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Variable response of three *Trifolium repens* ecotypes to soil flooding by seawater

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1 Abstract

Background and Aims Despite concerns about the impact of rising sea-levels and
 storm surge on coastal ecosystems, there is remarkably little information on the
 response of terrestrial coastal plant species to seawater inundation. Our aim was
 to elucidate responses of a glycophyte (*Trifolium repens*) to short duration soil
 flooding by seawater and recovery following leaching of salts.

Methods Using plants cultivated from parent ecotypes collected from a natural soil salinity gradient; we examined the impact of short-duration seawater soil flooding (8 or 24 hrs) on short-term changes in leaf salt ion and organic solute concentrations, and longer-term impacts on plant growth (stolon elongation), and flowering.

Results There was substantial Cl⁻ and Na⁺ accumulation in leaves, especially for
plants subjected to 24 h soil flooding with seawater, but no consistent variation
linked to parent plant provenance. Proline and sucrose concentrations also
increased in plants following seawater flooding of the soil. Plant growth and
flowering were reduced by longer soil immersion times (seawater flooding
followed by drainage and freshwater inputs), but plants originating from more
saline soil responded less negatively than those from lower salinity soil.

Conclusions The accumulation of proline and sucrose indicates a potential for
 solute accumulation as a response to the osmotic imbalance caused by salt ions,
 while variation in growth and flowering responses between ecotypes points to a
 natural adaptive capacity for tolerance of short-duration seawater soil flooding in
 T. repens. Consequently, we suggest that selection for tolerant ecotypes is

- possible should the predicted increase in storm surge flooding events frequencyunfold.
- 26 Key Words Flooding; Osmotic stress; Salinity; Saline soil waterlogging; Salt
- 27 ions; Sea-level rise; Stress metabolites; Storm surge; White Clover

29 Introduction

A combination of sea level rise and the increased likelihood of storm surge events 30 31 associated with anthropogenic climate change is likely to result in more frequent and 32 severe episodes of salt water inundation into low-lying coastal vegetation (Nicholls 33 and Cazenave, 2010; Martin et al., 2011; Zappa et al., 2013). Coastal habitats are 34 both economically and ecologically important. Not only do they provide protection 35 against the sea for urban areas and agro-ecosystems in-land (Hanley et al., 2014), 36 they offer refuge for many plant and animal species excluded by intensive 37 agriculture (Rhymer et al., 2010; Fisher et al., 2011). Consequently the ecological 38 response of these coastal habitats to seawater inundation may have important 39 ramifications not only for conservation, but also shoreline management.

40 The potential impact of storm surge events may be particularly acute for grazing 41 marsh, sand dunes and coastal pasture since unlike salt marshes, these ecosystems 42 are not naturally susceptible to the periodic intrusion of sea water. Salt stress and 43 inundation regimes are known to affect productivity and plant zonation in estuarine marsh/grassland transitions (Guo and Pennings, 2012; Janousek and Mayo, 2013), 44 45 and also influence plant survival, growth, and reproduction in freshwater 46 macrophytes (Van Zandt and Mopper, 2002; Van Zandt et al., 2003; Middleton, 47 2009; Pathikonda et al., 2010). However, our understanding of the impact of salt 48 stress on coastal mesophytes is limited to the effects of sea spray on cliff and 49 strandline vegetation (Malloch et al., 1985; de Vos et al., 2010; Rogers and Wiser, 50 2010). This contrasts markedly with a rich literature documenting salinity tolerance

in halophytes (Zhu, 2001; Flowers and Colmer, 2008; Wetson *et al.*, 2012) and the
impact of salinity on growth and yield of crop species (Munns and Tester, 2008;
Flowers *et al.*, 2010).

54 The tolerance of wetland plants to freshwater flooding has been studied extensively 55 (see reviews by Bailey-Serres and Voesenek, 2008; Colmer and Voesenek, 2009), but knowledge of plant response to seawater flooding is limited (Colmer and 56 57 Flowers, 2008; Bennett et al., 2009; Hanley et al., 2013) and for the most part 58 focusses on how increased tidal flooding affects halophytes in salt marsh systems 59 (e.g., Janousek and Mayo 2013; Valentim et al., 2013). In addition to impeded gas 60 exchange and chemically-reduced compounds in flooded soils (Armstrong, 1979), 61 flooding by seawater also imposes osmotic and ionic stresses; the osmotic stress 62 limits the plant's ability to absorb water and the ionic stress can result in toxicity through the accumulation of Na⁺ and Cl⁻ in tissues (Munns and Tester, 2008). In 63 64 order to cope with the challenge of salinity the plant must prevent or alleviate 65 damage caused by the high concentration of salt ions (Na⁺ and Cl⁻) and re-establish homeostatic function (e.g. for K^+). Salt tolerance is often achieved by the 66 67 accumulation of stress metabolites (e.g. compatible solutes) and the regulation of 68 tissue ions to exclude or compartmentalize the potentially damaging Na^+ and Cl^- , but 69 even if successfully achieved, this can impose a cost on plant growth (Munns and 70 Tester, 2008).

