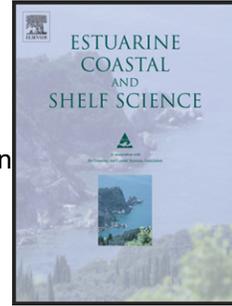


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Effects of salinity on seed germination and early seedling growth of the Mediterranean seagrass *Posidonia oceanica* (L.) Delile

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Abstract

Previous research has demonstrated the low tolerance of *Posidonia oceanica* mature shoots to salinity variability but there is no information about the response of its seeds and seedlings to this impact. In the present study, two independent experiments were conducted to investigate the effects of salinity variation on germination, development and survival of *P. oceanica* seeds and seedlings under laboratory-controlled conditions. Maximum *P. oceanica* seed germination occurred at the control salinity (37), while mortality was lowest for this treatment. However, no significant differences were detected with other salinity levels (39-49). In contrast, salinity appears to play an important role in seedling development. The number of blades, the length of the primary root and the maximum length of leaves of seedlings were significantly reduced with increased salinities. The results observed in the present study are consistent with those obtained for mature *P. oceanica* shoots, and suggest that hypersalinities, such as those associated with brine discharge of a desalination plant, may limit *P. oceanica* seedling recruitment, affecting the expansion and recovery of *Posidonia* meadows.

Key words: *Posidonia oceanica*; Seeds; Germination; Seedlings; Salinity effects; Desalination impact

1. Introduction

In seagrasses, the simplest and most frequent form of multiplication is asexual clonal reproduction (Clarke and Kirkman, 1989; Duarte and Sand-Jensen, 1990; Procaccini and Mazzella, 1998). However, sexual reproduction plays a key role in maintaining genetic diversity, dispersal and colonization of new coastal habitats (Buia and Mazzella, 1991; Orth et al., 1994; Procaccini and Mazzella, 1998; Waycott et al., 2006).

Posidonia oceanica is an endemic Mediterranean seagrass that exhibits both vegetative and sexual reproduction. Sexual reproduction in this species is considered a rare and sporadic phenomenon (Caye and Meinesz, 1984; Buia and Mazzella, 1991; Balestri and Cinelli, 2003; Balestri, 2004), although some episodic mass flowering has been observed associated with extreme summer temperatures (Mazzella et al., 1983; Diaz-Almela et al., 2006). Flowering occurs from September to December, depending on the water depth, and fruits are produced four months later, from late January to early March (Buia and Mazzella, 1991). The mature buoyant fruits, which contain a single seed, are released from the plant and are dispersed by water currents from March to June (Buia and Mazzella, 1991). The seed sinks after fruit dehiscence from the split pericarp and lacks dormancy, as germination has probably begun within the fruit prior to dehiscence (Belzunce et al., 2005).

There is little information available on *P. oceanica* seedling establishment and survival, generally seedling success is considered low (Gambi et al., 1996; Piazzini et al., 1999; Balestri and Cinelli, 2003), although a recent study reports a large recruitment event of this species (Balestri and Lardicci, 2008). In contrast, seed germination and seedling development in the laboratory are considered very successful (Caye and Meinesz, 1989; Balestri et al., 1998a; Piazzini et al., 1999; Balestri and Bertini, 2003; Belzunce et al.,

2008). For other seagrasses, seed germination and development may be affected by several abiotic factors, such as temperature, salinity, light, oxygen concentration and sediment burial (Orth and Moore, 1983; Moore et al., 1993; Brenchley and Probert, 1998). Among them, salinity can be considered as one of the main limiting factors in the germination of many seagrass species (Caye and Meinesz, 1986; Caye et al., 1992; Orth et al., 2000). Salinity stress affects seed germination either through osmotic effects, by delaying or inhibiting germination, or through ion toxicity, which can make the seeds unviable (Brenchley and Probert, 1998). Extreme salinities also affect growth and survival of seedlings of several seagrasses (Walker and McComb, 1990; Kahn and Durako, 2006).

Optimal germination in seeds of seagrasses often occurs under hyposalinity conditions and germination of most species is reduced and delayed with a salinity increase (Philips et al., 1983; Hootsmans et al., 1987; Loques et al., 1990; Harrison, 1991; Caye et al., 1992; Conacher et al., 1994), but the response may vary considerably with species. Several studies have shown little or no effect of salinity on germination (McMillan, 1981, 1983; Churchill, 1983; Orth and Moore, 1983; Conacher et al., 1994; Probert and Brenchley, 1999), but some of these studies have found differences in optimum salinity range between seed and seedling stages, implying that the ability of seagrasses to deal with a salinity stress may change during their development (Hootsmans et al., 1987).

The salinity of the open waters of the Mediterranean Sea is generally constant, but the recent development of new seawater desalination facilities can discharge significant hypersaline effluents into coastal areas that may affect benthic organisms (Fernández-Torquemada et al., 2005, 2009). *Posidonia oceanica* meadows represent an ecosystem susceptible to being affected by these desalination effluents (Boudouresque and Meinesz, 1982; Phillips and Meñez, 1988). Previous studies have shown that *P.*

oceanica mature shoots are relatively sensitive to salinity increases (Fernández-Torquemada and Sánchez-Lizaso, 2005; Gacia et al., 2007; Ruíz et al., 2009) and led to recommendations on critical salinity thresholds for this species to minimize the impact of brine discharges from desalination plants (Sánchez-Lizaso et al., 2008). However, to date, there is no information about the response of its seeds and seedlings to this impact.

The main aim of the present study is provide the first information on the tolerance of *P. oceanica* young phases (seeds and seedlings) to salinity changes and to establish their possible range of salinity tolerance. Experiments were conducted to investigate the effects of different levels of salinity on germination, development and survival of seeds and seedlings under laboratory-controlled conditions.

