzyme RuBisCo, suggesting that unaltered photosynthetic functioning and carbon fixation are maintained in these plants by an increase in the activity of the carbon-fixing enzyme phosphoenolpyruvate carboxylase (PEPcase; Beer et al., 1980). The proteomic analysis also revealed an overall decrease in mitochondrial and chloroplast adenosine triphosphate (ATP) synthases, pointing to salinity-induced alterations in oxidative and photo-oxidative phosphorylation processes, which could be the reason for the respiratory inhibition observed in some studies (e.g. Sandoval-Gil et al., 2012b). Hypersalinity-stressed *C. nodosa* increased the levels of key glycolytic enzymes likely to counteract the lack of these energy-carrying molecules (Piro et al., 2015).

Regarding ionic adjustment and osmoacclimation, Cozza and Pangaro (2009) applied *in situ* hybridisation techniques in *P. oceanica* and found strong overexpression of genes encoding the tonoplast aquaporin PoTIP; 1 in response to hypersalinity. The involvement of aquaporins in the response of seagrasses to hypersalinity has also been derived from immunofluorescence methods (Serra et al., 2013) and further supported using an RT-qPCR approach by overexpressing the aquaporin-related genes PIP1/PIP2 (Serra et al., 2012). These findings suggest that aquaporins play an important role in seagrass-water relations, as these proteins are membrane channels involved in the bidirectional transfer of water and small solutes across cell membranes. On the other hand, overexpression of the tonoplast-specific intrinsic protein pyrophosphate-energised inorganic pyrophosphatase [(H(+)-PPase) in

hypersalinity-stressed *C. nodosa* plants suggests compartmentalisation of sodium ions ( $\text{Na}^+$ ) in vacuoles to prevent ionic imbalance in seagrasses (Piro et al., 2015). In addition, the transcriptome of several seagrasses (Kong et al., 2014; Chen et al., 2021) and the genome of *Zostera marina* (Olsen et al., 2016) have shown that gene families encoding potassium ion ( $\text{K}^+$ ) channels and transporters,  $\text{Na}^+$ /hydrogen ion ( $\text{H}^+$ ) exchangers (NHX) and  $\text{Na}^+$ / $\text{H}^+$  pumps, all with osmoregulatory roles and involved in salt tolerance, are expanded in seagrasses compared with terrestrial plants. Taken together, these findings indicate that vacuoles may play a key role in seagrass cellular osmoacclimation by facilitating important functions such as  $\text{Na}^+$  and chloride ion ( $\text{Cl}^-$ ) sequestration and detoxification and the maintenance of intracellular water balance, as has been demonstrated in land plants and macroalgae (Munns, 2002; Muramatsu et al., 2002).

The cell wall composition of seagrasses also appears to contribute to their successful osmoacclimation as it includes many celluloses, pectins and alga-like polysaccharides with osmotic adjustment properties (Olsen et al., 2016). In fact, analysis of the *Z. marina* genome has shown that the species has regained its ability to produce sulphated polysaccharides, which are anionic electrolytes that maintain ionic balance in algal cells (Olsen et al., 2016).

Finally, the use of whole-genome gene expression profiling (e.g. RNA-seq) to study the response of seagrasses to hypersalinity has so far only been applied to the seagrasses *C. nodosa* and *Posidonia australis*. The transcriptomic profile of *C. nodosa* subjected to short-term hypersalinity showed the early and strong induction of the serine/threonine kinase RLK1, which is involved in plant salt-stress signal transduction and in the regulation of ion accumulation under salt stress (Malandrakis et al., 2017b). In a different study, transcriptomic analyses of salt-stressed *C. nodosa* plants also revealed the overexpression of genes related to cell elongation factors and protein kinases, likely associated with alterations in the cell ultrastructure (Tsioli et al., 2021). Interestingly, Booth et al. (2022) provide evidence of a significant differentiation in gene expression profiles among tissue types (leaf tissue, leaf meristem and root) of *P. australis* growing across salinity gradient in Shark Bay (Western Australia); for these authors, the low number of differentially expressed genes found between salinities was likely indicative of a general homeostatic response to living in higher salinity, instead of a true stress response.

### 3.2. Ultrastructural responses

Few studies have evaluated the ultrastructure of seagrass leaves in relation to salinity. Early studies on *Thalassia testudinum*, *Z. marina* and *Ruppia maritima* indicated that presence of invaginated plasmalemma-mitochondrial transport in epidermal cells and the absence of  $\text{Cl}^-$  ions in this cytoplasmic region. These observations were considered evidence of the role of epidermal cells in osmoregulation and of the existence of a secretion or exclusion mechanism for this ion (Jagels, 1973, 1983). Subsequently, Jagels and Barnabas (1989) demonstrated greater development of these plasmalemma invaginations in *R. maritima* adapted to high salinity levels, together with smaller central vacuoles and more chloroplasts and mitochondria. Some of these ultrastructural modifications were also observed in *Zostera capensis* in response to hypersalinity, although the authors of this study proposed that invaginated plasmalemma-mitochondria might be related to photosynthate transport (Iyer and Barnabas, 1993). Chloroplast swelling, mitochondrial structural damage and reduced vacuole volume are other ultrastructural symptoms associated with cellular water loss and salt stress in seagrasses (Iyer and Barnabas, 1993).

More recently, Al-Arbash et al. (2016) observed damaged grana and fewer plastoglobuli in chloroplasts of *Halodule uninervis* under hypersalinity, as well as less invagination of the plasmalemma towards the outer epidermal wall, which is in contrast to previous findings. Rhizome tissues also show severe damage signals, such as detachment of the epidermal layer, and the loss of structural integrity of the exodermis,

endodermis and cortical parenchyma cells.

Tsioli et al. (2021) have recently demonstrated that hypersalinity causes visible disorder in the structure of microtubule cytoskeleton and cell organelles in meristematic portions of juvenile leaves of *C. nodosa*. Tubulin immunofluorescence revealed a short, fragmented microtubule cytoskeleton, including thin bundles with random orientation. There were larger and more chloroplasts in salt-stressed plants presented in, but there were fewer thylakoids and undifferentiated grana. In addition, the leaf cells were highly vacuolated and had a high number of mitochondria with aberrant shapes (e.g. broken and dilated cristae), likely reflecting an increased demand for ATP or for mitochondrial stress proteins.

### 3.3. Plant–water relations

Plant–water relations are critical aspects of the biology of higher plants that have been widely studied in relation to drought and salinity (Lambers and Oliveira, 2019). Interestingly, there is comparatively little knowledge available on seagrasses, which is surprising considering the key role these processes play in their adaptation to the marine environment and in their acclimation to salinity changes (Ye and Zhao, 2003; Kuo and den Hartog, 2000). At the physiological level, changes in plant–water relations represent the first barrier to cope with hypersaline stress, as these changes may induce direct and indirect effects on other levels of plant metabolism (e.g. protein expression, photosynthesis, respiration and growth).

The very early studies on seagrass–water relations were conducted in the 1980s by S.D. Tyerman on Australian species (Tyerman, 1982, 1989; Tyerman et al., 1984). However, it is not until decades later when optimisation of thermocouple psychrometry methods allowed relatively

simple determination of plant tissue descriptors such as water potential ( $\Psi_w$ ), osmotic potential ( $\Psi_\pi$ ) and turgor pressure ( $\Psi_p$ ). The study of these descriptors has begun to unravel the different osmoacclimatisation strategies used by seagrasses to avoid dehydration in the face of increased salinity (Van Diggelen et al., 1987; Murphy et al., 2003; Koch et al., 2007b).

