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Macroalgal spore dysfunction: ocean acidification delays and weakens adhesion

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List of abbreviations: FHL, Friday Harbor Laboratories; OAEL, Ocean Acidification Environmental Laboratory

Early life stages of marine organisms are predicted to be vulnerable to ocean acidification. For macroalgae, reproduction and population persistence rely on spores to settle, adhere and continue the algal life cycle, yet the effect of ocean acidification on this critical life stage has been largely overlooked. We explicitly tested the biomechanical impact of reduced pH on early spore adhesion. We developed a shear flume to examine the effect of reduced pH on spore attachment time and strength in two intertidal rhodophyte macroalgae, one calcified (*Corallina vancouveriensis*) and one non-calcified (*Polyostea robusta*). Reduced pH delayed spore attachment of both species by 40-52% and weakened attachment strength in *C. vancouveriensis*, causing spores to dislodge at lower flow-induced shear forces, but had no effect on the attachment strength of *P. robusta*. Results are consistent with our prediction that reduced pH disrupts proper curing and gel formation of spore adhesives (anionic polysaccharides and glycoproteins) via protonation and cation displacement, although experimental verification is needed. Our results demonstrate that ocean acidification negatively, and differentially, impacts spore adhesion in two macroalgae. If results hold in field conditions, reduced ocean pH has the potential to impact macroalgal communities via spore dysfunction, regardless of the physiological tolerance of mature thalli.

The establishment and persistence of benthic macroalgal communities depend upon the successful settlement and attachment of algal spores (Chamberlain 1976, Santelices 1990, Steinhoff et al. 2011, Padilla-Gamiño et al. 2016), yet the impact of environmental perturbation on spore attachment is poorly understood (but see Fletcher and Callow 1992, Taylor et al. 2010). In the intertidal zone of rocky shores, spores must contend not only with hydrodynamic forces imposed by breaking waves, but also with environmental stresses, such

as increased temperature, desiccation and, perhaps, seawater pH. Thus, initial survival of algal spores (and thus algal thalli) depends upon the speed and strength of spore attachment.

Settled spores must resist shear forces imposed by a gradient in water velocity within the boundary layer to avoid being dislodged (Vogel 2003). To aid in attachment, spores are initially released from sporangia enveloped by mucilage composed of acidic sulphated polysaccharides (Chamberlain and Evans 1973, Boney 1975, Chamberlain 1976, Boney 1981, Fletcher and Callow 1992). This mucilage initially adheres spores to the substrate, and the strength of this mucilage determines attachment time (Boney 1975, 1981). Once attached, spores discharge a second type of adhesive mucilage, composed mainly of glycoproteins (Chamberlain and Evans 1973, Pueschel 1979), which spreads out to form a pad, permanently securing spores to the substrate (Chamberlain 1976, Fletcher and Callow 1992). The adhesive strength of this secondary mucilage increases through time (Charters et al. 1973, Chamberlain 1976), likely due to increasing ionic cross-linkages of the adhesive (Jones et al. 1982). In general, cross-linking is required for the proper formation of marine hydrogels, including spore mucilages, and shifts in ocean pH may affect ionic linkages (Verdugo et al. 2004, Lee and Mooney 2012, Li et al. 2013). Reduced pH can cause carboxylate and sulphate groups on anionic polysaccharides to become protonated (Lee and Mooney 2012, Li et al. 2013), weakening hydrogen bonds with water, displacing stabilizing cations (e.g., Ca^{2+}, K^{+}, Na^{+}), and potentially causing polymers to collapse and entangle (Bu et al. 2005). In low pH conditions, gel viscosity generally increases (Bu et al. 2005, Lee and Mooney 2012), making stiffer, more compact gels, which may also be less deformable (Picone et al. 2011). Although the chemical composition (Boney 1981, Fletcher and Callow 1992), curing rate (Moorjani and Jones 1972), and production rate (Gunn et al. 1984) of spore mucilages vary across taxa and are often poorly understood, given the generalized response of anionic hydrogels to reduced pH, we hypothesized that spore adhesion may be affected by ocean acidification.

Sporophytic specimens of macroalgae *Polyostea robusta* (formerly *Pterosiphonia bipinnata*) and *Corallina vancouveriensis* were collected from the intertidal zone on San Juan Island, WA (Deadman Bay: 48°30'48.93" N, 123°8'56.14" W), immediately transported to Friday Harbor Laboratories (FHL), and maintained in an outdoor seawater table before testing. Reproductive parent fronds were held at ambient levels of seawater pH and were exposed to experimental conditions while releasing spores. Spore release was performed in the Ocean Acidification Environmental Laboratory (OAEL) at FHL, allowing precise control of pH and temperature in a flow-through system (O'Donnell et al. 2013). Two pH treatments were established by bubbling CO_2 (7.75, 7.30 total scale) at 11°C and confirmed with carbonate water chemistry analyses according to Dickson (2007; Tables S1, S2 in the Supporting Information). Ambient pH values for the Salish Sea are approximately 7.8, and range seasonally from 7.6 to 8.0 (Murray et al. 2015). However, pH as low as 7.3 has been documented in nearshore environments in Washington (Wootton and Pfister 2012).