2. Material and methods

Two independent sets of experiments were conducted to analyse the effects of salinity on *P. oceanica* seed germination and seedling development under laboratory-controlled conditions. The first experiment focused on evaluating survival and germination of *P. oceanica* seeds and obtaining some preliminary data about early seedling development. In the second experiment, we considered the effects of a broader range of salinities on the seedling development over a period of 50 days.

2.1. Fruit collection and seed extraction

An exceptionally productive flowering event was observed in the Western Mediterranean meadows of *P. oceanica* in the autumn of 2003. *P. oceanica* shoots with mature fruits were collected by SCUBA diving from a shallow meadow (-10 m) in El Campello (Alicante, SE Spain; 38°28'12"N, 000°18'43"W) at the end of April 2004. Once collected, fruits were removed from the reproductive shoots and were transported

to the laboratory in a cooler, containing ambient seawater from the collection site.

Fruits with no signs of damage were surface-sterilized by rinsing in ethanol (Balestri et al., 1998a) and maintained in aerated aquaria with autoclaved seawater. Immediately prior to an experiment, fruits were cut in half and seeds were carefully extracted. Then seeds of homogeneous size were selected and were randomly assigned to each treatment.

2.2. Seed germination experiment

This experiment was conducted to evaluate the effect of exposure to different high salinity treatments on seed germination, survival and early seedling development of *P. oceanica*. Ten seeds were placed on filter paper in 12 cm glass Petri dishes submersed with different salinity treatments for 14 days, exposure time in which all seeds were able to germinate (Balestri et al., 1998a). The salinity levels tested were 37 (control), 40, 43, 46 and 49, with three Petri dishes per salinity treatment. The lowest salinity treatment used was the field salinity measured (37) when fruits were collected in May 2004; higher salinity concentrations were chosen to cover a broad range of variations associated with a desalination discharge (Fernández-Torquemada et al., 2005, 2009). These salinity treatments were achieved by adding natural salt, produced by Santa Pola saltworks from concentrating seawater, to ambient coastal seawater (37). Salinity during the experiment was measured using a Thermo Orion conductimeter. Salinity was adjusted with distilled water to avoid evaporative changes and the solutions were changed every three days. Petri dishes were placed in a culture chamber (ASL Aparatos Científicos M-92004, Madrid, Spain), and subjected to a regime of 12 h of light (400-700 nm, $475\text{-}520 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) and 12 h of dark at a constant temperature of 22°C for 14 days. Replicates were placed in two rows, which were rotated daily within

the incubator to compensate for any variation in light levels at the front and back of the chamber. Germinated seeds were counted, and dead seeds were counted and removed every two days. Seed germination was defined as root or shoot emergence and seeds were considered dead when they were necrotic. At the end of this experiment (14 days), measurements of primary root length, maximum leaf length and number of leaves were made with a vernier caliper for all seedlings.

2.3. Seedling growth experiment

This second experiment was performed to determine seedling response during 50 days to a higher range of salinity treatments using seedlings cultivated from seeds germinated in the laboratory. Seeds were extracted from fruits and germinated in a seawater medium in the laboratory 2 weeks prior to this experiment, as described above. Healthy seedlings of a similar size were then selected for this experiment. At the time of the experiment, seedlings had 4.0-5.0 leaves of 0.88-1.16 cm of maximum length and roots of 0.39-0.47 cm.

Individual seedlings were randomly transferred into glass culture tubes (150 x 20 mm) containing 5 ml of solid agar media and 10 ml of liquid treatment. Ten seedlings were randomly assigned to one of 14 salinity treatments (25-51, separated by 2 salinity units). These levels were chosen to cover a broad range of salinity due to the lack of knowledge about the salinity tolerance of *P. oceanica* seedlings. Salinity treatments were made with DI water and Santa Pola salts and the liquid media in each tube was changed every 2 days. Culture tubes were closed and sealed with Parafilm to avoid evaporation and were maintained in a plant growth chamber under the same conditions described above.

Morphometric characteristics were measured weekly for the duration of this experiment

(50 days). Individual root and leaf-blade lengths and total leaf number were measured. A seedling was considered dead when all blades fell from the sheath or all blades were necrotic.

2.4. Statistical analyses

The effect of salinity on the response of the different seagrass variables at the end of the first experiment was statistically analysed by nested ANOVAs (with three petri plates replicated for each salinity treatment). Analysis of variance with repeated measures (ANOVAR) was applied to determine differences in some seagrass variables across time and between salinity treatments. Normality was previously verified using the Shapiro-Wilk test of normality, and homogeneity of variance was tested using the Cochran test (Underwood, 1997). When analysis of variance identified a significant difference, the *post-hoc* test, SNK (Student-Newman-Keuls), was applied to identify specific treatment differences. All statistical analyses were implemented using Statistica packages with a minimum significance level established at $p < 0.05$.

3. Results

3.1. Effects of hypersalinity on seed germination and early seedling development

Mean length and fresh weight of seeds used in this experiment were 1.43 ± 0.04 cm and 0.35 ± 0.03 g. These *P. oceanica* seeds were able to germinate, survive and grow on all the salinity treatments tested here. Cumulative germination of *P. oceanica* seeds (Figure 1) shows that after 9 days, the highest percent of seed germination (90%) was reached at the control salinity (37), but the ANOVA did not detect significant differences (Table 1) with the remaining treatments (63.3-80%). Hypersalinities also seemed to affect mortality percentage (Fig. 1). Mortality values were the lowest for the control treatment

(13.3%), increasing considerably at salinities above 46 (53.3-73.3%), but again differences were not significant (Table 1).