Submerged macrophytes are osmotically stable in natural saline conditions through inherent *osmoadaptive properties* (sensu Kirst, 1989). These properties are mainly based on the maintenance of lower (i.e. more negative)  $\Psi_w$  and  $\Psi_\pi$  in plant tissues than in seawater, which allows the maintenance of positive water balances, and thus relative cellular water content and turgescence (Fig. 3). Under increased salinity, the water balance between plant tissues and the surrounding seawater may be reduced and/or disrupted (Tyerman, 1989; Touchette, 2007). Therefore, seagrasses should *osmoacclimate* by reducing their leaf  $\Psi_w$  to avoid alterations of the water status and eventually dehydration (Sandoval-Gil et al., 2012a). The osmoacclimative responses in seagrasses, named *dehydration avoidance strategies* in land plants (Verslues et al., 2006), are based on two main mechanisms: i) intracellular solute accumulation and ii) cell wall hardening processes (Fig. 3).

Osmoregulation via accumulation of intracellular osmotically active solutes or osmolytes, such as ions and organic solutes, is a primary process in marine macrophytes to cope with increased salinity (Karsten, 2012; Hurd et al., 2014). Intracellular accumulation of osmolytes results in a reduction in the leaf  $\Psi_\pi$ , and thus of  $\Psi_w$ , which stabilises  $\Psi_p$  and provides the metabolic state required for optimal plant growth (Zimmerman, 2017; Hasegawa et al., 2000). As an immediate response, seagrasses adjust their leaf  $\Psi_w$  under increasingly saline conditions through a consistent increase in ions (Koch et al., 2007b; Sandoval-Gil et al., 2012a, 2014a,b; Khalafallah et al., 2013; Marín-Guirao et al.,

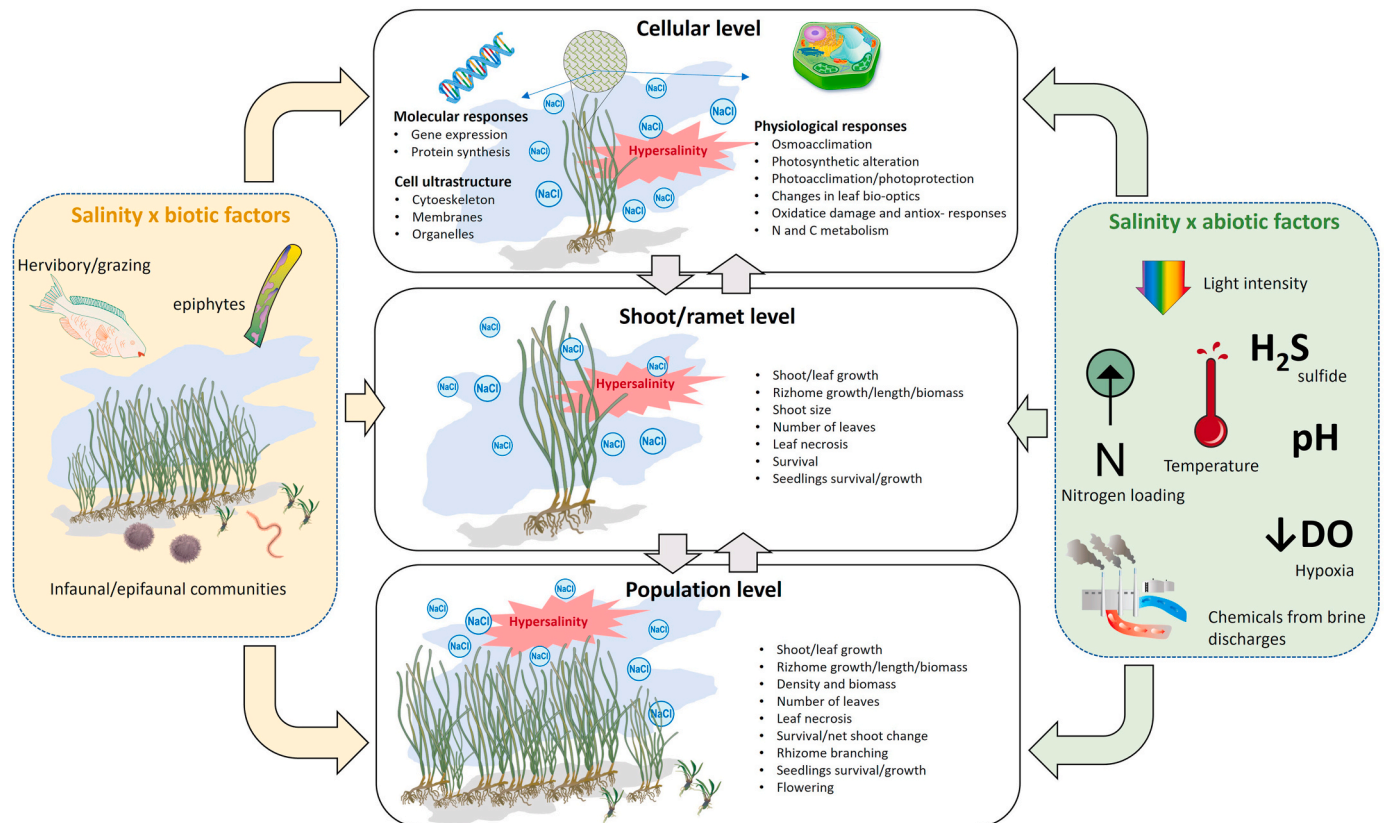


Fig. 3. Schematic representation of the different dehydration avoidance strategies – (1) osmoregulation and (2) cell wall hardening – of marine plants exposed to hypersaline conditions. The vertical panels on the left show these responses at the cellular level of leaf tissues. The vertical panels on the right provide a numerical example of osmoacclimation adjustments.  $\Psi_w$  = leaf water potential,  $\Psi_\pi$  = leaf osmotic potential,  $\Psi_p$  = leaf turgor pressure,  $\Psi_{sw}$  = water/osmotic potential of seawater, RWC = relative water content. The values are theoretical and in megapascals (MPa). The seagrass image was obtained from <http://ian.umces.edu/>.



**Fig. 4.** On the left: summary of the physiological responses of seagrasses to hypersalinity reported in the literature, showing the percentage of cases each variable increased (green), decreased (red), did not change (white) or even increased/decreased in the same study and species (grey). On the right: pie graphs describing the contribution of studies based on different physiological traits for each seagrass species (n = number of publications). See Table A1 in the Appendix for further details.