Any change in the frequency and severity of seawater flooding might therefore be
expected to influence greatly the vegetation of low-lying coastal ecosystems.
Nonetheless, our ability to predict the impact of anthropogenically-induced changes

74 in storm surge scenarios on coastal vegetation is limited by the paucity of 75 information on how component species respond to salt water inundation (Hoggart et 76 al., 2014; but see Redondo-Gómez et al., 2011; Hanley et al., 2013). Here we 77 investigate how one common and economically important coastal grassland species, 78 Trifolium repens L., responded to simulated short-term seawater inundation of the 79 soil. Although some coastal populations of this species have been shown to be 80 relatively tolerant of salinity, all previous work has exposed plants to a range of salt 81 concentrations imposed for several weeks (Ab-Skukor et al., 1988; Rogers et al., 82 1997), rather than looking at the effects of a simulated, short-duration seawater soil 83 flooding event and subsequent recovery. Given the lack of information on plant 84 response to salinity stress following storm surge events, our primary goal was to 85 elucidate how exposure to a relatively short pulse of seawater soil flooding affected 86 plant survival, immediate onward growth, and flowering. In addition, as plant 87 performance and response are likely influenced by the accumulation of salt ions and organic solutes (Munns and Tester, 2008; Flowers and Colmer, 2008), we 88 89 investigated post-immersion changes in the tissue concentrations of Na⁺, Cl⁻ and K⁺ 90 and organic solutes (sugars, sugar alcohols, and proline).

We also examined whether response to seawater immersion varied for plants cultivated from clonal fragments taken from a natural salinity gradient, since we hypothesised that likely natural adaptation for salt tolerance (Munns and Tester, 2008) would influence the response of *T. repens* plants to short-duration soil immersion in seawater. Environmental gradients across even small distances can facilitate the evolution of local ecotypes; i.e., genetically distinct populations

97	adapted to local environmental conditions (Turesson, 1922). Population-specific
98	variation in morphological or physiological responses to environmental factors such
99	as climate are well-known (Fernández-Pascual et al., 2013; Quilot-Turion et al.,
100	2013), but our understanding of ecotype-specific variation in response to salinity
101	stress is largely confined to halophytes (Huiskes et al., 1985; Blits and Gallagher,
102	1991 – but see Ab-Skukor et al., 1988; Van Zandt et al., 2003). Ours is the first
103	study to examine ecotypic variation in response to simulated seawater soil flooding
104	in a terrestrial, coastal glycophyte.

105 Materials and Methods

106 *Plant species, collection and cultivation*

107 Naturally distributed throughout Europe, North Africa and Asia, T. repens has been 108 widely introduced elsewhere as a pasture forage plant (Grime et al., 2007). It is also 109 a very common component of coastal grasslands and dune systems likely to be 110 affected by sea-level rise and storm-surge events. In June 2011, we collected parent 111 plants from grassland pasture at South Efford Marsh near Aveton Gifford, Devon, 112 England (50°18'14"N, 3°50'59"W). Plants were collected at locations approximately 113 25, 200, and 700 m away from the most southerly point of the site. At 25 m, seepage 114 under the sea wall favoured the development of a semi-halophytic community 115 (containing Puccinellia maritima and Spergularia media) as well as common 116 pasture species like T. repens and Agrostis stolonifera. At 200 m the plant 117 community was more typical of a terrestrial pasture (dominated by A. stolonifera and Ranunculus repens), and at 700 m, the sward was dominated by similar species, 118

but also including common pasture grasses such as *Holcus lanatus* and *Cyanosurus cristatus*. The variation in the plant community with distance from the sea wall suggested a salinity gradient (confirmed by electrical conductivity measurements of the soil - described below), with which to test the hypothesis that natural adaptation for salt tolerance would influence plant response to short-duration soil immersion in seawater.

