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FEATURE ARTICLE

Effects of sediment sulfides on seagrass *Posidonia oceanica* meristematic activity

Neus Garcias-Bonet[1,*], Núria Marbà[1], Marianne Holmer[2], Carlos M. Duarte[1]

[1]Institut Mediterrani d'Estudis Avançats (CSIC-UIB), Miquel Marquès 21, 07190 Esporles, Spain
[2]Institute of Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark

ABSTRACT: Meristematic activity response of *Posidonia oceanica* shoots was assessed along a gradient of sediment sulfate reduction rates (SRR) and sediment sulfide pools (SSP) to test if meristematic activity could be used as an early indicator of seagrass health. The percentage of nuclei in the G2 phase of the cell cycle was used as a proxy of the cell division rate and therefore of the meristematic activity. The variability observed in the percentage of dividing cells (i.e. those containing nuclei in the G2 phase) in *P. oceanica* meristems was closely (>80% of total variance) coupled to variability in SSP and SRR. The percentage of nuclei in the G2 phase exponentially declined with increasing SSP and SRR, reaching the lowest values (<5%) when plants were growing on sediments with SSP >0.001 mol AVS (acid volatile sulfides) m^{-2}. These results strongly suggest that the meristematic activity can be used as an early warning indicator of seagrass stress.

KEY WORDS: *Posidonia oceanica* · *Caulerpa* spp. invasions · Sediment sulfate reduction rate · Sediment sulfide pools · Seagrass decline · Meristematic activity

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Increased sediment sulfides due to *Caulerpa* invasions decreases meristematic activity of the seagrass *Posidonia oceanica*.

Photo: Marianne Holmer

INTRODUCTION

Seagrass meadows rank amongst the most productive ecosystems on Earth, growing along tropical and temperate coasts, where they have important structural and biogeochemical functions (Hemminga & Duarte 2000). Seagrasses are clonal plants, which expand into new areas vegetatively by adding modules produced by the meristems located at the rhizome apices, rendering seagrass demographics and productivity closely dependent on rhizome meristematic activity (Tomlinson 1974).

Currently, seagrass meadows are undergoing a worldwide decline, as reflected by losses in cover and density during the 20th century (e.g. Orth et al. 2006). Excess inputs of nutrients and organic matter have been identified as the main drivers of this decline (e.g. Orth et al. 2006). Organic carbon and nutrient inputs to the sediment stimulate bacterial activity, increasing sediment oxygen demand and the production of bacterial metabolites such as sulfides, which are toxic for seagrasses (Terrados et al. 1999). Several die-off events in subtropical *Thalassia testudinum* (Borum et al. 2005) and temperate *Zostera marina* (Goodman et al. 1995) seagrass meadows have been linked to episodes of high sediment sulfide concentrations. It has been suggested that *Caulerpa* spp. invasions accelerate the decline in *Posidonia oceanica* meadows, since these macroalgae species cause the

*Email: neus.garcias@uib.es

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sediment sulfate reduction rate (SRR) to increase by more than 3-fold, when compared with those in seagrass meadows (Holmer et al. 2004). The rate of decline in *P. oceanica* meadows is closely coupled to sediment sulfide pools (SSP), increasing when sulfide porewater concentration exceeds 10 µM H_2S (Calleja et al. 2007). There is evidence of sulfide intrusion in *P. oceanica* tissues when this species is growing in sediments with a high SRR (Frederiksen et al. 2007). The intrusion of sulfide into the meristematic zone is considered detrimental for the plants, as it inhibits cytochrome oxidase even at low concentrations (1 to 10 µM, Raven & Scrimgeour 1997). Hence, seagrass health is compromised by anoxia and sulfide concentration in the rhizosphere (Terrados et al. 1999, Duarte et al. 2005), since the activity of seagrass meristems growing in sediments with high sulfide concentrations may be affected.