2013a; Garrote-Moreno et al., 2015a; Piro et al., 2015; Al-Arbash et al., 2016; Cambridge et al., 2017, 2019). While Na<sup>+</sup> and Cl<sup>-</sup> increased steadily in leaf tissues with increasing salinity, K<sup>+</sup> and calcium ions (Ca<sup>2+</sup>) showed inconsistent patterns (Marín-Guirao et al., 2013a; Garrote-Moreno et al., 2015a, 2016), most likely due to differential ion uptake and/or ion selectivity by competition phenomena (Cramer, 2002; Carpaneto et al., 2004; Kongrueang et al., 2018). This different ionic behaviour has the potential to cause ionic imbalances, usually expressed in lower K<sup>+</sup>/Na<sup>+</sup> and Ca<sup>2+</sup>/Na<sup>+</sup> ratios, and severe metabolic alterations because K<sup>+</sup> and Ca<sup>2+</sup> have critical metabolic functions other than as osmotic (e.g. cofactors for signalling cascades and ATPase activity; Bisson and Kirst, 1995). Moreover, the intracellular accumulation of other ions with toxic potential and negligible contribution to cellular osmolality (e.g. Fe, Mn, Zn or Mg) can also contribute to the occurrence of metabolic dysfunction in seagrasses (Tsioli et al., 2021).

Subsequently, ions must be excluded from the cell cytoplasm, compartmentalised in specific cell organelles or stored in other plant tissues to avoid excessive accumulation and interference with cell metabolism (Hasegawa et al., 2000). The capacity to efflux Na<sup>+</sup> via H<sup>+</sup>/Na<sup>+</sup> antiporters, and the low permeability of cell membranes for this ion compared with others (e.g. K<sup>+</sup>), is a recognised characteristic in seagrasses (Fernández et al., 1999; Carpaneto et al., 2004; Rubio et al., 2011). Ion exclusion and/or ion translocation mechanisms between plant tissues are other suggested mechanisms in marine plants (see subsection 3.1), such as *P. oceanica* and *C. nodosa* (Garrote-Moreno et al.,

2015b). As ions are removed from the cell cytoplasm, they must be gradually replaced by organic solutes to sustain lower  $\Psi_p$ . Soluble non-structural carbohydrates and free amino acids (mainly proline) act as major compatible organic solutes which progressively increase in seagrass tissues exposed to hypersaline conditions (Brock, 1981; Pulich, 1986; Tyerman, 1989; Adams and Bate, 1994; Murphy et al., 2003; Koch et al., 2007b; Marín-Guirao et al., 2013a; Sandoval-Gil et al., 2014a; Garrote-Moreno et al., 2015a). In contrast, the osmotic functionality of starch is not yet well understood in marine plants. Starch granules can regulate the intracellular osmotic volume in land plants (Ackerson and Hebert, 1981), while their utilisation/mobilisation in seagrasses can be activated to cope with energy demands for osmoregulation, with the potential to erode the energy status of plants and their survival in the long term (Ogata and Takada, 1968; Drew, 1978; Gacia et al., 2007; Ruiz et al., 2009b; Sandoval-Gil et al., 2014a).

The activation of *cell wall hardening processes* is an alternative to intracellular osmolyte accumulation to avoid tissue dehydration in seagrasses (Fig. 3). These processes are dependent on cell wall elasticity, which is usually quantified by the so-called *elastic modulus*,  $\epsilon$  (Kramer and Boyer, 1995; Verslues et al., 2006). Specifically, this osmoacclimation strategy is based primarily on the notion that more rigid and/or thicker cell walls (i.e. higher  $\epsilon$ ) cannot accommodate a reduction (even small) in cell volume. Consequently, a small shrinkage of the cell volume without substantial reductions in the relative water content can lead to a significant decrease in  $\Psi_p$ . This reduction in turgor pressure ultimately



results in a more negative  $\Psi_w$ , which is considered more energy conservative than osmolyte accumulation (Bisson and Kirst, 1995; Kramer and Boyer, 1995; Verslues et al., 2006). In fact, the activation of cell wall hardening mechanisms has been demonstrated in highly salt-tolerant terrestrial and wetland plants, as well as in seaweeds adapted to fluctuating saline environments (Bisson and Kirst 1995; Touchette et al., 2007; Lambers and Oliveira, 2019). In seagrasses, different  $\epsilon$  values have been correlated with the adaptation of species to different salinity regimes, including their vertical distribution within the intertidal belt (Tyerman, 1982; Touchette, 2007; Sandoval-Gil et al., 2014b). The activation of a cell wall hardening strategy under increased salinity has been observed in *C. nodosa*, a finding that is consistent with the euryhaline nature of this species (Sandoval-Gil et al., 2012a; Garrote-Moreno et al., 2015a).

Contrary to the expected decrease in turgor associated with dehydration, some evidence has shown that  $\Psi_p$  increases during osmoacclimation of seagrasses to increased salinity (Marín-Guirao et al., 2013a; Sandoval-Gil et al., 2014a; Piro et al., 2015). Although the physiological significance of this atypical response remains unclear, it could reflect the functioning of *turgor sensing mechanisms*, which are involved in the activation of mechanoreceptor-operated ion channels and downstream signalling cascades in seaweeds and land plants (Kirst, 1989; Bisson and Kirst, 1995; Ringli, 2010). These mechanisms may also operate in intertidal *Zostera noltii* plants, as a secondary response to tolerate long periods of emersion (Sandoval-Gil et al., 2014b).

### 3.4. Photobiological responses

The photosynthetic sensitivity to increased salinity varies inter- and intraspecifically in seagrasses depending on the salinity regime that plants have experienced in their native environments along their evolutionary history (Dunton, 1996; Shafer et al., 2011; Sandoval-Gil et al., 2012). Variables related to photosynthesis and carbon metabolism are among the most prevalent in the literature of seagrasses-hypersalinity interactions (Fig. 4). Reduced photosynthetic performance is a common effect of hypersaline stress in seagrasses and is usually the result of ionic toxicity, alterations in the structure and functioning of the photosynthetic apparatus (e.g. PSI and PSII proteins and RuBisCo) and/or pigment degradation (Buapet, 2017).

A widespread effect of hypersaline stress on seagrasses is alteration of the ability to convert light energy into chemical energy (i.e. photochemical efficiency). Measurements of chlorophyll *a* fluorescence using pulse amplitude modulated (PAM) fluorometers have revealed reduced values of maximum ( $F_v/F_m$ ) and effective ( $\Phi_{PSII}$ ) quantum yields and electron transport rates (ETR) in a number of seagrasses (Kamermans et al., 1999; Kahn and Durako, 2006; Koch et al., 2007b; Pagès et al., 2010; Howarth and Durako, 2013a; Marín-Guirao et al., 2013b; Garrote-Moreno et al., 2015a; Cambridge et al., 2017, 2019; Kongrueang et al., 2018; Tsioli et al., 2021, 2019). These photochemical alterations are likely the consequence of hypersalinity-induced ultrastructural changes in chloroplasts and thylakoids (see subsection 3.2), or the uncoupling of electron flow between photosystems or between them and final electron acceptors, such as  $NADP^+$ . Altered photochemistry often results in a decrease in maximum photosynthetic rates ( $P_{max}$ ) and photosynthetic efficiency ( $\alpha$ ) in salt-stressed seagrasses, as determined by oxygen evolution measurements (Ogata and Matsui, 1964; Kerr and Strother, 1985; Fernández-Torquemada et al., 2005b; Koch et al., 2007b; Marín Guirao et al., 2011; Shafer et al., 2011; Sandoval-Gil et al., 2012; Sandoval-Gil et al., 2014a; Johnson et al., 2020). By using  $O_2$ -micro-sensors, Johnson et al. (2020) and Koch et al. (2022) found that hypersalinity alter internal seagrass  $pO_2$  and  $O_2$  flux to the water column, leading to a drop in oxygen pressurisation in the aerenchyma of leaves and meristems, and facilitating hydrogen sulphide ( $H_2S$ ) intrusion and its phytotoxicity. In response to reduced photosynthetic performance, salt-stressed plants can increase their photosynthetic efficiency at sub-saturating irradiances ( $\alpha$ ) and reduce their light compensation ( $E_c$ )

and light saturating ( $E_k$ ) irradiances to compensate for reduced carbon fixation (Kahn and Durako, 2006; Marín Guirao et al., 2011; Shafer et al., 2011; Sandoval-Gil et al., 2014a; Cambridge et al., 2019; Koch et al., 2022). These adjustments are critical for stabilising the plant carbon balance as they maximise the daily period of irradiance-saturated photosynthesis ( $H_k$ ) and the daily period in which photosynthetic productivity compensates carbon loss by respiration ( $H_c$ ) (Dennison and Alberte, 1985).