125 In mid-June 2011 we sampled 12 individual plants per distance, collecting large, 126 branched (circa 100 mm diameter) fragments with multiple rooting points. All 127 parent plants were at least 5 m apart and taken from distinct patches to reduce the 128 likelihood of collecting from the same individual (Ab-Shukor et al., 1988). The 129 plant fragments were transplanted into $110 \times 110 \times 120$ mm plastic pots containing 130 John Innes No. 2 potting compost and cultivated in a sheltered outdoor area. Three 131 soil cores (10 cm depth, 5 cm diameter) per distance were also collected at random 132 intervals to quantify soil electrical conductivity. Three 20 g subsamples were taken 133 from each core and mixed with 100 ml deionised water on a rotational mixer to 134 provide a 1:5 soil:water extract (British Standards Institution, 1997). An electrical 135 conductivity reading was then obtained for each subsample using a WTW Cond 330i 136 handheld conductivity meter with a WTW TetraCon 325 probe (Wissenschaftlich-137 Technische Werkstätten GmbH, Weilheim, Germany). One-way ANOVA was used 138 to determine variation in soil electrical conductivity from these samples.

139 *Experimental treatments*

In November 2011 we removed 10 stolons (about 20 mm long with 1 root node) from each of 12 parent 'stock' plants per distance group and individually transplanted these into 70 x 70 x 80 mm pots containing John Innes No. 2 potting compost. Plants were subsequently grown in a naturally lit, heated greenhouse in Plymouth, England (mean daily temperatures varying between 10.7 °C (\pm 0.2) min and 26.3 °C (\pm 0.6) max, with daily watering with tap water), until late April 2012.

On April 24th 2012, established ramets were selected such that each of 12 parent 146 147 'stock' plants was represented by up to 6 clones, each uniform in size and 148 appearance. Clones were allocated to a different treatment group such that we aimed 149 to subject two ramets from each parent 'stock' plant to the same soil immersion 150 treatment with seawater collected from Plymouth Sound (electrical conductivity = 151 47.9 mS/cm at 23.8 °C) for 8, or 24 h, or were retained as untreated controls. This 152 arrangement ensured that genetic variation across all soil immersion treatments was 153 minimised within all distance classes. However, due to some plants failing to 154 establish, replication fell below the target of 6 clones per parent for the 200-m and 155 700-m distance groups, affecting the number of replicates used in the onward 156 growth and flowering experiment (see below). By immersing to pot-level (in large 157 plastic tubs) we simulated short-term soil waterlogging. Although we recognise that 158 seawater inundation following a storm-surge event would potentially also result in 159 shoot submergence, our approach allowed us to focus on the effect of ionic 160 imbalance in the root-zone rather than the combined effects that could also arise if 161 shoots experienced oxygen deficiency by submergence. In addition, while seawater 162 flooding following storm surge events might also be expected to persist longer than

163 24 h, records from along the UK coast suggest a 1-d long seawater flooding event is164 typical (Environment Agency 2014).

165 Immediately after immersion in seawater, pots were allowed to fully drain before 166 being arranged randomly on a wire mesh-topped bench inside the greenhouse; the 167 wire mesh allowed free drainage and prevented cross-contamination of any leachates 168 between pots.

169 *Tissue ion and metabolite analyses*

One fully expanded, non-senescent leaf was removed from each of 12 individual clones per ecotype per immersion treatment 2 d after soil immersion. These samples were frozen in liquid nitrogen and freeze-dried. Samples were then stored in sealed containers with desiccant in a freezer at -80 °C until analysis. These plants were then discarded.

Dried samples were digested in 5 mL dilute (0.5 M) nitric acid while suspended on a shaker in dark conditions for 2-d at room temperature prior to determination by flame photometry of Na⁺ and K⁺ and by chloridometry of Cl⁻ (Munns et al., 2010). This procedure was applied to 4 replicate leaf samples per soil immersion treatment/ecotype group (minimum sample mass = 26.7 mg). A reference tissue taken through the procedures confirmed the reliability of these measurements.

181 The remaining leaf samples were bulked (by adding two samples together) such that 182 4 replicate samples were created for each soil immersion treatment/ecotype group 183 (minimum sample mass = 54.3 mg). Tissue metabolites were extracted from these

184 using 5% (w/v) perchloric acid and neutralised extracts were analysed using HPLC 185 (Fan et al. 1993). Neutralised extracts were filtered (0.22 µm) and stored at -80 °C. 186 The initial HPLC analysis of organic solutes (glycinebetaine, proline, prolinebetaine 187 and trigonelline), soluble sugars (fructose, glucose, sucrose) and sugar alcohols 188 (sorbitol, mannitol and pinitol) was adapted from Slimestad and Vågen (2006). The 189 HPLC system (Waters, Milford, MA) consisted of a 600E pump, 717plus 190 autosampler and a 996 photo-diode array detector (PDA). As detection of fructose, 191 glucose and sucrose with the PDA at 195 nm is very insensitive, an Alltech 192 (Deerfield, IL, USA) evaporative light scattering detector (ELSD) was also used to 193 improve sensitivity by minimum 100 fold. Separation was achieved at 22 ± 1.0 °C 194 on a Prevail ES Carbohydrate column (250 x 4.6 mm i.d. with 5 µm packing; 195 Alltech) using a gradient elution profile of acetonitrile (Eluent A) and water (Eluent B) at 1 ml min⁻¹. Samples in the autoinjector were held at 10 °C, the ELSD drift tube 196 197 held at 85 °C and eluent nebulisation with high purity N₂ gas at a flow rate of 2.61 \min^{-1} . 198