The activity of plant meristems can be analysed by measuring the percentage of dividing cells at a specific time point, as demonstrated for intact tissues of *Nicotiana tabacum* (Galbraith et al. 1983) and for cell suspensions of *Solanum aviculare* (Yanpaisan et al. 1998). Flow cytometry has been extensively used to quantify the DNA content of cells or isolated nuclei suspensions (e.g. Galbraith et al. 1983, Le Gall et al. 1993). Eukaryotic cells have a cell division cycle that is divided into the interphase and mitosis phase. The interphase consists of a G1 phase (post-mitotic phase), when the cell grows and synthesizes proteins and RNA; an S phase (DNA synthetic phase), when the DNA is replicated; and a G2 phase, when the cell has doubled the DNA content and the nuclear proteins and is preparing to enter the mitotic phase. The percentage of nuclei in the G2 phase provides information on the percentage of meristematic cells that are dividing. Hence, the percentage of nuclei in the G2 phase could be used as an indicator of meristematic activity and, thus, at declining percentages as an indicator of seagrass stress.

In the present study, we quantify the percentage of nuclei in each phase of the interphase of the cell cycle in rhizome meristems of the dominant Mediterranean seagrass *Posidonia oceanica*, growing alone or mixed with the native *Caulerpa prolifera* and invasive *C. taxifolia* or *C. racemosa*, in 3 meadows from the Balearic Islands (Spain). The *P. oceanica* examined grew on sediments encompassing the broad range of sediment sulfide concentrations and SRR reported for the Balearic Islands region (Holmer et al. 2003, Calleja et al. 2007). The percentage of nuclei in the interphase phases was quantified by using flow cytometer techniques and cell cycle analysis. We compare the variability found for the percentage of dividing cells, or nuclei in the G2 phase, with SSP and SRR to explore the potential use of meristematic activity as an early warning indicator of seagrass stress.

MATERIALS AND METHODS

Study site. This study was conducted at 3 *Posidonia oceanica* meadows in Mallorca (Balearic Islands, Spain) that were partially colonised by the autochthonous macroalgae *Caulerpa prolifera* in Cala Llonga (39° 22.03′ N, 3° 13.73′ E), and the invasive *C. taxifolia* or *C. racemosa* in Cala d′Or (39° 22.164′ N, 3° 13.88′ E) and in Cala Estancia (39° 32.13′ N, 2° 42.65′ E), respectively. The *P. oceanica* meadow at Cala Llonga, located in a sheltered bay, at the entrance of a marina, is declining, as evidenced by large areas with dead rhizomes. It grows at 3 m depth and receives high sedimentary inputs (10 g dry weight [DW] m^{-2} d^{-1}, M. Holmer et al. unpubl. data). The *P. oceanica* meadow at Cala d′Or is in steady-state growth condition (N. Marbà unpubl. data); it grows at 6 m depth and receives low sedimentary inputs (2 g DW m^{-2} d^{-1}, M. Holmer et al. unpubl. data) and has been invaded by patches of *C. taxifolia* since 1992 (T. Grau pers. comm.). The *P. oceanica* meadow at Cala Estancia grows at 3 m depth in front of a highly developed coastal area, receiving about 5 g DW m^{-2} d^{-1} sedimentary inputs (M. Holmer et al. unpubl. data), and the presence of *C. racemosa* has been recorded since 1998 (T. Grau pers. comm.). In all meadows studied *Caulerpa* spp. grow as monospecific patches on sandy areas, on dead *P. oceanica* rhizomes, as well as mixed with *P. oceanica*. The study was conducted during July 2005, the time of the year when the biomass of all 3 *Caulerpa* spp. (Terrados & Ros 1991, Thibaut et al. 2004, Ruitton et al. 2005) and bacterial activity are the highest (Hobbie & Cole 1984).