Salinity did appear to play an important role in early seedling development. The number of leaves, the length of the primary root and the maximum length of leaves of seedlings differed significantly among treatments (Table 1, Fig. 2). Seedlings at control salinity produced an average of 4 blades, their longest leaves averaged 1.25 cm and the length of their primary roots was 0.87 cm. These values decreased with increased salinities (3-3.5 blades per seedling, 0.33-0.73 cm and 0.05-0.27 cm of leaf and root length, respectively).

3.2. Effects of salinity on seedling development

At the start of this experiment, *P. oceanica* seedlings had 4.0-5.0 leaves of 0.88-1.16 cm of maximum length and roots of 0.39-0.47 cm. In this experiment, the highest survival and seedling development was observed for seedlings growing on control salinity (37).

Mortality at the end of the experiment was higher in all reduced salinities than in the control (Fig. 3). Seedlings at control salinity exhibited only 10% mortality, whilst those subjected to salinity of 25-35 exhibited more than 50% of mortality. In contrast, hypersalinities did not produce mortality values as elevated as hyposalinities ($\leq 40\%$ at 39-51).

The number of produced leaves differed among salinity levels and exposure times (Table 2, Fig. 4), initially 4.0-5.0, reached maximum values in the control and low salinities (7.0-8.0 leaves per seedling) than in the higher salinity levels after 32 days (5.6-7.6 leaves per seedling). Also, a significant decrease in leaf length occurred in seedlings at high salinity treatments after 10 days of hypersalinity exposure (Table 2,

Fig. 5). Maximum leaf length was higher at control and hyposalinity conditions (2.9-4.2 cm) when compared to hypersalinities (1.5-2.6 cm). Significant effects of salinity were also detected on root seedling growth (Table 2, Fig. 6). At the end of this experiment (49 days), seedlings at the control salinity developed a root of 1.4 ± 0.2 cm, significantly higher than at elevated salinities (0.5-0.8 cm at 39-51), while root lengths at lowest salinities were similar to the control (0.6-1.6 cm).

4. Discussion

In the present study, final *P. oceanica* seed germination (90%) and survival (86.7%) at the control salinity (37) was within the range reported for this species in the field (Buia and Mazzella, 1991; Balestri et al., 1998b; Piazzini et al., 1999) and under laboratory conditions (Balestri et al., 1998a.; 2009; Balestri and Bertini, 2003; Belzunce et al., 2008; Celdrán and Marín, 2011). However, significant differences in germination and survival with elevated salinities were not detected, although a clear trend was observed. Maximum germination occurred at the control salinity and lower values were observed for all of the higher salinities tested (63.3-80%). Survival percentages were also highest for the control treatment, decreasing at salinities above 40 (< 50%).

Numerous studies have observed that an increase of salinity induces both a reduction in the number of seeds germinated and a delay in the initiation of germination (Philips et al., 1983; Hootsmans et al., 1987; Loques et al., 1990; Harrison, 1991; Caye et al., 1992; Conacher et al., 1994). These negative responses might be related to the inhibitory effect of osmotic stress or to the decline in mobilization of food reserves. Our lack of a significant salinity effect may be explained by the high variability observed in this study, which obscured any significant effect or that *P. oceanica* seeds initially may depend on stored nutrient reserves (Caye and Meinesz, 1989; Belzunce et al., 2005; Balestri et al., 2009), allowing seed germination and survival in unpredictable

conditions.

In this same experiment, significant salinity effects were detected on foliar and root growth of seeds recently germinated. Seedlings at control salinity had produced on average 4 leaves, their longest leaves averaged 1.25 cm and the length of their primary roots was 0.87 cm, similar to data observed elsewhere (Balestri and Bertini, 2003; Belzunce et al., 2008; Balestri et al., 2009). However, seeds germinated in hypersaline conditions produced a lower number of leaves and their maximum length and root elongation were significantly reduced by salinities higher than 40. These results were consistent with data obtained in the second seedling-growth experiment.

In the second experiment, *P. oceanica* seedling survival at the control treatment was 90%, similar to values recorded in other studies carried out with this same species (Balestri et al., 1998a; Balestri and Bertini, 2003). Higher mortality at hyposaline conditions ($\geq 50\%$) was observed. However, the number of blades, maximum length of leaves and roots at the reduced salinities were similar to values obtained in the control treatment. In the hypersaline treatments, a lower mortality on seedlings ($\leq 40\%$) was detected, but these seedlings had a lower number of blades, shorter leaf length and shorter roots. This suggests that *P. oceanica* seedlings surviving exposure to low salinities (25-35) are relatively more tolerant to hyposalinity. In contrast, while seedling survival was greater at the highest salinities surviving seedlings exhibited slower development, which may reduce their eventual survival at longer exposure times than tested here.

The inhibitory effect of high salinity on leaf development may be critical due to any resultant effect on photosynthesis to maintain the seedling once its seed reserves are depleted (Belzunce et al., 2005, 2008). In addition to the negative effects of elevated salinity on the seedling leaf system, we also observed a reduction in root growth. This

effect is also important since the establishment of seedlings in situ depend on rooting in their initial recruitment phases (Belzunce et al., 2008), thus any reduction in the root development may cause a decline in the number of seedlings successfully establishing in the field.

This reduction of the seedling root and leaf biomass may be explained by the toxicity of elevated salinities on meristematic tissues, by an increment on carbon demands as compatible solute required for osmoregulatory functions (Sandoval-Gil et al., 2012) or by a decrease in photosynthetic activity (Zhu, 2001). Celdrán and Marín (2011) have recently confirmed the importance of photosynthesis at seedling stage to compensate the respiration and to avoid exhaustion of the seed starch reserves.