Quantification was based on PDA peak area for organic solutes, and ELSD peak area for soluble sugars and sugar alcohols. Calibration curves were generated from peak area versus the mass of standard analyte injected, with linear relationship for the PDA and a power relationship for the ELSD output. A standard was analysed every 10 samples to check for any instrument/detector drift. Retention times of standards were used to identify analytes in the sample extracts with the PDA spectral data and peak purity used to confirm organic solutes. Typical sample

injections were 20 μ l and runtime was 20 min per sample with EmpowerTM 2 software (Waters) used for data acquisition and processing.

Due to the fact that pinitol co-eluted with fructose, a second HPLC approach was needed to separate and quantify these two analytes and the method described by Naidu (1998) was followed. The Sugar-Pak column ($300 \times 6.5 \text{ mm i.d.}$) was held at $90 \pm 0.5 \text{ °C}$ and separation achieved using a mobile phase of 2.5 mg l⁻¹ Ca-EDTA at 0.6 ml min⁻¹. Detection and quantification of pinitol was undertaken with the PDA as this offered good sensitivity as well as peak spectral and purity comparisons with the standards.

Following application of the Cochran test for homogeneity of variance, and (log_N) data transformation where necessary (Underwood, 1997), the effect of immersion on tissue ion and solute concentrations was examined using a two-way ANOVA with 'Immersion Time' and 'Distance' as factors, with the tests for an 'Immersion Time' \times 'Distance' interaction included to examine any evidence for distance-specific variation in response to immersion duration.

221 Plant survival, growth and flowering

Plants not used for chemical analysis were watered to capacity 48 h after salt water immersion (using tap water), and then every two days thereafter for a further 70 d (5th July 2012). Greenhouse air temperatures during this phase of the experiment were: 11.3 °C (\pm 0.3) min and 29.9 °C (\pm 0.9) max. Due to some clones failing to establish before immersion, replication fell below the target of 12 plants for the 200-

227 m (0-hr = 9 plants, 8-hr = 9 plants, 24-hr = 8 plants) and 700-m (24-hr = 11 plants) 228 distance groups.

The number of inflorescences on each plant were monitored weekly, so that the total number of inflorescences produced per plant ('Flowering Effort') and the percentage of plants that produced open inflorescences ('Percentage Flowering') could be quantified at the end of the experiment. Elongation of a representative, randomly selected shoot on each plant, measured from a node marked with loosely tied cotton thread, was quantified at 14 days and 42 days ('Stolon Growth'). Plant mortality was recorded daily.

236 Subsequently, the effects of 'Immersion Duration' and 'Distance' on 'Flowering 237 Effort' were examined using a two-way ANOVA with 'Immersion Duration' and 238 'Distance' as factors, with the tests for an 'Immersion Duration' \times 'Distance' 239 interaction included to examine any evidence for distance-specific variation in 240 response to immersion duration. The effects of 'Immersion Duration' and 'Distance' 241 on 'Stolon Growth' at 14 d were analysed in the same way. Data transformation of 242 the 42 d 'Stolon Growth' data could not avoid deviations from normality and 243 homogeneity of variance; therefore Kruskal Wallis tests were applied. Pearson's chi-244 squared test was performed on 'Percentage Flowering' data to assess the effects of 245 immersion within 'Distance' groups, where assumptions of the statistical test were 246 met.

In order to quantify the leaching of salts from the potting compost, four pots were randomly chosen from each soil immersion treatment and water passing through collected and electrical conductivity measured as described above for 5 weeks.

250 **Results**

251 *Electrical conductivity of soil samples from transects*

252 One-way ANOVA indicated significant variation in soil electrical conductivity (of 253 1:5 soil:water extract, EC_{1:5}) between sample sites ($F_{(2,27)} = 39.61$, P <0.01). Post-254 hoc SNK comparisons revealed that soil electrical conductivity declined with 255 distance from the sea wall embankment, with a mean EC_{1:5} of 6.96 (\pm 1.35 SE) 256 mS/cm at 25 m, 1.29 (\pm 0.40 SE) mS/cm at 200 m and 0.19 (\pm 0.02 SE) mS/cm at 257 700 m. We therefore conclude that soil salt concentrations were higher at 25 m due 258 to likely sea water seepage through the sea wall and/or deposition of salts from sea 259 spray.