Sampling. At each meadow we selected 2 sampling sites (hereafter called stations), one where *Posidonia oceanica* was growing monospecifically, and one where *P. oceanica* and *Caulerpa* spp. were growing together. At each station, we collected 3 sediment cores of 4.5 cm diameter, 10 cm long, to estimate sediment density and porosity, and 3 sediment cores of 2.6 cm diameter, 20 cm long, to quantify SSP and SRR. At each station, we harvested 12 *P. oceanica* vertical shoots that were transported to the laboratory in seawater, where their meristematic activity was analysed immediately.

SRR and SSP. These were quantified according to the 2-step procedure (acid volatile sulfides, AVS, and chromium reducible sulfur, CRS; Fossing & Jørgensen 1989). Sulfide concentrations were measured by the spectrophotometric method (Cline 1969), and SSR were calculated according to Jørgensen (1978). Only rates and pools from the AVS fraction are used here (SRR_{AVS} and SSP_{AVS}).

Meristematic activity. The activity of *Posidonia oceanica* rhizome meristems was estimated by quanti-

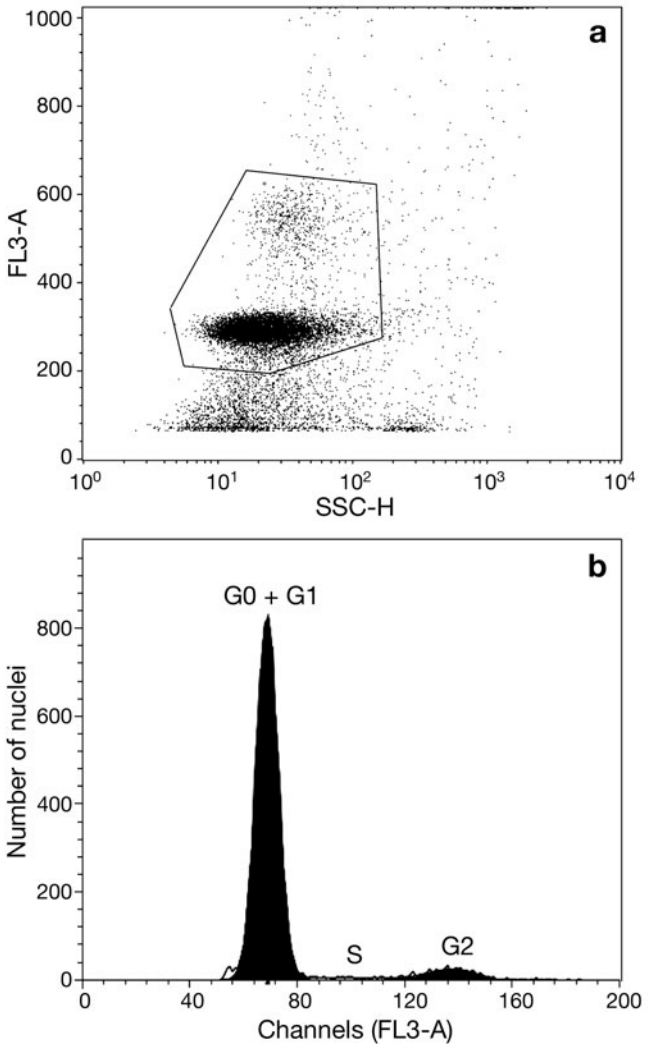

Fig. 1. *Posidonia oceanica*. (a) Red fluorescence (FL3-A) vs. side scatter (SSC-H) of the nuclei extracted from the *P. oceanica* meristematic zone, measured with the flow cytometer; the polygon encloses the nuclei in the sample. (b) Histogram of nuclei fluorescence (FL3-A) used to estimate the percentage of nuclei in cell cycle phases (G0 + G1, G2, S)

fying the percentage of nuclei in each phase of the cell cycle (i.e. G0 + G1, S, G2). Samples of 0.5 cm × 1 cm from the rhizome meristematic zone were cut. The nuclei were isolated using a Partec® extraction kit and stained with propidium iodide (PI) for 1 h in darkness at 4°C. The number of nuclei in each phase of the cell cycle (i.e. G0 + G1, S, G2) was quantified using a Beckton-Dickinson flow cytometer equipped with an argon-ion laser measuring the red fluorescence emitted by the PI. For each meristem 10 000 nuclei were analysed (Fig. 1a). The histograms of nuclei fluorescence obtained from the flow cytometer were analyzed using cell cycle analysis software (ModFit), which provided the percentage of nuclei in G0 + G1, S, G2 phases of each meristem sampled (Fig. 1b).