Tolerance to salinity variations also may differ significantly between young and mature plants (Hootsmans et al., 1987; Tyerman, 1989), with young stages, such as seeds and seedlings, being more sensitive to stressors than mature ones. This has been observed with mature *Amphibolis antarctica*, which tolerates high salinities (58-70) in Shark Bay (Walker, 1985; Kendrick et al., 1988), while Walker and McComb (1990) observed that *A. antarctica* seedlings die at a salinity of 65. Phillips et al. (1983) also observed that seed germination in *Z. marina* was increased in salinities lower than those of the environment in which the mature plant grew. In a study with *P. oceanica*, Balestri et al. (1998a) reported that the salinity of a seawater medium seemed supra-optimal for its germination, indicating that *Posidonia* seed germination may have a lower salinity range than that for mature shoot growth. Other studies have suggested that young plants often acclimate to salinity variations, as do older plants. Kahn and Durako (2006) reported that *Thalassia testudinum* seedling survival and development was similar to ranges reported by previous works involving adult *T. testudinum* shoots, with significantly reduced growth in both hypo- and hypersaline conditions (Zieman, 1975;

Doering and Chamberlain, 1998; Lirman and Cropper, 2003).

The present study showed an analogous situation in that the optimum salinity range observed here for seedlings was consistent with that obtained by previous laboratory and field studies done with mature *P. oceanica* shoots, with decreased growth and survival under hypersaline conditions (Fernández-Torquemada and Sánchez-Lizaso, 2005; Gacia et al., 2007; Ruiz et al., 2009). The present study indicates that once *P. oceanica* seeds germinate, they grow better under hyposaline conditions than under hypersaline conditions, although the lowest salinity tested was 25. Hence the absence of *P. oceanica* from areas under the influence of freshwater pulses from rivers is probably not related to salinity, but is due to other factors such as turbidity.

Salinity effects on germination and seedling development of this species may be crucial given the low frequency and extent of sexual reproduction in *P. oceanica* (Caye and Meinesz, 1984; Balestri and Cinelli, 2003), despite the importance of this mechanism for genetic diversity and population maintenance (Buia and Mazzella, 1991; Procaccini and Mazzella, 1998), and the very slow vegetative growth and recovery of adult plants (González-Correa et al., 2005).

Our study suggests that the hypersalinities, such as those associated with desalination plant discharge, may negatively influence the distribution of *P. oceanica* meadows and could decrease the recruitment and expansion of this species into brine-affected areas. Our data also agree with the previous salinity thresholds established to minimize the impact of brine discharges from desalination plants on the Western Mediterranean sea (Sánchez-Lizaso et al., 2008). Furthermore, the present study is focused solely on the effects of salinity on *Posidonia* young stages, and future studies should therefore investigate the effects of increases in salinity in combination with other important environmental factors, such as temperature or pH, that can vary and interact on the

habitat of this species.

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Figure and table captions:

Table 1. Summary of the repeated measures ANOVA (A) and nested ANOVA (B) testing the effects of salinity treatments (37, 40, 43, 46 and 49) on *P. oceanica* seed germination and early seedling development parameters (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, P(Sal.) = 3 replicate Petri plates)

Table 2. Summary of the repeated measures ANOVA testing the effects of salinity treatments at different times on seedling development parameters: number of leaves, maximum leaf length and primary root length (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Fig. 1. Accumulative germination (%) and mortality (%) of *P. oceanica* seeds subjected to the different salinity treatments over the experimental period (bars represent standard errors).

Fig. 2. Morphometric characteristics of viable *P. oceanica* seedlings measured at the end of the first experiment (14 days). Means with different letters are statistically different at $p < 0.05$ (bars indicate standard errors).

Fig. 3. Accumulative mortality (%) of *P. oceanica* seedlings subjected to the different salinities over the experimental period.

Fig. 4. Mean number of leaves per seedling over the experimental period at the different salinity treatments (bars represent standard errors). Asterisks indicate the statistically different treatments at $p < 0.05$

Fig. 5. Maximum leaf length (cm) of viable *P. oceanica* seedlings over the experimental period for the different salinity treatments (bars represent standard errors). Asterisks indicate the statistically different treatments at $p < 0.05$.

Fig. 6. Maximum root length (cm) of viable *P. oceanica* over the experimental period at the different salinity treatments (bars represent standard errors). Asterisk indicates the

statistically different treatment at $p < 0.05$.