The spore settlement apparatus consisted of a carriage system that slowly (1-2.5 cm · hr^{-1}) drove reproductive algal thalli across a glass plate (0.6 x 7.6 x 60 cm) in stationary water, while releasing spores (Fig. 1a). The working section (14 x 1.5 cm) was defined at one end of the plate. Spores landing in the working section experienced a decreasing gradient of attachment time, determined by the rate parent thalli were driven across the plate, which resulted in a gradation of attachment time across the working section and the time the plate was allowed to set. After spore release and settlement, a shear flume was attached and this flume released a tall column of water that flushes quickly across the entire glass plate. Water column height was varied to create a range of five shear stresses (Fig. 1b), and the stresses generated by each water column height were calculated according to Schultz et al. (2000). Prior to each test, the shear flume was fitted on the glass plate with clamps and the released

spores were photographed using a microscope (Steindorff SXC, New York Microscopes) connected to a camera (Nikon Coolpix S3300).

For the attachment time assay, a low shear stress (1 Pa) was applied after spores were allowed to attach in stationary seawater and the remaining spores were photographed and counted. Attachment success as a function of time was determined from the number of remaining spores using a generalized linear model logistic regression (R v. 3.1.2; Fig. 2, a and b; Table S3 in the Supporting Information). Reduced pH delayed the attachment of both *C. vancouveriensis* and *P. robusta* spores. However, in *P. robusta* there was a significant interaction between time and pH; spores that were allowed to attach for longer than 35 h were not negatively affected by pH. (Fig. 2, a and b; Table S3). Although the delay in attachment in *C. vancouveriensis* was less acute than in *P. robusta*, the magnitude of this difference was similar between the two species; spores of both species took 40-52% longer to attach in reduced pH seawater relative to ambient conditions.

For the attachment strength assay, spores from *P. robusta* were allowed to set in stationary water for 35-48 h and those from *C. vancouveriensis* for 5-10 h to maximize attachment of each species according to the logistic regression analyses of attachment over time from the attachment time assay (Fig. 2, a and b). Attached spores were then exposed to increasing shear stresses (1, 4, 7, 17 and 20 Pa; multiple treatments, see Fig. 1b), representing boundary layer velocities of $0.2 – 4 \cdot ms^{-1}$, similar to intertidal field conditions (Hata 2015). At each position and at each shear stress applied, spores were counted through photo-analysis in ImageJ (v. 1.48; U.S. National Institutes of Health, Bethesda, MD). With each successive application of shear stress, we expected an increasing percentage of spores to detach from the settlement plate, up to a maximum value assuming some spores would be stronger than our assay. The effect of shear stress on spore detachment in each species was tested using an exponential rise-to-maximum non-linear regression (SigmaPlot 11.0; $R^2 = 0.84 - 0.99$; Fig. 2,

c and d, Table S4 in the Supporting Information), which provided estimates of two parameters: the maximum percentage of spores detached and the initial dependence of detachment on shear stress. The first derivatives of fitted, non-linear regressions were plotted to estimate frequency distributions of spore attachment strength (Fig. 2, e and f). Reduced pH did not affect attachment strength in *P. robusta*; a maximum of 50-55% of spores were dislodged regardless of seawater pH (Table S4; Fig. 2, c and e). However, reduced pH significantly weakened the attachment strength of *C. vancouveriensis* spores (Table S4; Fig. 2, d amd f). In low pH seawater, an additional 24% of the spore population dislodged (Fig. 2d), due primarily to 77% more spores being dislodged under moderate shear (4 Pa; Fig. 2d), leading to an increased observed frequency of the modal attachment strength (Fig. 2f).

Reduced pH negatively impacts spore attachment in two species of red algae and in two distinct phases of the attachment process. Spores of *C. vancouveriensis* attach much more rapidly (5-10 h) than spores of *P. robusta* (35-43 h), yet fewer of these spores may be able to survive high shear stresses in reduced pH conditions, due to their weakened attachment. In contrast, spore attachment in *P. robusta* was greatly delayed in reduced pH seawater – perhaps increasing the likelihood of being cast ashore or washed out to sea – but those that attach, despite being delayed, are likely to have an attachment strength that is insensitive to reduced pH . In this manner, differing effects of ocean acidification on attachment times and strengths of algal spores have the potential to impact the future composition, diversity and abundance of macroalgal populations.