Photosynthetic reductions in terms of oxygen evolution can also occur without signs of hypersaline stress-induced photochemical disruptions, which can be explained by the activation of alternative electron sinks that enhance electron flow in stressed plants (Marín-Guirao et al., 2011, 2013a; Sandoval-Gil et al., 2014a; Piro et al., 2015). The overreduction of electron transport carriers, together with the enhanced oxygen consumption estimated by some of these alternative pathways (e.g. photorespiration and the Mehler reaction) result in the overproduction of reactive oxygen species (ROS) with the potential to cause oxidative stress (Niyogi, 2000; Alric and Johnson, 2017; Dumanović et al., 2020). Therefore, safe dissipation of excess photons and the activation of antioxidant responses in salt-stressed plants are required to protect the photosynthetic apparatus and other cell components from (photo-)oxidative damage (Bischof and Rautenberger, 2012; Hanelt and Figueroa, 2012; Bhagooli et al., 2021). Signs of oxidative stress, such as lipid peroxidation and protein damage, have been detected in *P. oceanica* plants under the influence of brines from a desalination station, which in turn activate the antioxidant defence machinery by increasing the activity of antioxidant enzymes (e.g. glutathione peroxidase) and the concentration of antioxidants such as polyphenols and glutathione (Capó et al., 2020). The photoprotective mechanism operated by the xanthophyll cycle offers additional protection against photo-oxidative damage through non-radiative thermal dissipation of absorbed excitation energy within pigment-protein complexes of thylakoid membranes (Govindjee, 2014). The operation of this mechanism, evidenced as increments in non-photochemical quenching (NPQ) by chlorophyll *a* fluorescence measurement, has been demonstrated in several seagrasses subjected to hypersalinity (Trevathan et al., 2011; Marín-Guirao et al., 2013a; 2013b; Howarth and Durako, 2013b; Garrote-Moreno et al., 2015a).

PAM fluorometric imaging for two- and three-dimensional analyses of photosynthesis at the whole-plant level is a promising tool, but its use is still very limited in marine plants compared with terrestrial plants (Gao et al., 2011; Jarvis and Bielmyer-Fraser, 2015). Fluorometric imaging has been used to map the spatial variability of photosynthesis along the leaves of just a few seagrass species (Ralph et al., 2005); regarding saline stress, this technique has been applied only in *P. oceanica* (Alfonso-Avellán et al., 2010). This study revealed that the effects of saline stress vary with tissue age, showing that the stress-response of the whole-shoot is more complex and variable than previously thought, as recently demonstrated in this species under heat stress (Ruocco et al., 2019b).

### 3.5. Pigments and leaf bio-optical responses

The concentration of photosynthetic pigments (chlorophyll *a*, chlorophyll *b* or carotenoids) and their distribution within cells or tissues (e.g. packaging level) determines, together with other factors such as the presence of scattering structures within leaf tissues, the optical properties of seagrass leaves and thus their capacity to harvest solar energy (Enríquez, 2005; Vásquez-Elizondo et al., 2017). This capacity is generally expressed in terms of absorbance (*A*) and pigment-specific absorption coefficients ( $a^*$ ), which denote the proportion of incident photosynthetically active radiation (PAR) that can be potentially used in the photosynthetic process (Vásquez-Elizondo et al., 2017). Hypersalinity can lead to pigment degradation in seagrasses with the potential to impact their photosynthetic performance (McMillan and Moseley, 1967; Koch and Dawes 1991; Ralph 1998, 1999; Trevathan et al., 2011;



Howarth and Durako, 2013b; Khalafallah et al., 2013; Kongrueang et al., 2018; Tsioli et al., 2021; 2019). Some studies have also reported increased leaf pigmentation and leaf absorbance as a compensatory mechanism to balance the plant's energy status in situations of altered photosynthesis and/or accelerated respiratory metabolism (Marín-Guirao et al., 2011, 2013a, 2013b; Sandoval-Gil et al., 2012b, 2014a; Oscar et al., 2018; Spinelli, 2018). Other pigment responses to salt stress include increased carotenoid content and increased xanthophyll:chlorophyll ratios, indicating the activation of photoprotective (enhanced NPQ) and antioxidant mechanisms (Marín-Guirao et al., 2013b; Sandoval-Gil et al., 2014a).

Changes in morphological traits such as the leaf mass per unit area (LMA) also influence leaf optical properties in seagrasses (Cayabyab and Enríquez, 2007). Under increased salinity, LMA slightly decreases in *C. nodosa*, with reduced pigment packaging and a more efficient distribution of pigments in photosynthetic structures (Sandoval-Gil et al., 2012b, 2014a). This response actually increases  $A$  and  $\alpha$ , as well as the mass-specific absorption coefficient ( $a^*_w$ ) and the chlorophyll-specific absorption coefficient ( $a^*$ ), clearly reflecting the increased efficiency of these plants in capturing PAR. In contrast, LMA increases in *P. oceanica* under similar hypersaline conditions, pointing to increased leaf biomass packaging that ultimately leads to a proportional reduction in light absorption efficiency per unit of leaf biomass. This means that there is a reduction in the energy return per unit of tissue produced, and hence the carbon turnover of those plants (Marín-Guirao et al., 2013a; Sandoval-Gil et al., 2014a).

Leaf spectral reflectance [ $R(\lambda)$ ] is an apparent bio-optical property used as a non-destructive method to detect plant stress, and to map and monitor seagrass meadows by remote sensing techniques (Thorhaug et al., 2007). Leaf  $R(\lambda)$  responds to changes in leaf pigments and regulates plant energy transduction (Sims and Gamon, 2002). Pigment reductions together with increased spectral reflectance are typical plant responses to stress and have been described in hypersalinity-stressed seedlings of the seagrass *T. testudinum* (Durako and Howarth, 2017). Increased  $R(\lambda)$  is likely due to an increase in the leaf anthocyanin content promoted by stressful salinity levels; this phenomenon is consistent with a significant reduction in the photochemical efficiency of salt-stressed seedlings.