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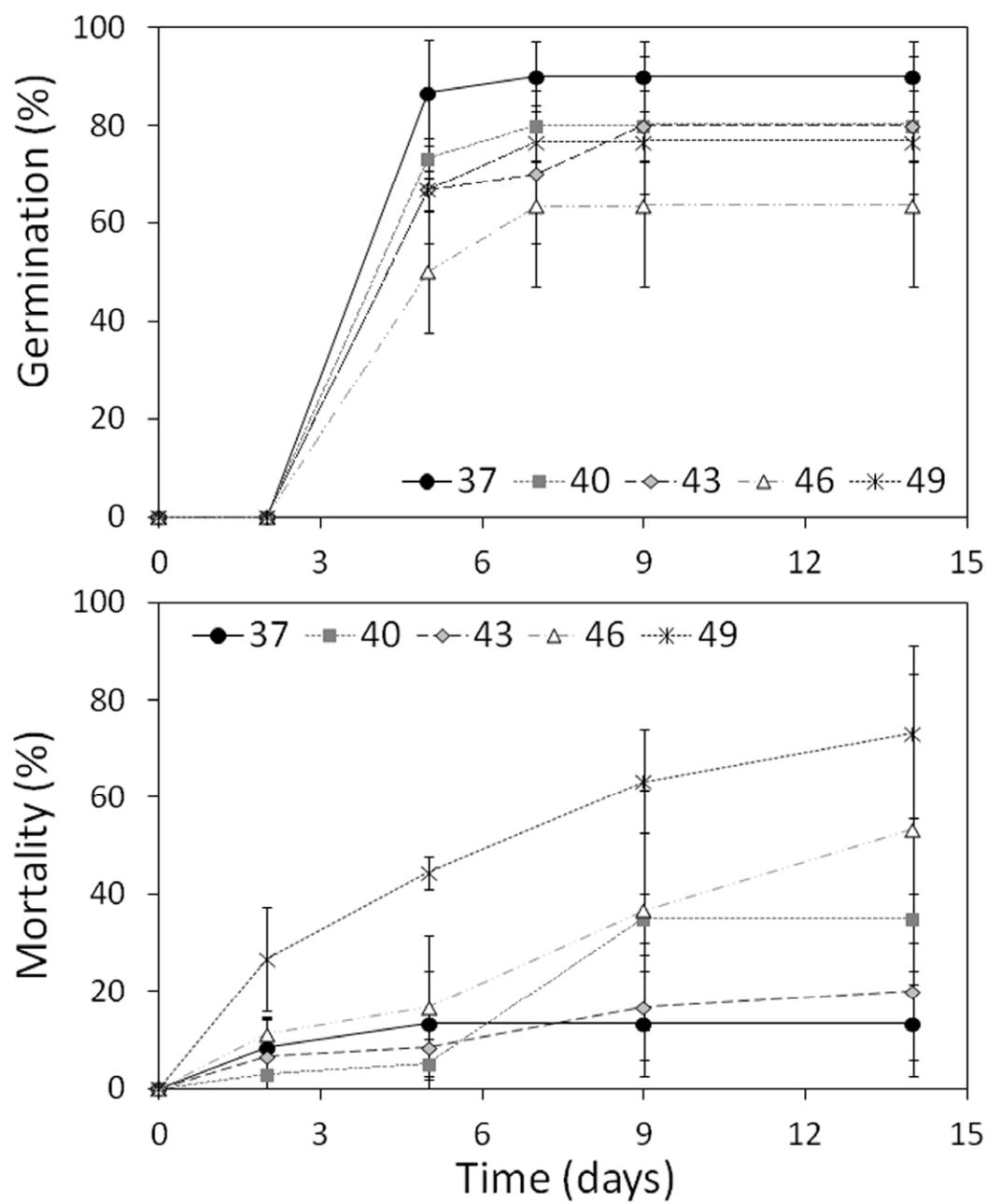
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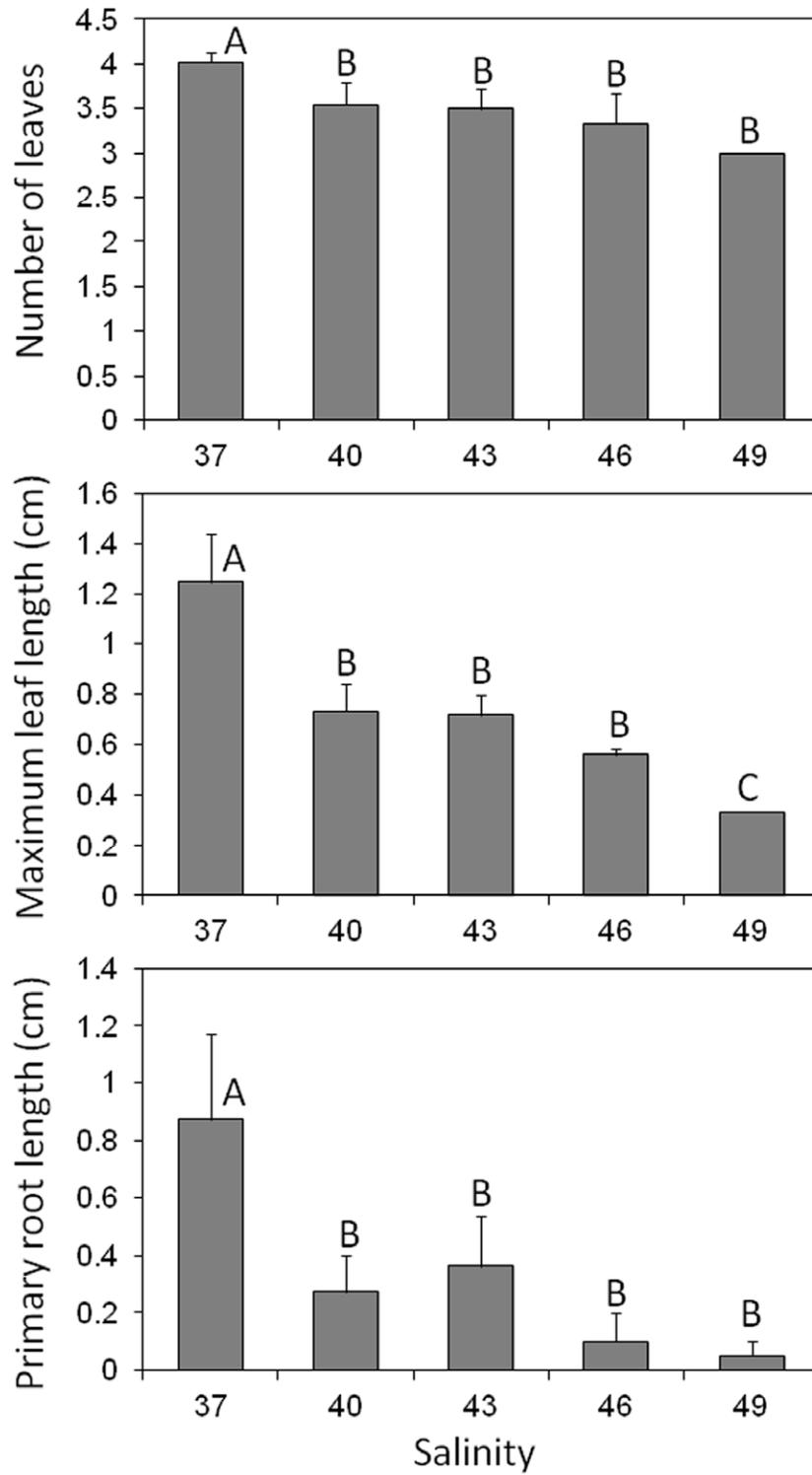