Statistics. We assessed the statistical significance of the variability in the percentage of *Posidonia oceanica* nuclei in each cell cycle phase, and sediment AVS pools and SRR, among stations (i.e. presence/absence of *Caulerpa* sp.) and meadows using a 2-way ANOVA. The significance of differences between stations and meadows was assessed using Tukey's post-hoc test. We tested the relationships between *P. oceanica* meristematic activity and SSP and SRR using least square regression analysis on log-transformed variables to meet the requirements of the analysis.

RESULTS

SSP_{AVS} and SRR_{AVS} ranged between 0.029 and 226 mmol SSP_{AVS} m^{-2}, and 0.086 and 64.37 mmol SRR_{AVS} m^{-2} d^{-1} across stations and meadows. SSP_{AVS} were significantly higher in the sediments colonised by *Caulerpa* spp. at Cala Llonga when compared with those from the rest of stations and meadows (Table 1, Fig. 2). The differences in SSP_{AVS} observed across meadows and stations reflected differences in SRR_{AVS}, as the highest rates were observed at Cala Llonga when sediments were colonised by *Caulerpa* spp., and the lowest ones in both sediment types at Cala d'Or (Fig. 2). SRR_{AVS}, in *Caulerpa* spp. sediments at Cala Llonga and Cala Estancia were significantly higher than those at the rest of stations and meadows (Fig. 2, Table 1). The sediments colonised by *Caulerpa* species mixed with *Posidonia oceanica* exhib-

Table 1. Summary of the factorial 2-way ANOVA to assess significant differences in meristematic variables of *Posidonia oceanica* (cell cycle phases G0 + G1, S, G2) and sediment variables (sediment sulfide pools [SSP_{AVS}] and sulfate reduction rates [SRR_{AVS}]) between stations (presence/absence of *Caulerpa* spp.) and meadows (Cala Llonga, Cala d'Or and Cala Estancia). p provided by a univariate *F*-test

Variable	Effect	df	*F*	p
SSP_{AVS}	Station	1	28.61	<0.0005
	Meadow	2	0.20	ns
	Station × Meadow	2	9.59	<0.005
SRR_{AVS}	Station	1	15.95	<0.005
	Meadow	2	0.11	ns
	Station × Meadow	2	4.33	<0.05
G0 + G1	Station	1	0.17	ns
	Meadow	2	2.38	ns
	Station × Meadow	2	1.51	ns
G2	Station	1	0.56	ns
	Meadow	2	11.75	<0.0001
	Station × Meadow	2	0.52	ns
S	Station	1	0.46	ns
	Meadow	2	2.67	ns
	Station × Meadow	2	0.84	ns