### 3.6. Respiratory responses

Under hypersaline stress conditions, the respiratory activity ( $R$ ) of seagrasses tends to increase, although the pattern of the response can vary inter- and intraspecifically, making this response inconsistent. The stimulated respiratory metabolism observed in seagrasses probably indicates an increased energy demand to fuel the activation of repair, protective and acclimative mechanisms to withstand hypersalinity (Berns, 2003; Shafer et al., 2011; Marín-Guirao et al., 2011, 2017; Sandoval-Gil et al., 2014a; Spinelli, 2018). In fact, an increased number of mitochondria has been reported in seagrass plants subjected to increased salinity (see subsection 3.2), a finding that supports the need for accelerated metabolism to cope with the hypersalinity-associated energy. This implies accelerated consumption of carbon reserves that could impair the energetic status of plants in the mid/long term, deteriorating their health and eventually leading to plant death (Alcoverro et al., 2001; Touchette, 2007). Starvation of carbon substrates, or their utilisation/mobilisation to cover osmoregulatory and osmoprotective functions, may in turn lead to inhibition of respiration, ultimately counteracting dysfunctional carbon metabolism in salt-stressed plants (Biebl and McRoy, 1971; Ogata and Takada, 1968; Fernández-Torquemada et al., 2005b; Kahn and Durako, 2006; Trevathan et al., 2011; Johnson et al., 2020). Both stimulation and inhibition of  $R$  have been observed in *C. nodosa* as a function of hypersalinity (Sandoval-Gil et al., 2012b), although the pattern of respiratory response may vary due to the interaction of salinity with other abiotic factors, as recently observed in *Halophila ovalis* under salt and temperature stress (Ontoria et al.,

2020).

### 3.7. Vegetative responses: from shoot to meadow

Vegetative deterioration of seagrasses subjected to hypersalinity has been documented in terms of an increased incidence of leaf necrosis, reduced plant growth and size (leaf length/number) and shoot mortality, among others. These alterations are probably the result of direct saline effects on the plant physiology (e.g. increased intracellular toxic salts), as well as of the metabolic costs associated with physiological adjustments and cellular damage repair (Koch et al., 2007b; Fernández-Torquemada and Sánchez-Lizaso 2011; Khalafallah et al., 2013; Oscar et al., 2018; Tsioli et al., 2019).

Leaf necrosis is symptomatic of hypersaline stress, and the appearance of necrotic marks on the central and basal parts of the leaves is usually the result of ionic toxicity caused by salt accumulation (Fernández-Torquemada and Sánchez-Lizaso 2005; Gacia et al., 2007; Ruiz et al., 2009b). Alternatively, increased necrosis in the upper part of the leaves could be due to stimulated leaf senescence promoted by accelerated metabolism or reabsorption processes.

Clonal integration allows reabsorption and translocation of resources between connected ramets to manage the metabolic costs of the whole clone, thus improving stress tolerance (Dodd and Douhovnikoff, 2016; Liu et al., 2016). In seagrasses, clonal integration has been demonstrated to buffer physiological performance against different environmental stressors (Olivé et al., 2009; Tuya et al., 2013), and even under extreme adverse conditions it could allow the activation of a 'sacrificing response' to allow the survival of some ramets at the expense of others (Ruocco et al., 2020). A reduction in the shoot density may also reflect a structural adjustment at the population level to optimise whole-plant carbon balance (Enríquez et al., 2019). Despite the above phenomenon, it is undeniable that when hypersalinity exceeds the tolerance thresholds of species and populations, it can lead to plant mortality with consequences on the meadow structure (i.e. shoot density and/or meadow cover; Vermaat et al., 2000; Fernández-Torquemada and Sánchez-Lizaso, 2006; Ruiz et al., 2009a; Garrote-Moreno et al., 2014; Cambridge et al., 2017).

Interestingly, positive effects of hypersalinity on vegetative reproduction have been reported in some cases, such as the stimulation of shoot production in *H. ovalis* (Ontoria et al., 2020). Likewise, Garrote-Moreno et al. (2014) observed the proliferation of branching and the production of new ramets in *C. nodosa* in response to increased salinity, as well as an increase in the proportion of horizontal (plagiotropic) shoots, which probably reflects an 'escape mechanism' to avoid unfavourable hypersalinity.

Hypersaline stress can also affect sexual reproduction of seagrasses, impairing the success of different stages of the process, including seed germination and seedling development (Fernández-Torquemada and Sánchez-Lizaso 2013; Xu et al., 2016). Although to our knowledge there is no available information about the effects of hypersalinity on seagrass flowering induction, experimental exposure to reduced salinity significantly increased the reproductive effort of a multi-specific meadow (Rasheed, 2004), while it severely reduced flowering in the Indo-Pacific seagrass *H. ovalis* (Collier et al., 2014). Flowering is still a largely unknown process in seagrasses, and the bloom event observed by Rasheed (2004) has been associated with a stress-induced response (Cabaco and Santos, 2012). In their review, the authors found that seagrasses markedly increase their reproductive effort when subjected to disturbances, regardless of the origin and type of disturbance. This view has been supported by the recent transcriptomic profile of *P. oceanica* plants that massively flowered under manipulated heat-stress conditions (Marín-Guirao et al., 2019).

Seed germination and survival rates decrease progressively with increasing salinity in the seagrass species *P. oceanica*, *Z. marina* and *Zostera japonica* (Fernández-Torquemada and Sánchez-Lizaso, 2013; Xu et al., 2016; Yue et al., 2019). Hypersalinity also affects seedling

establishment and growth because it impacts the development of their leaves and roots (Xu et al., 2016; Cambridge et al., 2019). When compared with adult plants, the resistance of seagrass seedlings to hypersalinity does not show a clear pattern. On one hand, the higher sensitivity of *P. oceanica* and *Amphibolis antarctica* seedlings to hypersalinity compared with adult plants has been associated with the lack of clonal integrity and lower resistance to osmotic stress in the early life stages of the plants (Walker and McComb, 1990; Serra et al., 2012; Fernández-Torquemada and Sánchez-Lizaso, 2013). On the other hand, the higher resistance of *T. testudinum* and *P. australis* seedlings to increased salinity with respect to adults has been associated with the mobilisation of resources stored in seeds, which makes the young seedlings relatively independent from external conditions (Kahn and Durako, 2006; Cambridge et al., 2019). Although not generalisable, these effects on seagrass seedlings could seriously compromise recruitment success and survival in seagrass meadows under the influence of hypersaline waters, and thus their potential recovery as long as saline conditions remain adverse (Tomasello et al., 2009; Fernández-Torquemada and Sánchez-Lizaso, 2011).

At the shoot size level, salinity stress could result in undersized plants by decreasing growth rates, increasing defoliation and altering leaf morphology (Fernández-Torquemada and Sánchez-Lizaso, 2005, 2011; Gacia et al., 2007; Ruiz et al., 2009b; Tomasello et al., 2009; Garrote-Moreno et al., 2014; Tsioli et al., 2021; Capó et al., 2020). A hypersalinity-induced reduction in root growth, as observed in *P. australis* (Cambridge et al., 2017), could be behind this plant size reduction because it may limit plant nutrient acquisition. Interestingly, it has been argued that plant size reduction could alternatively reflect a carbon conservation strategy to persist under prolonged hypersalinity, as documented for some *P. oceanica* meadows influenced for months/years by hypersaline waters (Gacia et al., 2007; Ruiz et al., 2009; Marín-Guirao et al., 2017) or growing in natural hypersaline systems (Tomasello et al., 2009; Spinelli, 2018). In fact, and based on studies on land plants (Lichtenthaler, 1996), these undersized vegetative structures could be seen as a stress-coping mechanism, as it implies a reduced demand for metabolic resources needed to maintain plant growth. Whatever the case, the reduction in plant size and density has an impact on the functioning and physical structure of seagrass meadows and, thus, on the ecosystem services they provide. However, we are not aware of any studies that have explored how hypersalinity affects the range of goods and ecological services that these valuable ecosystems provide to humanity.