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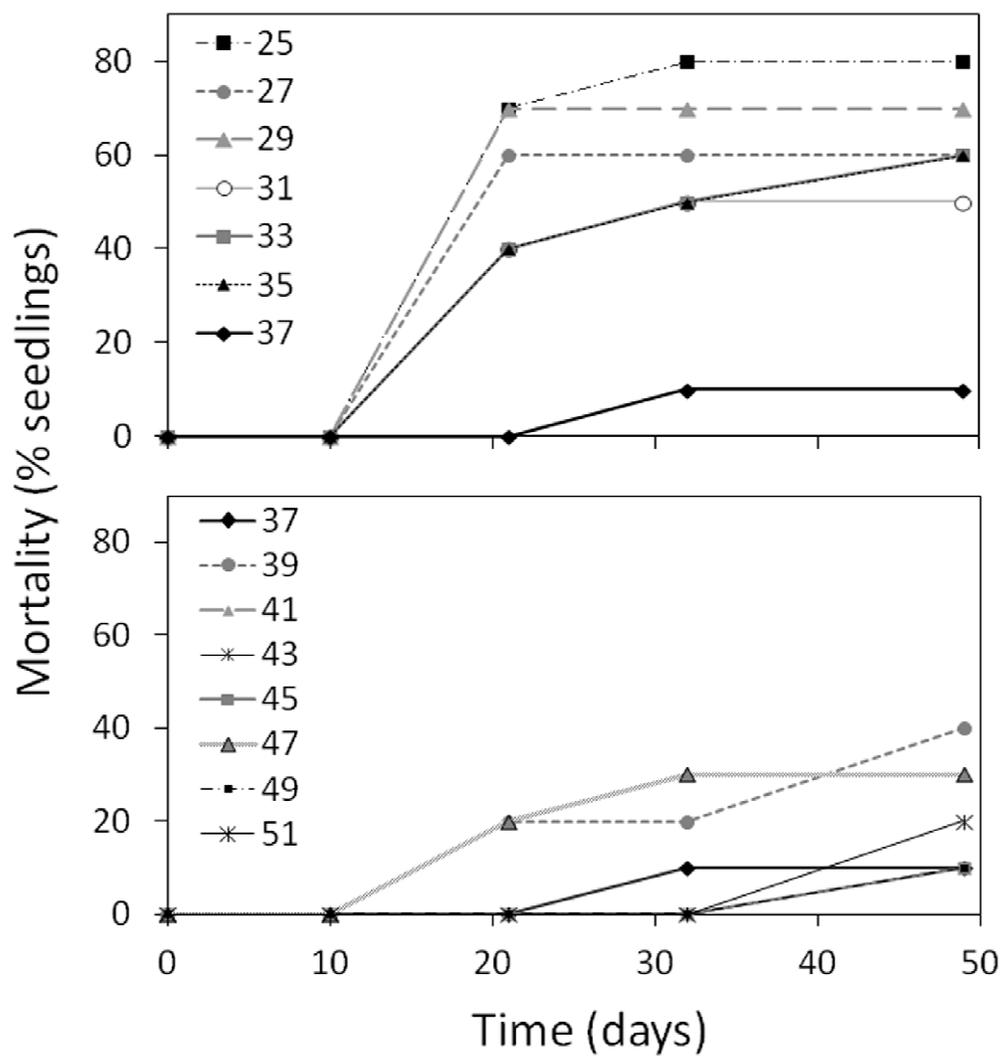
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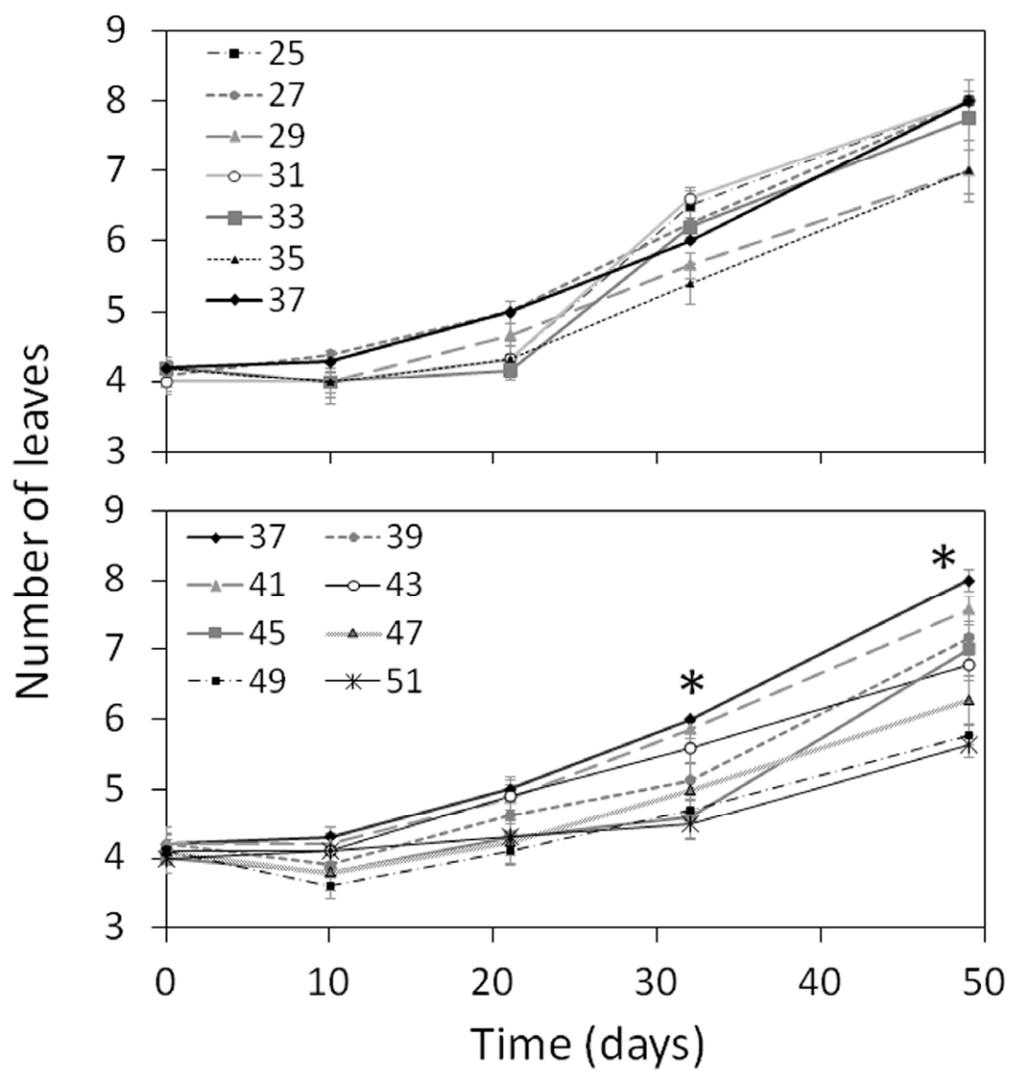