260 Accumulation in leaves of salt ions and stress metabolites

There was substantial accumulation of Cl⁻ and Na⁺ in leaves following soil immersion in sea water (Table 1). Post-hoc SNK tests showed that leaf tissue Cl⁻ concentrations significantly increased following 24 h sea water immersion compared with both control and 8 h treatments, irrespective of original parental distance from the sea wall. There was also a significant increase in Cl⁻ concentrations in the 8 h treatments compared with control plants. A similar effect of 'Immersion Time' was evident for Na⁺ although there were also additional 'Distance' and 'Immersion

Time' × 'Distance' effects. However, SNK tests indicated that these were a manifestation of relatively high leaf Na⁺ levels in 200 m control plants, and generally relatively low leaf Na⁺ levels in 25 m plants following soil immersion. There was no effect of 'Immersion Time' on K⁺ concentrations; the significant 'Distance' effect denoted the inherently higher leaf K⁺ concentrations in plants cultivated from the 200 m parent population.

We found detectable amounts of the amino acid proline, the sugars fructose, glucose and sucrose, and the sugar alcohol pinitol, in leaf extracts (Table 2). Of these, proline and sucrose increased in all distance groups following soil immersion, but no other treatment-specific trends were located for organic solutes.

278 *Plant survival, growth, and flowering*

Water collected from control plants remained at an average electrical conductivity (EC_w) of 2.37 ± 0.03 mS/cm. Leachates from pots immersed for 8 and 24 h recorded similar levels of EC_w 2 d post-immersion (12.53 ± 3.28 mS/cm and 11.05 ± 5.61 mS/cm respectively) and declined at a similar rate until 35 d. At this point, stabilisation of EC_w occurred in both treatments (Fig.1).

Only four plants died during the experiment, these were all in the 24 h sea water soil immersion treatment; three from the 700 m ecotype and one from the 200 m ecotype. All plants from the 25 m ecotype survived 24 h soil immersion. At 14 d after the short-durations of soil flooding, 'Stolon Growth' was greatly reduced by 'Immersion' for all distance groups ($F_{(2,91)} = 50.25$, *P* <0.01), with post-hoc SNK tests indicating the greatest reduction after 24 h soil immersion treatment (*P* < 0.01)

(Fig. 2a). 'Distance' also had a significant effect on 'Stolon Growth' ($F_{(2,91)} = 3.40$, 290 291 P < 0.05; likely due to a generally greater stem growth of 200 m plants and much 292 reduced growth of 700 m plants following soil immersion. There was no interaction 293 between 'Immersion' and 'Distance' ($F_{(4.89)} = 1.78$, P > 0.05). The loss of cotton 294 thread markers used to identify stolons reduced effective replicate number (see 295 Figure 2b) at 42 d, nevertheless the 'Immersion' effect remained apparent ($H_{(2)}$ = 296 35.22, P < 0.01), but 'Distance' had no significant effect on 'Stolon Growth', as average growth of 25 m plants increased to similar rates as 200 m ($H_{(2)} = 2.15$, P > 2.15297 298 0.05). However, average stolon growth of 700 m plants appeared to be much 299 reduced even at 42 d after the longer duration (24 h) of soil immersion (Fig. 2b).

300 While both 'Immersion' ($F_{(2,92)} = 14.18$, *P* <0.01), and 'Distance' ($F_{(2,92)} = 6.12$, *P* 301 <0.01) had a significant effect on 'Flowering Effort' (Fig. 3), there was no 302 interaction between 'Immersion' and 'Distance' ($F_{(4,90)} = 0.69$, P > 0.05). Post-hoc 303 SNK tests revealed that the mean number of inflorescences per plant ('Flowering 304 Effort') produced by the end of the experiment were reduced in both the 8 and 24 h 305 soil immersion treatments (P < 0.01). The significant 'Distance' effect could be 306 explained by greater number of inflorescences produced by the 200 m and 700 m 307 control plants compared with the 25 m control plants, suggesting that these 308 populations naturally produce more flowers. However, 'Flowering Effort' in the 200 309 m and 700 m plants was nonetheless greatly reduced by 24 h soil immersion. Similar 310 patterns were observed for 'Percentage Flowering' (Fig. 4); 700 m plants produced 311 more open flowers than plants in the groups collected at 200 m and 25 m. Pearson's 312 chi-squared test revealed that 'Percentage Flowering' in the 25-m plants was

reduced by soil immersion ($X^{2}_{(36,2)} = 16.94$, P < 0.01). Although the same test could not be performed on 200 m and 700 m plants (over 50% of cells in the test matrix had expected values of less than 5), there was a trend for reduced flowering in both these distance groups following soil immersion.