#### 4. Interaction of hypersalinity with other biotic and abiotic factors

The effects of hypersalinity on seagrasses may be modulated by interactions with other biotic and abiotic factors (Fig. 2). Desalination of seawater by RO involves several processes and the use of diverse chemicals (such as anti-fouling agents, acidifiers and disinfectants) that alter the physicochemical characteristics of the brine effluent. The discharge of these brines modifies the conditions of the receiving coastal waters, including changes in temperature and pH (Lattemann and Höpner, 2008; Portillo et al., 2014; Falkenberg et al., 2015). Changes in seawater pH can determine the speciation of dissolved inorganic carbon and disrupt electrochemical gradients in cell membranes, thus altering important biological processes such as photosynthesis and nutrient acquisition (Beer et al., 2014). However, little is currently known about the interaction between hypersalinity and low pH in seagrasses. Experiments under controlled laboratory conditions have shown that this interaction does not cause a cumulative reduction in leaf growth of *P. oceanica* (Fernández-Torquemada and Sánchez-Lizaso, 2003), while it decreases photosynthetic productivity in *Halophila johnsonii* (Fernández-Torquemada et al., 2005b).

In terms of the interaction between hypersalinity and increasing temperatures, neutral or positive effects on the photosynthetic

performance of *H. johnsonii* and growth of *C. nodosa* have been reported (Fernández-Torquemada et al., 2005b; Fernández-Torquemada and Sánchez-Lizaso, 2011). However, this interaction along with an increase in sediment pore water sulphide promoted by hydrological modifications seems to have been the most likely cause of critical *T. testudinum* die-off events in a semi-enclosed estuary in Florida Bay (Koch et al., 2007a, 2007b). In addition, warming can cause additive physiological stress for seagrasses under hypersaline stress, as demonstrated at the photophysiological level in *H. ovalis* from shallow sheltered and intertidal waters (Ralph, 1999), although recent findings seem to contradict these results (Ontoria et al., 2020). Moreover, Tsioli et al. (2021) have recently found both antagonistic and synergistic effects of these two factors on the quantum yield ( $\Phi_{PSII}$ ) and chlorophyll *a* content of *C. nodosa* depending on the exposure time, which corroborates that the complexity of this interaction is still poorly understood. Given that the effects of hypersalinity and warming in seagrass meadows are expected to overlap more frequently as a consequence of climate change (Nguyen et al., 2021), we need to increase our knowledge on the effects of this interaction to protect seagrass meadows in the coming decades.

Due to the presence of chemicals in desalination effluents, the occurrence of stress symptoms in seagrasses exposed to desalination brines is accelerated and intensified compared with purely hypersaline treatments (Cambridge et al., 2019). Portillo et al. (2014) evaluated the effects of hypersalinity and sodium metabisulphite, a chemical reagent used to clean RO membranes, on the vitality of *C. nodosa*. The study showed that this chemical causes acidification and hypoxia of the seawater, enhancing the harmful effects of hypersalinity on this euryhaline seagrass species. On the other hand, the use of groundwater contaminated with agricultural runoff in desalination processes may result in nitrogen-enriched hypersaline effluents that can amplify the deleterious effects of hypersalinity, as reported in *P. oceanica* meadows (Gacia et al., 2007). Nitrate uptake may be facilitated under hypersalinity by a sodium-dependent nitrate transport at the plasma membrane of leaf cells (García-Sánchez et al., 2000; Rubio et al., 2005; 2007, 2018), but can also reduce the activity of the enzyme glutamine synthetase and promote the toxic accumulation of intracellular ammonia (Pulich 1986; Gacia et al., 2007).

Light and temperature gradients associated with depth can also modify the responses and sensitivity of seagrasses to hypersalinity. Shallow and deep stands of *P. oceanica* have shown different acclimation and adaptation strategies to daily light fluctuations (Procaccini et al., 2017) and contrasting physiological tolerance to changes in light and temperature (Marín-Guirao et al., 2016; Dattolo et al., 2017). Accordingly, meadows of this species from contrasting depths have shown different sensitivity to hypersalinity, with deep (~20 m) plants experiencing greater carbon balance reduction than shallow (5 m) plants (Sandoval-Gil et al., 2014a). Similarly, different resistance to hypersalinity has been observed among species and ecotypes inhabiting different saline environments, such as coastal lagoons and estuarine systems (Koch et al., 2007a; Fernández-Torquemada and Sánchez-Lizaso, 2011; Shafer et al., 2011; Salo and Pedersen, 2014; Spinelli, 2018; Suykerbuyk et al., 2018). Fernández-Torquemada and Sánchez-Lizaso (2011) provided an enlightening example. They demonstrated that hypersalinity (up to 50) affected *C. nodosa* plants from coastal open waters (salinity ~37) in the western Mediterranean more negatively than those from an adjacent coastal lagoon with higher salinity levels (~42–47).

While salinity changes may affect seagrasses at multiple levels of biological organisation, they may also modify biotic interactions between the plant/meadow and associated communities, as well as among organisms within those biocenoses. The impact of desalination brines on infaunal and epifaunal seagrass-associated communities involves changes in their structure and composition, with the potential to amplify the consequences for the whole ecosystem (Del Pilar Ruso et al., 2007; Fernández-Torquemada et al., 2013; De-la-Ossa-Carretero et al., 2016). Brine-induced alterations in the composition of benthic microbial

communities and seagrass root microbiomes can alter plant nutrient acquisition and its nutritional status, but also important seagrass-associated functions such as nutrient cycling (Seymour et al., 2018; Frank et al., 2019). On the other hand, nitrogen enrichment associated with brine discharges can trigger the development of a high epiphyte load and stimulate herbivory activity (Gacia et al., 2007), which can lead to significant changes in seagrass biomass and meadow structure, as observed along a natural salinity-stress gradient (Bell et al., 2019). The strong correlation between the wasting disease pathogen *Labyrinthula zosterae* and salinity on *Z. marina* also suggests the likely influence of changing salinities on the presence/abundance of pathogens, and/or the health status of the plants to cope with infections (Jakobsson-Thor et al., 2018).

## 5. Phenotypic plasticity of seagrasses under hypersalinity

Phenotypic plasticity is an individual property that allows species' phenotypes to accommodate and react to environmental changes and stressful conditions (Pazzaglia et al., 2021a,b). It represents the change in a specific phenotypic trait, or a set of traits, as a response of the organisms to acclimate (and eventually to adapt) to the new conditions (Pfennig, 2021; Schlichting, 2021). Therefore, phenotypic plasticity is the ability of a single genotype to produce different phenotypes when exposed to different environments (Hollaway, 2002). Because environmental changes alter complex networks of diverse traits at multiple levels of plant biological organisation, it is challenging to integrate and interpret the end result of such changes in the final acclimatisation of individuals, and their potential for species evolution (Nicotra et al., 2010; Merilä and Hendry, 2014). The clonal nature of seagrasses makes it even more complex to quantify, integrate and interpret their plastic responses and how these contribute to the resulting phenotype (Auld et al., 2010). Seagrasses act as macro-organisms in which responses to environmental changes are highly interrelated due to clonal integration among connected ramets, which allows a top-down cascade of responses, from changes in meadow structure to metabolic adjustments in plants (Ruocco et al., 2020). For example, increased branching of rhizomes and particular adjustments at the level of meadow structure (e.g. plant density and the aboveground-to-belowground biomass ratio) have been described as strategies to optimise whole-plant carbon balance under hypersaline or low-light stress conditions (Tomasello et al., 2009; Garrote-Moreno et al., 2014; Marín-Guirao et al., 2017; Enríquez et al., 2019).