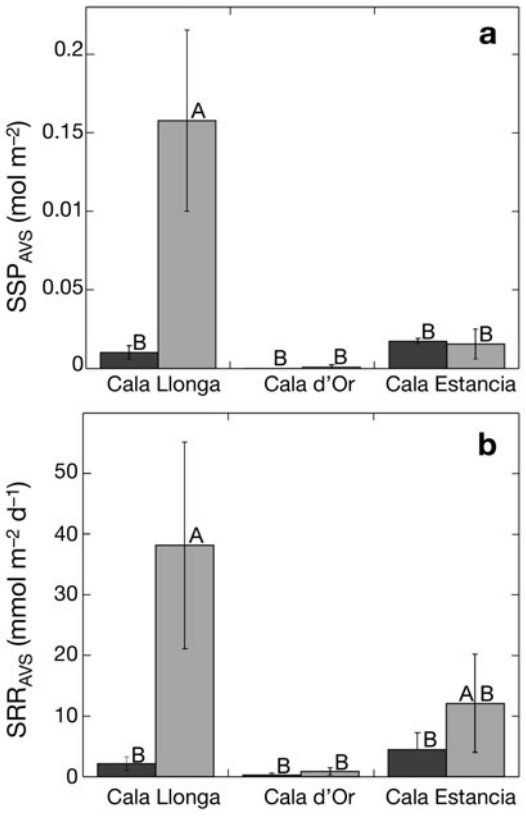

Fig. 2. *Posidonia oceanica*. Mean ± SE of (a) sediment sulfide pools (SSP_{AVS}) and (b) sulfate reduction rates (SRR_{AVS}) in the top 10 cm sediment where *P. oceanica* was growing monospecifically (black bars) and mixed with *Caulerpa* species (grey bars) (n = 3). A, B indicate significantly different SSP_{AVS} and SRR_{AVS} across meadows and stations (Tukey post-hoc test)

ited SSP_{AVS} up to 15.2-fold higher than those colonised only by *P. oceanica*. SRR_{AVS} in sediments colonised by *Caulerpa* species mixed with *P. oceanica* were between 2.7 to 17.5-fold higher than those in adjacent sediments colonised by *P. oceanica* alone.

The meristems of *Posidonia oceanica* presented between 86.0 and 90.9% of nuclei in phase G0 + G1, 2.0 and 7.0% in phase S, and 4.7 and 10.2% in phase G2 of the cell cycle. There were no significant differences (Table 1) in the percentage of nuclei in phases G0 + G1 and S across *P. oceanica* meadows. Similarly, the presence of *Caulerpa* species did not affect significantly the percentage of nuclei in G0 + G1 and S phases (Fig. 3, Table 1). Conversely, the percentage of nuclei in phase G2 in the meristems of *P. oceanica* growing at Cala d'Or was significantly 1.5-fold higher than those colonizing Cala Llonga and Cala Estancia (Fig. 3, Table 1). The percentage of nuclei in phase G2 in the meristems of *P. oceanica* tended to decrease when growing with *Caulerpa* species (Fig. 3), although this trend was not statistically significant (Table 1).

The variability observed in the percentage of dividing cells (i.e. containing nuclei in phase G2) in *Posidonia oceanica* meristems was closely (>80% of total variance) coupled to variability in SSP_{AVS} and SRR_{AVS} (Fig. 4). The percentage of nuclei in G2 phase declined with increasing SSP_{AVS} and SRR_{AVS}, reaching the lowest values (<5%) when plants were growing on sediments with SSP_{AVS} greater than 0.001 mol AVS m^{-2} (Fig. 4). No relationship was observed between the variability in the percentage of nuclei in G0 + G1 and S phases and sediment sulfur dynamics (regression analysis, $p > 0.05$).

DISCUSSION

The percentage of nuclei in G2 in *Posidonia oceanica* meristems is low, particularly when compared with estimates for other plants. For instance, the percentage of dividing cells in leaves of *Nicotiana tabacum* is 22 to 27% (Galbraith et al. 1983, Chen et al. 2001). The low fraction of dividing cells in *P. oceanica* rhizome meris-