317 **Discussion**

318 Our experiment to simulate the effects of transient saline soil waterlogging yielded 319 consistent ecophysiological and plant performance responses; two days after soil 320 immersion, all plants exhibited higher leaf concentrations of Na⁺ and Cl⁻, 321 concentrations of some organic solutes significantly changed (e.g. sucrose and 322 proline increased), and longer-term plant growth and flowering potential were 323 reduced. The fact that growth and flowering responses also varied according to the 324 location of the parent plants along a natural salinity gradient, suggests that our study 325 plants were displaying natural ecotypic variation to the short-duration salinity stress.

326 The accumulation of Na⁺ and Cl⁻ in leaf tissues is commonly observed in salt-327 stressed plants which in-turn leads to the accumulation of organic solutes to counter 328 the resulting osmotic imbalance (Wyn Jones and Gorham, 2002; Flowers and 329 Colmer, 2008). It was interesting to note, however, that despite having different 330 growth and flowering responses to seawater soil immersion, the accumulation of 331 Na⁺ and Cl⁻ in leaf tissues did not vary for plants from different locations along the natural salinity gradient. Unlike Rogers et al. (1997) who documented that tolerance 332 333 of T. repens to longer exposures to salinity was associated with 'exclusion' of Cl from the shoot, the apparent salinity tolerance of plants we collected from the 25 m 334

335 population could not be linked to differences in leaf ion concentrations (the trend in 336 the present study for lower leaf Na^+ and Cl^- in ecotypes collected from closer to the 337 sea wall was not statistically significant - Table 1). Moreover, in addition to 338 regulation of leaf ion concentrations, other traits such as differences in "tissue 339 tolerance" can also influence overall plant salinity tolerance (Munns and Tester, 2008). Maintenance of tissue K^+ concentration, which can decrease in plants 340 341 exposed to excess Na^+ , to retain a favourable K^+/Na^+ ratio of the cytoplasm for 342 enzyme functioning, is also of importance for salt tolerance (Maathuis and 343 Amtmann, 1999), but for the short-term salinity exposures in the present experiment 344 on T. repens there was no significant treatment effect on leaf K^+ (Table 1). In 345 addition to regulation of leaf ion concentrations, organic solutes that are compatible 346 with enzymes and therefore accumulated in the cytoplasm are also of importance for 347 adaptation to salinity (Munns and Tester, 2008; Flowers and Colmer, 2008). Organic 348 solutes in plants can include various sugars, sugar alcohols, betaines, and amino 349 acids, many of which accumulate with increasing plant exposure to NaCl, but the 350 types and concentrations vary in different species (Flowers and Colmer, 2008). T. 351 repens leaves contained constitutively high concentrations of pinitol, whereas 352 proline and sucrose concentrations rose dramatically in the salt-stressed plants 353 (Table 2). The increased organic solute accumulation is likely a response to provide the osmotic balance needed in the cytoplasm for Na⁺ and Cl⁻ accumulation in the 354 355 vacuole.

The reduction in plant growth reported here mirrors that of plants in salinized agricultural systems (Bennett *et al.*, 2009; Flowers *et al.*, 2010). Simulated seawater

358 soil flooding not only reduced vegetative growth, measured here for T. repens as 359 stolon growth, but had the added effect of causing reduced flowering potential. 360 Exposure of the roots to seawater is expected to exert a negative effect on plant 361 growth and reproductive allocation via ionic and osmotic stresses; salt stress impacts 362 on cell division and photosynthesis as an osmotic imbalance leads to stomatal 363 closure and reduced CO_2 uptake and so less photosynthesis (Zhu, 2001). Therefore, 364 greater stress will be exerted on plants enduring longer root immersion as Na⁺ and 365 Cl⁻ ions have more time to accumulate in leaf tissues (as was observed here for all 366 plants subject to 24 h soil immersion when compared to the 8 h treatment). This is 367 reflected by the greatest reduction in stolon growth and flowering potential of T. 368 repens occurring as a result of 24 h seawater soil immersion despite the fact that salt 369 levels in the growing media were roughly comparable for 8 and 24 h treatments after 370 removal from seawater and subsequent leaching due to watering.