Sandoval-Gil et al. (2014a) analysed and compared the plastic responses of two seagrass species with contrasting biological and ecological attributes, namely *P. oceanica* and *C. nodosa*, to increased salinity by using the relative distance plasticity index (RDPI) developed by Valladares et al. (2006). Both species showed similar plasticity (i.e. similar RDPI values), which was unexpected because the eurybiontic nature of *C. nodosa* presupposes greater plasticity compared with the stenohaline *P. oceanica* (see 'generalist' versus 'specialist' species in Dudley, 2004). However, *C. nodosa* showed a higher resistance to hypersaline conditions, which was partially explained by the activation of physiological strategies combining adjustments in water relations and plant photobiology that were different than those of *P. oceanica*. These results highlighted that, rather than the degree of plasticity, the integration of particular plant traits and their functional consequences, as well as the costs and benefits for plant homeostasis, are responsible for the species-specific resistance to hypersalinity (Fig. 5), as has been described for terrestrial plants (DeWitt and Scheiner, 2004; Dudley, 2004; Ghalambor et al., 2007; Pugnaire and Valladares, 2007).

Phenotypic plasticity can be adaptive, neutral or non-adaptive (maladaptive) depending on whether it enhances the plants' resistance to the new environment, or whether it has deleterious effects on their fitness (Dudley, 2004; Ghalambor et al., 2007). Seagrasses have shown simultaneous adaptive and maladaptive plasticity under hypersalinity, both of which are tightly integrated. Hypersalinity may comprise

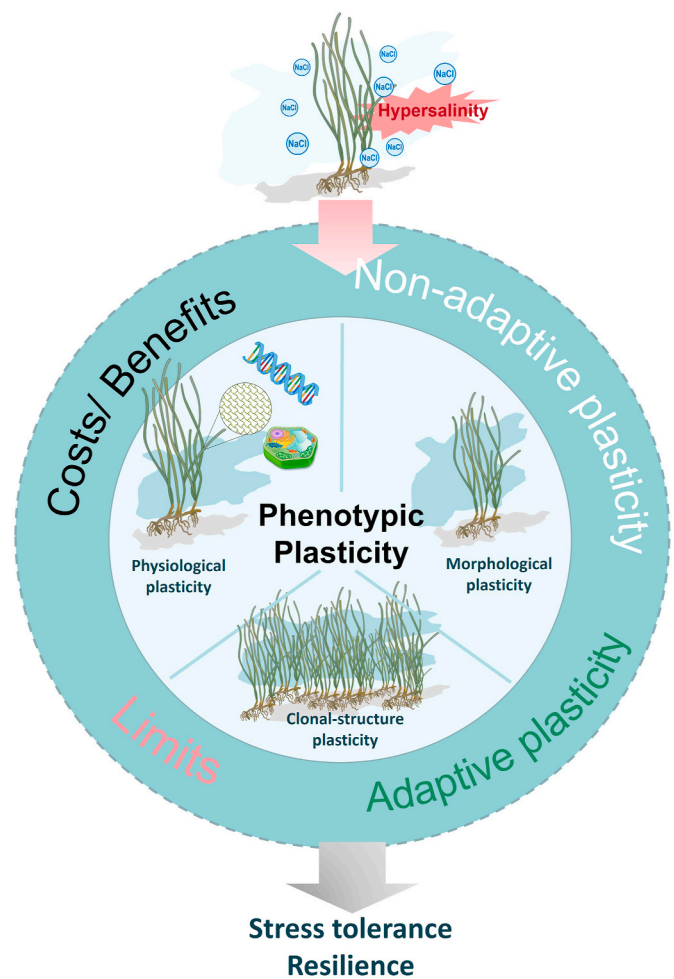


Fig. 5. A schematic representation of the phenotypic plasticity of seagrasses under hypersalinity and related key conditioning factors (see section 6).

'active' responses involving activation of regulatory pathways, as well as 'passive' phenotypic alterations as a consequence of biological susceptibility to hypersaline stress. The active accumulation of intracellular ions to reduce leaf  $\psi_w$  and the potential ionic toxicity over metabolic processes are clear examples of both kind of responses during seagrass osmoacclimation (Marín-Guirao et al., 2013a; Garrote-Moreno et al., 2016; Cambridge et al., 2017; Kongrueang et al., 2018). In general, it can be extremely difficult to determine whether a plastic response is adaptive or maladaptive, and in some cases plasticity for a particular trait can be interpreted in both directions. For example, increased respiration may provide photoprotection and metabolic energy under hypersaline stress, but it may also lead to long-term plant carbon imbalances (Marín-Guirao et al., 2017; Spinelli, 2018). In this sense, it can be deduced that the 'speed of induction of phenotypic plasticity' and its consequences at different time scales, and across levels of biological organisation, are additional factors conditioning seagrass tolerance to hypersaline stress.

In addition to the integration of the plastic response of different plant traits, the associated metabolic costs and limits also determine the tolerance of seagrasses to hypersalinity (DeWitt et al., 1998; Auld et al., 2010). Some seagrass species are more efficient than others in dealing with the 'costs' associated with salinity-induced plastic responses. This stems from the fact that, despite showing similar physiological responses (e.g. reduced  $\psi_w$ , accumulation of osmolytes, altered photosynthesis and respiration), the deleterious effects on plant growth and survival vary strongly among species (Fernández-Torquemada and Sánchez-Lizaso, 2005, 2006; Ruiz et al., 2009b; Marín-Guirao et al.,



2011; Sandoval-Gil et al., 2012b, 2014a).

Finally, and paraphrasing DeWitt et al. (1998) and Auld et al. (2010), the ‘limits’ of plasticity are defined as evident when facultative development cannot produce a trait mean as near the optimum as fixed development can; hence, they are detected when a plastic genotype cannot express the same phenotype as a non-plastic genotype. A limitation in the plastic response of seagrasses to hypersalinity may be imposed or accentuated by the influence of other abiotic/biotic factors (see section 4), which act as ‘ecological limits’ according to the definition of DeWitt et al. (1998). On the other hand, when the potential of a response to changes in the environment depends on previous responses and exposure to similar changes, especially at early ontogenetic stages, this constraint falls into the category of ‘plasticity-history limits’ (DeWitt et al., 1998). Interestingly, this limitation to plasticity might be related to the ‘stress memory’ recently observed through intragenerational and transgenerational phenotypic plasticity in heat-stressed seagrasses (DuBois et al., 2020; Nguyen et al., 2020; Jueterbock et al., 2020). However, there is a lack of empirical evidence of a hypersaline stress memory in seagrasses.