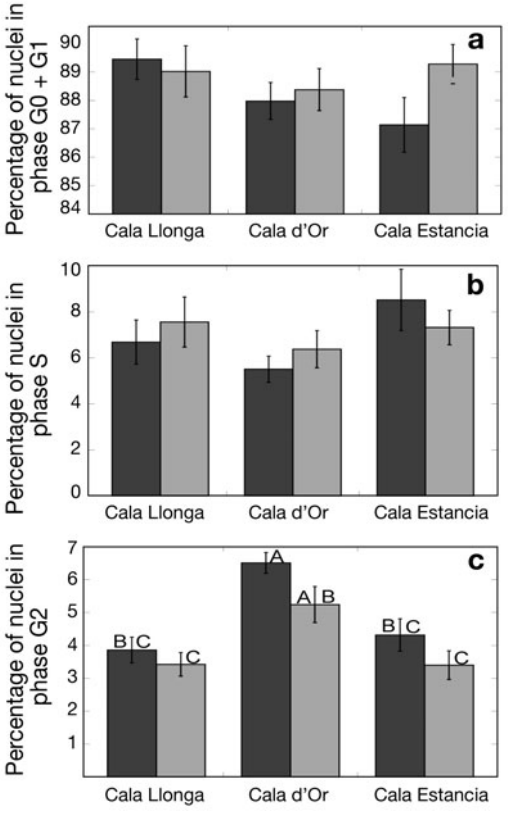

Fig. 3. *Posidonia oceanica*. Mean ± SE percentage of nuclei of *P. oceanica* meristems in the (a) G1 + G0 phase, (b) S phase and (c) G2 phase growing monospecifically (black bars) and mixed with *Caulerpa* species (grey bars) at the study sites (n = 12). A, B, C indicate significantly different percentage of nuclei in G2 phase across meadows and stations (Tukey post-hoc test); variability in G0 + G1 and S phases across meadows and stations was not statistically different

tems is consistent with the slow growth of this species (rhizome growth rate: 4.2 cm yr^{-1}, leaf growth: 1.3 mm $leaf^{-1}$ d^{-1}; Duarte 1991), which ranks amongst the slowest-growing angiosperms (Nielsen et al. 1996).

Despite the low fraction of dividing cells in *Posidonia oceanica* meristems, the fraction of cells in the G2 phase varied by up to 50% across stations and meadows (Fig. 3). The variability in the percentage of dividing cells in *P. oceanica* meristems mainly reflected differences between sites (Table 1), the meristems in plants at Cala d'Or exhibiting a larger fraction of nuclei in the G2 phase than those at Cala Llonga and Cala Estancia. Despite the fact that the presence of *Caulerpa* spp. did not have a statistically significant effect on the percentage of nuclei in the G2 phase in *P. oceanica* meristems (Table 1), the activity of seagrass meristems displayed lower values when growing with *Caulerpa* spp. than in monospecific stations at all 3 sites (Fig. 3). Recently, M. Holmer et al. (unpubl. data) found that *Caulerpa* spp. enhance sulfate reduction rate and anoxia in the sediments they colonise, and a strong coupling between *Caulerpa* spp. biomass and sulfate reduction rate and sediment anoxia. The lower percentages of G2 nuclei in meristems of *P. oceanica* in the presence of *Caulerpa* spp. as opposed to seagrass growing alone, albeit not statistically significant (Fig. 3), suggests that *P. oceanica* health is compromised by *Caulerpa* spp. invasions, due to the increase in sediment sulfides, and it supports the hypothesis that *Caulerpa* spp. act as ecological engineers (M. Holmer et al. unpubl. data), excluding *P. oceanica* by deteriorating sediment conditions .