371 Growth and flowering responses to seawater soil immersion also varied according to 372 the location of the parent plant along a natural salinity gradient. After 24 h of 373 seawater soil immersion, stolon growth of 700 m plants was severely limited, while 374 25 m and 200 m plants were less affected and seemed to return to similar rates of 375 growth by 42 d. Similarly, although 'Flowering Effort' and the percentage of plants 376 with flowering inflorescences were reduced for all distances, the effects were 377 particularly marked for 700 m plants. Ecotype-specific variation in flowering as a 378 consequence of long-term salinity exposure is well known for halophytes like Aster 379 tripolium (Gray et al., 1979) and Sporobolus virginicus (Blits and Gallagher, 1991), 380 but ours is this first study to demonstrate this effect for a glycophyte from a natural

salinity gradient when exposed to short-duration seawater soil flooding. Our results indicate that the 25 m- and to some degree the 200 m-ecotype, were resilient to transient seawater soil immersion presumably as a consequence of a local population adaptation to the higher soil salinity near to the sea wall embankment. Interestingly, a trade-off between salinity tolerance and reproductive allocation might be evident, as flowering potential in the 25 m ecotype was lower than in the 700 m population.

387 Plant adaptation to salinity has been shown for species in many hypersaline semi-388 arid/arid areas (Munns and Tester, 2008), but such local adaptation also occurs in 389 coastal plant populations exposed to salt spray and long-term accumulation of salt 390 ions in the soil (Lowry et al., 2009), including T. repens (Ab-Shukor et al., 1988; 391 Rogers et al., 1997). Our study is considerably different, however, in that we applied 392 a one-off, short-term, salinity pulse via soil flooding in an attempt to mirror seawater 393 inundation in a storm surge event. Consequently, our plants experienced an 394 immediate salinity shock followed by a gradual reduction of salt ions around the 395 roots as freshwater diluted and leached salt from the potting media. In flooded 396 ecosystems, the dissipation of salt ions over time may also allow plants to recover 397 from the initial impact of ionic and osmotic stress, an effect not only likely to vary 398 between plant species and ecotypes, but also with season and local weather 399 conditions. The effects of seasonality and species identity remain to be elucidated, 400 but we demonstrate here population-level variation in plant response to seawater soil 401 flooding. Although ecotype-specific response to salinity stress has been 402 demonstrated at the regional scale for T. repens (Ab-Shukor et al., 1988), our study 403 evidences the presence of different salinity-tolerant ecotypes over relatively small distances within a single coastal site. If salinity-tolerant ecotypes are a widelydistributed feature of coastal plant species, supra-littoral vegetation may be naturally buffered to some extent against predicted sea-level rise and increased incidence of storm-surge events over coming decades. The existence of flooding-tolerant ecotypes may also be important in developing salt-tolerant cultivars for use in saline soils (Ab-Shukor *et al.*, 1988; Rogers *et al.*, 1997; Bennett *et al.*, 2009), and any coastal pastures susceptible to future episodes of seawater flooding.

411 Given the increased likelihood of storm surge events over coming decades (Martin 412 et al., 2011; Zappa et al., 2013), the international conservation importance of low-413 lying coastal vegetation and the role these areas play in coastal defence (Rhymer et 414 al., 2010; Fisher et al., 2011; Hanley et al., 2014), a more detailed understanding of 415 the structural and functional responses of coastal vegetation to periodic seawater 416 flooding is particularly pressing. We demonstrate here that one common plant 417 species component of many coastal grasslands and dune systems, T. repens, 418 responds poorly to simulated seawater soil flooding, but that the response may be 419 population-, i.e. ecotype-, specific and that the species consequently has an adaptive 420 capacity to withstand short periods of soil inundation by seawater. Whether the 421 likely increased selection pressure for plants resistant to seawater flooding impacts 422 on other plant traits (e.g. growth, N-fixing capacity, anti-herbivore defence, 423 reproductive potential) and thus the ecological role and economic value of this 424 species is worthy of future attention. In addition, the impact of short-duration 425 seawater flooding on multi-species mixtures and subsequent community assembly 426 and function could yield many useful insights into the likely responses of coastal

- 427 vegetation to rising sea-levels and the anticipated increased frequency and severity
- 428 of saline flooding events.

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Distance	Treatment	N	Chloride (µmol g ⁻¹ DM)		Potassium (µmol g⁻¹ DM)		Sodium (µmol g⁻¹ DM)	
			Mean	SE	Mean	SE	Mean	SE
	Control	4	134 ^A	12	476	41	216 ^A	18 3
25 m	8 h	4	966 ^B	47	383	82	663 ^B	46 4
	24 h	4	1261 ^c	233	502	81	1049 ^c	129_
	Control	4	138 ^A	16	797	30	273 ^A	29 ⁵
200 m	8 h	4	1025 ^B	77	712	112	929 ^B	34 ₆
	24 h	4	1598 ^c	67	693	76	1126 ^c	30
	Control	4	110 ^A	17	466	66	202 ^A	28 /
700 m	8 h	4	1047 ^B	36	457	66	814 ^B	39 ₈
	24 h	4	1805 ^C	59	518	20	1454 ^C	49
	Factor		F	Р	F	Р	F	Р
Results of	Distance (DF = 2,27)		1.1	0.358	14.9	0.000	5.0	0.014
ANOVA	Immersion time (DF =	471.0	0.000	0.7	0.498	289.6	0.0 00	
	Interaction (DF = 4,27)		2.3	0.084	0.5	0.753	3.2	0.027 13