## 6. Future research directions

Despite the progress made in recent decades, additional research on seagrass stress and acclimation responses to hypersalinity is needed to unravel the complexity of salinity–seagrass interactions. With the advent of new ‘omics’ techniques (e.g. genomics, transcriptomics, proteomics, metabolomics, phenomics and ionomics), data integration in life sciences and systems biology has undergone a revolution (Hu et al., 2020; Yang et al., 2021). In crop plants, the application of ‘multi-omics’ approaches to generate high-throughput data has allowed researchers to elucidate for the first time the complexity of metabolic networks involved in plant responses to a variety of biotic and abiotic stressors (Weckwerth et al., 2020; Yang et al., 2021). The application of these new techniques to seagrasses is still in its nascent stage, but it has great potential to identify the processes underlying the resistance and resilience of seagrasses to high salinity (Kuzhiumparambil et al., 2017; Malandrakis et al., 2017a).

Next-generation sequencing (NGS) techniques have recently enabled the first genomic studies in seagrasses. The complete genomes of *Z. marina* (Olsen et al., 2016) and *Zostera muelleri* (Lee et al., 2016), and the chloroplast genomes of three other seagrass species (*Z. japonica*, *Zostera nigricaulis* and *Phyllospadix iwataensis*; Chen et al., 2021), are providing valuable insights into the adaptive evolution of seagrasses. Indeed, these studies have revealed how the gain and loss of gene families have contributed to their physiological adaptation to a submerged lifestyle in seawater, and thus to their tolerance to salinity. However, because this evolutionary step from land to sea has occurred at least three times during the evolutionary history of seagrasses, genomic information derived from species of the genus *Zostera* may not reflect independent adaptive mechanisms of other seagrass lineages. Therefore, it is crucial to sequence the complete genomes of other seagrass species of different lineages to unravel whether they share a common pathway of adaptation to the ocean (Lee et al., 2018).

The identification of seagrass populations locally adapted to extremely high salinity (see Box 1) offers the possibility to examine genome-wide signals of adaptive divergence, and to identify traits under selection with significant heritability (i.e. loci) that could underlie plant tolerance to hypersaline stress. Genotyping by sequencing of single nucleotide polymorphisms (SNPs) now enables researchers to explore genome–environment interactions and to characterise both neutral and functional (adaptive) genetic diversity of organisms without a reference genome (Eklom and Galindo, 2011), as is the case of most seagrass species. The identification of putative heritable loci under selection for hypersaline stress could then be combined with experimentally manipulating saline stress to examine the resilience and the potential trade-offs of genotypes possessing such loci (Anderson et al., 2014). This

information may be useful to identify the processes that confer salinity tolerance to seagrasses and may have implications for improving salt tolerance of seagrasses and crop species, as it can guide genetic modification to produce individuals with improved salt tolerance through genetic engineering (e.g. CRISPR/CAS9) and selective breeding approaches.

Although the selective pressure exerted by hypersalinity through the selection of resistant genotypes may have favoured the rapid adaptation of populations to saline conditions exceeding species thresholds, it is also possible that this increased tolerance is a dose- and frequency-dependent issue. The increased tolerance (and resistance) of these populations could in fact be the result of progressive/recurrent exposure of plants to hypersaline mid-stress levels as plants can ‘learn’ and ‘memorise’ how to cope with persistent stressful conditions. This process, known as ‘stress imprint’ (Bruce et al., 2007), relies on alterations in signalling metabolites, key transcription factors and transgenerational heritable epigenetic modifications, which are the basis of the ‘intelligent behaviour’ and ‘stress memory’ in clonal plants (Latzel et al., 2016). Stress ‘priming’, by artificially exposing plants to a stress factor to increase their tolerance to subsequent exposures, is a rapidly expanding technique in agriculture to ensure food availability under climate change (Xiao et al., 2017). In contrast, stress priming is still an immature field in seagrasses and only a few recent studies have evidenced the existence of heat-stress memory, and the likely involvement of epigenetic modifications in the process (Entrambasaguas et al., 2021; Nguyen et al., 2021; Pazzaglia et al., 2022). These findings have raised expectations for the restoration, farming and conservation of marine macrophytes (Jueterbock et al., 2021; Pazzaglia et al., 2021a,b). Therefore, the development of osmo-priming strategies for improving the osmotic resistance of plants and populations is a promising topic in seagrass research.

With ongoing climate change, seagrass meadows exposed to local hypersalinity may simultaneously experience stressors acting on a global or regional scale (warming, acidification, sea level rise, etc.). Although some effort has been made to identify the concurrent effects of hypersalinity with other biotic and abiotic stressors (see section 4), more effort is needed to conduct studies that simulate realistic future scenarios to improve the management and conservation of these important marine ecosystems. For example, with the increasing incidence of marine heatwaves (and other extreme weather events), it is possible that hypersalinity tolerance thresholds established for certain species (Barrio et al., 2020) may be modified by synergistic effects caused by the interaction between hypersalinity and thermal stress. The use of electrochemical microsensors and Non-invasive Micro-test System (NMT) (Fang et al., 2020; Koch et al., 2022) can help to elucidate the *in vivo* effects of these combined stressors on carbon (and other metabolites) fluxes (e.g., carbon fixation/sequestration, carbon efflux, trophic cascades) in seagrass communities conditioning, for instance, their role as blue carbon system. These kinds of experimental approaches could be especially relevant in the field of seagrass ecology in the coming years (Zimmerman, 2017).

Due to the clonal structure of seagrasses, there are strong developmental and metabolic gradients within shoots/ramets (leaves-rhizome-roots) or between them (e.g. orthotropic [vertical] versus plagiotropic shoots; Ruocco et al., 2019a; 2019b; Jueterbock et al., 2020). Clonal integrity allows the translocation of resources like nutrients, hormones, photosynthates and secondary metabolites among physically connected ramets (Marbà et al., 2002, 2006; Liu et al., 2016). The clonal nature of seagrasses, and the functional differentiation of the different plant compartments acting as a cooperative system, have been highlighted as a source of variability in the responses of seagrasses to changing ambient conditions (e.g. light and temperature; Olivé et al., 2009; Jueterbock et al., 2020; Ruocco et al., 2020). Although there is some evidence that seagrass physiological responses to hypersalinity, such as photosynthesis, depend on tissue variability (Alfonso-Abellán et al., 2010), and that chronic exposure to hypersalinity could promote adjustments at the

clonal architecture level (Garrote-Moreno et al., 2014; Marín-Guirao et al., 2017), the understanding of these processes is still emerging and merits further investigation.

Finally, seagrasses that act as hosts of microorganisms (including bacteria, fungi and viruses) represent a complex functional unit named 'holobiont', in which important symbiotic, mutualistic, commensal and pathogenic interactions converge (Tarquinio et al., 2019; Conte et al., 2021). The presence of associated microbial communities is critical for seagrass health, conditioning primary functions like phytohormone and vitamin production, nutrient acquisition and defence against pathogens. The interaction between environmental factors and the physiological status of seagrasses leads to rapid changes in the associated microbiota, as observed in plants exposed to altered light regimes and acidification (Hassenrück et al., 2015; Martin et al., 2018). Regarding salinity, changes in benthic microbiomes have been found to occur in seagrass meadows growing along saline gradients (Fraser et al., 2018) and under the influence of brines from desalination plants (Frank et al., 2019), although the effects on plant health have not been separated from the effects of hypersaline stress. Identifying the core microbial community and assessing its changing components is therefore a key-point in understanding holobiont dynamics (seagrass-microbe interactions) in response to hypersalinity and its effects on plants. This, in turn, could provide useful monitoring tools through the use of ecological microbiome indicators (Conte et al., 2021).

#### 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.

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2022.105809>.

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