Past studies have demonstrated that acidified seawater delays germination, retards development, and reduces growth of macroalgal spores (Coelho et al. 2000, Hofmann et al. 2010, Chan et al. 2015, Bradassi et al. 2013, Gaitán-Espitia et al. 2014, James et al. 2014, but see Padilla-Gamiño et al. 2016, Leal et al. 2017). Here, we demonstrate negative biomechanical impacts of reduced ocean pH on early spore adhesion, which precedes and

perhaps supersedes any physiological response. Spore adhesion is fundamental to the colonization of macroalgal populations to new substrata (Chamberlain 1976, Boney 1981, Fletcher and Callow 1992). Even calcified macroalgae, which may (Kroeker et al. 2013, McCoy and Kamenos 2015, Roleda et al. 2015) or may not (Padilla-Gamiño et al. 2016, Leal et al. 2017) be highly susceptible to reduced pH, have uncalcified spores like other macroalgae that must adhere despite changing ocean conditions. That ocean acidification affects spores of both calcified and uncalcified macroalgae suggests that impacts on spore attachment may be widely distributed across species. Compromised spore attachment in acidified seawater would be consistent with reduced ionic cross-linking and hydration of protonated, anionic spore mucilages (Li et al. 2013), potentially slowing curing rates (Jones et al. 1982) or disrupting the interaction of mucilages with substrata (Callow and Fletcher 1994) perhaps via increased gel density and viscosity (Bu et al. 2005). Nevertheless, we cannot rule out the possible impact of reduced ocean pH on spore physiology, as sulphate uptake (Quatrano and Crayton 1973) or intercellular mucilage production (Pueschel 1979) may also be affected. Because the chemical composition, curing rate, and production rate of spore mucilages differ widely among macroalgal species (see Fletcher and Callow 1992) yielding differences in spore adhesion (see Fig. 1; Moorjani and Jones 1972), the impact of ocean acidification on spore adhesion is also expected to vary across species. Additional work is needed to clarify if the precise impact of reduced ocean pH on adhesion is generally biological or chemical in nature.

In summary, ocean acidification compromised spore attachment in two species of red macroalgae. Assuming results translate to field conditions and natural substrata, reduced pH has the potential to impact the life cycles of both calcified and fleshy species via spore dysfunction, despite the potential viability and resilience of mature algal thalli. If fitness is affected, compromised spore adhesion could cause declines in seaweed diversity and

abundance along our shores. Future studies should address whether the excessive number of spores produced by mature macroalgal thalli could compensate for increased spore mortality and identify tipping points beyond which documented impacts on spore adhesion would influence macroalgal population dynamics.

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Figure Captions

Fig. 1: (A) Spore settlement apparatus used to access spore attachment for variable amounts of time, as mature thalli were moved across the working section (14 x 1.5 cm) of the glass settlement plate (60 x 7.6 cm), and (B) the shear flume with five replaceable columns used to generate different shear stresses. The flume was mounted on top of the settlement plate where spores were released creating a channel over the working section through which the outflow of water passed.

Fig. 2: The effects of control pH (7.75 pH; black datapoints) and reduced pH (7.30 pH; red datapoints) on spore attachment time, dislodgement, and frequency distribution of attachment strengths. Attachment times of spores in (A) *Polyostea robusta* (n = 614-663) and (B) *Corallina vancouveriensis* (n = 335-477), where each point represents one spore scored as 0 (not attached) or 1 (attached) after an applied low shear stress (1 Pa). Solid lines are logistic regressions (ln[Y/(1-Y)] = a +bX), where a is the intercept, b is the slope, and X is time, and dashed lines are 95% confidence intervals. Detachment of spores in (C) *P. robusta* after 35-48 h of attachment time (n = 5-9, mean ± S.E.M.) and (D) *C. vancouveriensis* after 5-10 h of attachment time (n = 9-11, mean ± S.E.M.). Lines are exponential rise-to-maximum, non-linear regressions of percent spore detachment as functions of shear stress (y = a * (1-exp(b * x)), where a is the maximum detachment and b is the initial rate of increase of detachment with shear stress. Frequency distributions of spore attachment strengths in (E) *P. robusta* and (F) *C. vancouveriensis*, generated from first derivative calculations from fitted, non-linear regressions of detachment data.

Table Captions

Table S1: Average pH (total scale) and temperature (°C) of control and low pH treatments of *Corallina vancouveriensis* and *Polyostea robusta.* Before is value when assay was set up and after is value after assay was completed. Error is S.E.M. for each spore assay and pH treatment.

Table S2: Total alkalinity (µmol/kgSW) measurements from collected water samples over the course of spore attachment assays.

Table S3: Generalized linear model results for attachment times of algal spores of *Corallina vancouveriensis* and *Polyostea robusta.*

Table S4: Results of exponential rise-to-maximum, non-linear regressions on detachment of algal spores of *Corallina vancouveriensis* and *Polyostea robusta.* Parameter p-values reflect the significance of parameters in each regression, and treatment p-values reflect results from *t*-tests comparing parameter estimates in the two treatments (control and low pH).