Table 1: Ion concentrations of leaf tissues taken from *Trifolium repens* plants 2-d after root-zone immersion in seawater. Significant

differences (post hoc S-N-K tests, P < 0.05) between treatment means are denoted by different letters following two-way ANOVA

showing how immersion time (0, 8 or 24 h) and parent plant location along a natural salinity gradient (25 m most saline, 700 m least

saline; described in 'Results' section) affected accumulation of Cl^{-} , Na^{+} and K^{+} .

Distance	Treatment	N	Proline (µmol g⁻¹ DM)		Fructose (µmol g ⁻¹ DM)		Glucose (µmol g ⁻¹ DM)		Sucrose (µmol g ⁻¹ DM)		Pinitol (µmol g ⁻¹ DM)	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
	Control	4	ND	-	84	11	57	5	9 ^A	2	166	31
25 m	8 h	4	14 ^{AB}	8	74	16	75	12	155 ^B	19	138	18
	24 h	4	28 ^B	10	85	42	74	16	148 ^B	32	125	16
	Control	4	ND	-	42	5	36	3	14 ^A	2	160	28
200 m	8 h	4	16 ^в	3	68	12	63	8	54 ^B	10	148	13
	24 h	4	45 ^C	5	30	9	45	6	61 ^B	9	163	8
	Control	4	ND	-	77	33	55	15	21 ^A	7	137	11
700 m	8 h	4	22 ^B	4	61	18	57	6	142 ^B	15	131	9
	24 h	4	55 ^C	8	45	22	58	8	107 ^в	18	118	16
Results	Factor		F	Р	F	Р	F	Р	F	Р	F	Р
of two-	Distance ((DF = 2,27)	3.3	0.054	1.9	0.174	3.4	0.047	13.8	0.001	1.8	0.183
way	Immersion time	(DF = 2,27)	44.0	0.000	0.4	0.649	1.9	0.171	73.9	0.000	0.9	0.436
ANOVA		(DF = 4,27)	1.6	0.198	0.5	0.735	0.6	0.697	4.8	0.005	0.4	0.790

1

2 **Table 2:** Organic solute concentrations of leaf tissue taken from *Trifolium repens* plants 2-d after root-zone immersion in seawater.

3 Significant differences (post hoc S-N-K tests, P < 0.05) between treatment means for each solute are denoted by different letters

4 following two-way ANOVA showing how immersion time (0, 8 or 24 h) and parent plant location along a natural salinity gradient

- 1 (25 m most saline, 700 m least saline; described in 'Results' section) affected solute accumulation. ND denotes failure to detect any
- 2 quantity above the detection limit, which were: proline (12 μ mol g⁻¹ DM), fructose (14 μ mol g⁻¹ DM), glucose (20 μ mol g⁻¹ DM),
- 3 sucrose (6 μ mol g⁻¹ DM), and pinitol (36 μ mol g⁻¹ DM).

1 Figure Legends

2 Figure 1: Average electrical conductivity (mS cm⁻¹) of water passing through pots (i.e. of collected leachates following watering)

3 after sea water soil immersion for the durations of 0 (control), 8 and 24 h.

4 Figure 2: Mean stolon extension (mm ± SE) of *Trifolium repens* clones cultivated from parent plants from different locations along a

5 natural salinity gradient (25 m is most saline, 700 m least saline; described in 'Results' section), (a) 14 d and (b) 42 d after the root-

6 zones of plants were immersed in sea water.

Figure 3: The effects of immersion time on the mean (\pm SE) number of inflorescences ('Flowering Effort') produced by *Trifolium*

8 repens clones cultivated from parent plants from different locations along a natural salinity gradient (25 m is most saline, 700 m least

9 saline; described in 'Results' section) 70 d after root-zone immersion in sea water.

10 Figure 4: The effects of immersion time on the proportion of *Trifolium repens* plants that had flowered by 70 d after root-zone

- 11 immersion in sea water. Clonal fragments were cultivated from parents from different locations along a natural salinity gradient (25
- 12 m is most saline, 700 m least saline; described in 'Results' section).