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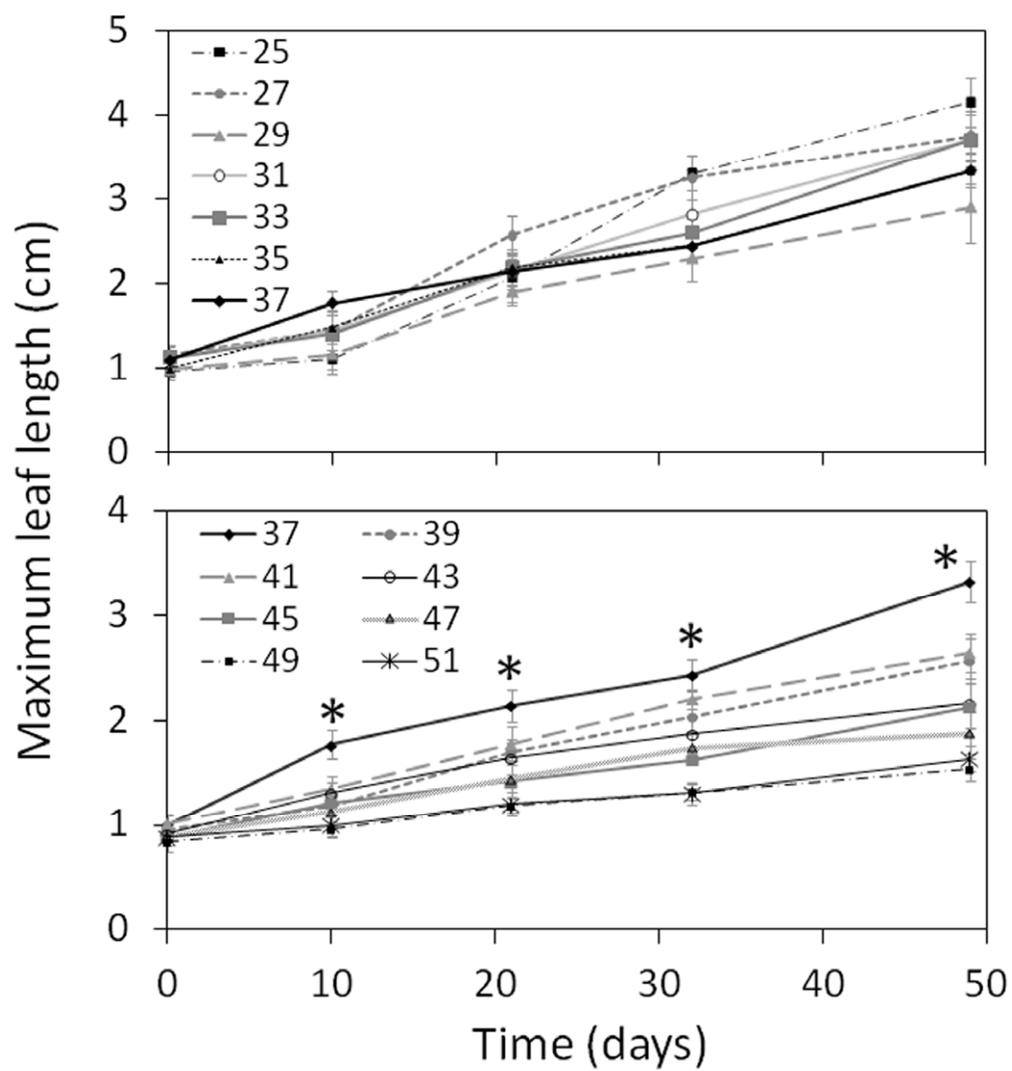
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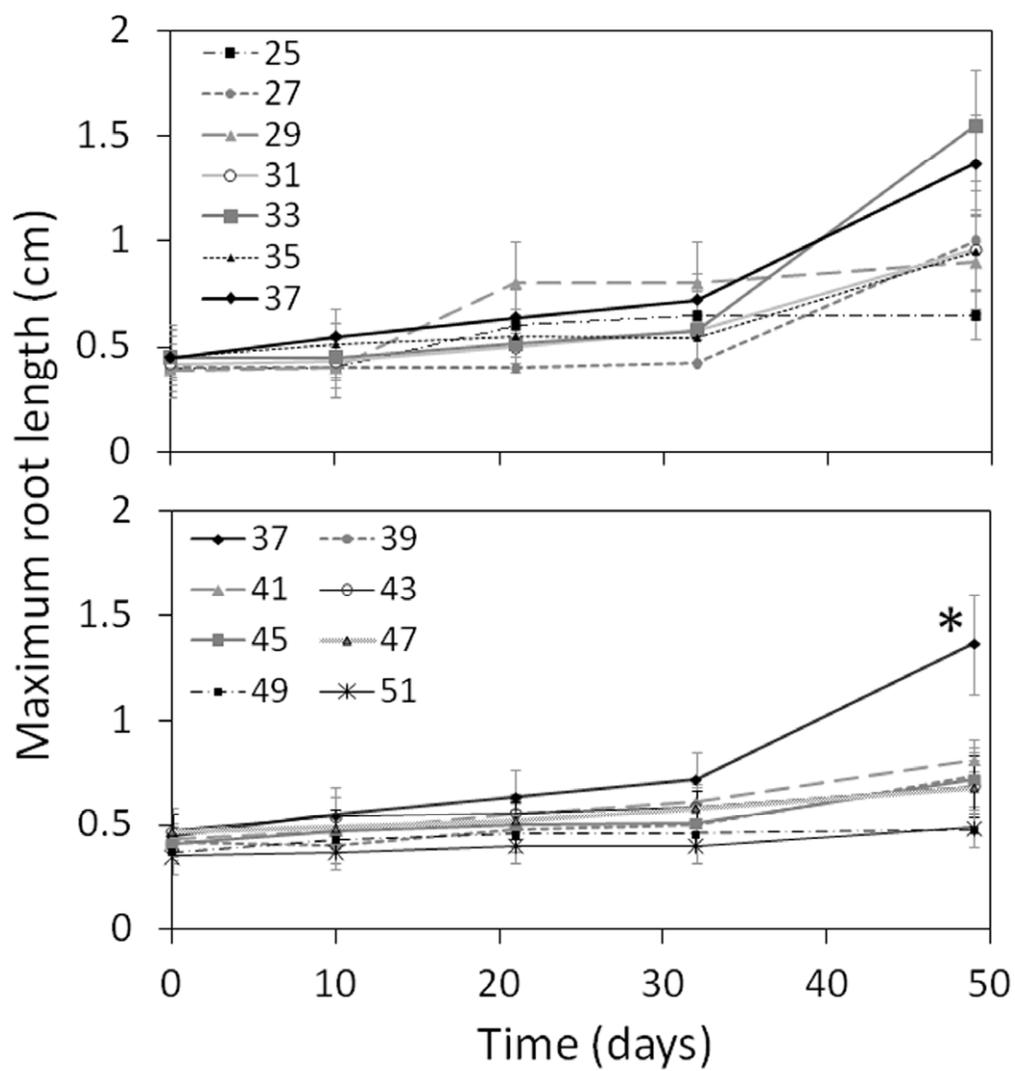