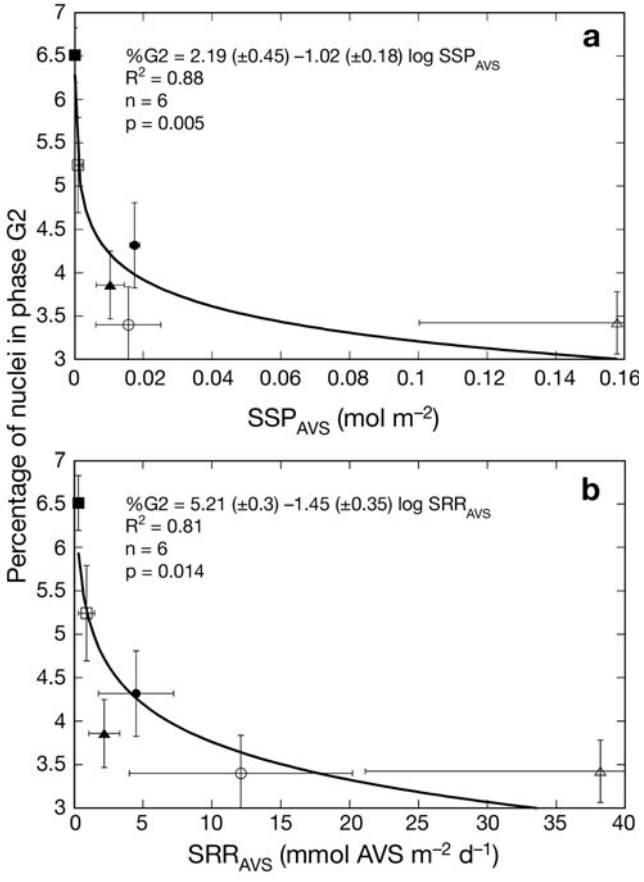

Fig 4. *Posidonia oceanica*. Relationship between the percentage of nuclei of *P. oceanica* meristems in the G2 phase and (a) sulfide sediment sulfide pools (SSP_{AVS}) and (b) sulfate reduction rates (SRR_{AVS}) in monospecific (black symbols) and mixed (open symbols) meadows at Cala Llonga (Δ), Cala d'Or (□) and Cala Estancia (○). Standard errors of the mean values are shown (n_{G2} = 12; n_{AVS} = 3; n_{SRR} = 3)

The close and negative relationship between the percentage of dividing cells in *Posidonia oceanica* meristems and sediment sulfide concentrations and production reflects the high sensitivity of seagrasses to sulfides. Sulfide intrusion in seagrass tissues has been observed in *Zostera marina* (Pedersen et al. 2004), *Thalassia testudinum* (Borum et al. 2005) and *P. oceanica* (Frederiksen et al. 2007, Marbà et al. 2007). Mass balance computations based on $\delta^{34}S$ signals in *P. oceanica* tissues and the potential sulfur sources (sediment porewater sulfides and seawater sulfate) showed that sulfide intrusion accounts for up to 40% of total plant sulfur at Cala Llonga (M. Holmer et al. unpubl. data), the location that supported the plants with meristems with the least percentage of dividing cells (Fig. 3).

The percentage of dividing cells in *Posidonia oceanica* meristems decreased sharply when SSP_{AVS} and SRR_{AVS} increased (Fig. 4). The stability of *P. oceanica* meadows is closely related to sediment sulfide pools, accelerating meadow decline rates when sediment sulfide concentration exceeds 10 µM (Calleja et al. 2007). Considering that 10 µM of H_2S equals 0.001 mol AVS m^{-2} (calculated using the water content and sediment density, and assuming that all SSP_{AVS} within the top 10 cm sediment layer was released as H_2S in the porewater), our results (Fig. 4) indicate that *P. oceanica* meristems with less than about 5% nuclei in the G2 phase are under sulfide stress. Hence, there is a potential for seagrass meristematic activity to be used as an early warning indicator of plant health and meadow decline.

In summary *Posidonia oceanica* meristems divide slowly, as the percentage of cells in the G2 phase was <7%, below that observed for meristems of any other plant. *P. oceanica* meristematic activity, assessed as the percentage of nuclei in the G2 phase, is closely coupled to sediment sulfur dynamics, the percentage of nuclei decreasing when SSP_{AVS} exceeds 0.001 mol AVS m^{-2}. These findings confirm that seagrass meristematic activity is highly sensitive to increased sulfide and points at seagrass meristematic activity as an indicator of plant stress and seagrass health, as well as an early warning indicator of seagrass decline.

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