Table 1

Summary of the repeated measures ANOVA (A) and nested ANOVA (B) testing the effects of salinity treatments (37, 40, 43, 46 and 49) on *P. oceanica* seed germination and early seedling development parameters (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, P(Sal.) = 3 replicate Petri plates).

Parameter	Effect	dF	MS	F	p	
A)	Germination	Salinity	4	1274.10	1.80	ns
		Error	10	709.50		
		Time	3	1056.60	33.27	***
		Sal*T	12	44.30	1.40	ns
		Error	30	31.80		
	Mortality	Salinity	4	1811.97	2.80	ns
		Error	10	646.81		
		Time	3	2336.67	14.62	***
		Sal*T	12	313.94	1.96	ns
		Error	30	159.83		
B)	Number of leaves	Salinity	4	2.051	5.20	***
		P(Sal.)	2	0.509	1.29	ns
		Error	76	0.394		
	Maximum leaf length	Salinity	4	2.196	33.78	***
		P(Sal.)	2	0.172	2.64	ns
		Error	76	0.065		
	Maximum root length	Salinity	4	2.239	18.41	***
		P(Sal.)	2	0.337	2.772	ns
		Error	76	0.122		

Table 2

Summary of the repeated measures ANOVA testing the effects of salinity treatments at different times on seedling development parameters: number of leaves, maximum leaf length and primary root length (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Parameter	Effect	dF	MS	F	p
Number of leaves	Salinity	13	4.906	3.22	***
	Error	72	1.526		
	Time	4	114.755	475.77	***
	Sal*T	52	0.973	4.03	***
	Error	288	0.241		
Maximum leaf length	Salinity	13	7.064	8.31	***
	Error	72	0.850		***
	Time	4	29.900	433.79	***
	Sal*T	52	0.391	5.68	
	Error	288	0.069		
Maximum root length	Salinity	13	0.532	0.88	ns
	Error	72	0.6035		
	Time	4	1.766	49.35	***
	Sal*T	52	0.113	3.16	***
	Error	288	0.036		