



Cell wall structural changes lead to separation and shedding of biofouled epidermal cell wall layers by the brown alga *Ascophyllum nodosum*

Laryssa Halat^{1,2} · Moira E. Galway¹ · David J. Garbary^{1,3}

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Abstract

Marine plants control the accumulation of biofouling organisms (epibionts) on their surfaces by various chemical and physical means. *Ascophyllum nodosum* is a perennial multicellular brown alga known to shed patches of epidermal material, thus removing epibionts and exposing unfouled surfaces to another cycle of colonization. While surface shedding is documented in multiple marine macroalgae, the cell and developmental biology of the phenomenon is almost unexplored. A previous investigation of *Ascophyllum* not only revealed regular cycles of epibiont accumulation and epidermal shedding but also stimulated the development of methods to detect the corresponding changes in epidermal (meristoderm) cells that are reported here. Confocal laser scanning microscopy of cell walls and cytoplasm fluorescently stained with Solophenyl Flavine 7GFE (Direct Yellow 96) and the lipophilic dye Rhodamine B (respectively) was combined with light and electron microscopy of chemically fixed or freeze-substituted tissues. As epibionts accumulated, epidermal cells generated thick, apical cell walls in which differentially stained central layers subsequently developed, marking the site of future cell wall separation. During cell wall separation, the outermost part of the cell wall and its epibionts plus the upper parts of the anticlinal walls between neighboring cells detached in a layer from multiple epidermal cells, exposing the remaining inner part of the cell wall to new colonizing organisms. These findings highlight the dynamic nature of apical cell wall structure and composition in response to colonizing organisms and lay a foundation for further investigations on the periodic removal of biofouling epibionts from the surface of *Ascophyllum* fronds.

Keywords *Ascophyllum nodosum* · Cell wall · Biofouling · Epidermis shedding · Phaeophyceae

Introduction

Like other marine organisms, the surfaces of multicellular marine macroalgae host diverse, dynamic, and interacting populations of epibionts, including bacteria, algae, protists, yeasts, and filamentous fungi, which may have neutral, beneficial or harmful effects on the basibiont (Wahl 1989; Wahl

et al. 2012; Egan et al. 2012; Da Gama et al. 2014). Together, the epibionts and their basibiont host seaweed might be considered as an integrated community of organisms, sometimes termed a holobiont (see O'Malley 2017 for a critical review). The study organism, *Ascophyllum nodosum* (Linnaeus) Le Jolis (hereafter *Ascophyllum*), is the ultimate host for a complex symbiotic community which includes an obligate endosymbiotic fungus and several epiphytic red and brown seaweeds (see Garbary et al. 2017a, b for review). Excessive epibiont growth (biofouling, hereafter fouling) on the macroalgal host reduces transmission of photosynthetically active radiation, decreases access to gases and nutrients, and increases mass and drag in flowing water, which may detach macroalgae from their substrates (Wahl 1989; Wahl et al. 2012; Da Gama et al. 2014). Thus, like other marine organisms, macroalgae such as *Ascophyllum* employ a variety of chemical, mechanical, and physical means to regulate epibiont growth (Wahl 1989; Egan et al. 2012; Da Gama et al. 2014; Halat et al. 2015).

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✉ Moira E. Galway
mgalway@stfx.ca

¹ Department of Biology, St. Francis Xavier University, Antigonish, Nova Scotia B2G 2W5, Canada

² Department of Botany, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada

³ Jack McLachlan Laboratory of Aquatic Plant Resources, St. Francis Xavier University, Antigonish, Nova Scotia B2G 2W5, Canada

Surface shedding (or sloughing), also referred to as epidermal, epithallial, meristodermal, cuticular, or “skin” shedding by different authors over the past 40 years, is one way for longer-lived marine macroalgae to rid themselves of epibionts. Since there is no standard terminology, surface shedding will henceforth be referred to as “epidermal shedding,” so the *Ascophyllum* surface (technically the meristoderm) will be the epidermis, and the individual epidermal cells will be the meristoderm cells. Epidermal shedding occurs in certain red, green, and brown marine macroalgal species (Da Gama et al. 2014). Epidermal shedding is relevant to seaweed cultivation and harvesting industries, to marine ecologists, and to plant and cell biologists. *Ascophyllum* shedding can contribute significantly to the organic detritus of coastal ecosystems (Halat et al. 2015), although the method used to estimate this contribution has been disputed by Ugarte et al. 2017 (see also response by Garbary et al. 2017b). The initiation and coordination of shedding is likely to involve signaling between epibionts and the basibiont as well as between cells of the basibiont itself, but at present, little is known beyond the mere existence of this phenomenon in various species.

The two major mechanisms of epidermal shedding are the detachment of whole epidermal cells or the detachment of an outer fouled layer of cell wall from the epidermal cells. Among crustose (coralline) red algae, either sheets of cells detach in a locally synchronized process or there is an unsynchronized detachment of cells from the apices of epithelial filaments, in both cases accompanied by indications of cell death (Keats et al. 1997 and references therein). In contrast, outer cell wall layers separate and detach in some green and red algae (Borowitzka and Larkum 1977; McArthur and Moss 1977; Sieburth and Tootle 1981; Gonzalez and Goff 1989 and references therein). However, outer cell wall separation is best documented among brown algal species (Moss 1982; Russell and Veltkamp 1984; Sokhi and Vijayaraghavan 1985, 1987; Pedersen and Sokhi 1990; Martinez and Correa 1993; Garbary and Galway 2013; Yamamoto et al. 2013; Rickert et al. 2016). These reports are consistent with an abscission-like process whereby an outer layer of cell wall and its epibionts separates from the remaining cell wall in multiple adjoining cells. This results in the shedding of discrete cell wall patches or flakes of varying dimensions (Sieburth and Tootle 1981; Moss 1982; Russell and Veltkamp 1984; Martinez and Correa 1993; Yamamoto et al. 2013; Rickert et al. 2016).

Ascophyllum is a brown, fucoid alga, which grows abundantly in the intertidal zone of Atlantic provinces of Canada and other temperate shores of the North Atlantic Ocean (Baardseth 1970), and is the basibiont for a complex community that includes mutualist, parasitic, and commensal symbionts (Garbary et al. 2017a). The plant (Fig. 1a) consists of long, flat, or rounded branching fronds, kept buoyant by a series of regularly positioned air bladders. Each spring a new bladder is produced at the apex of each branch which becomes separated from the

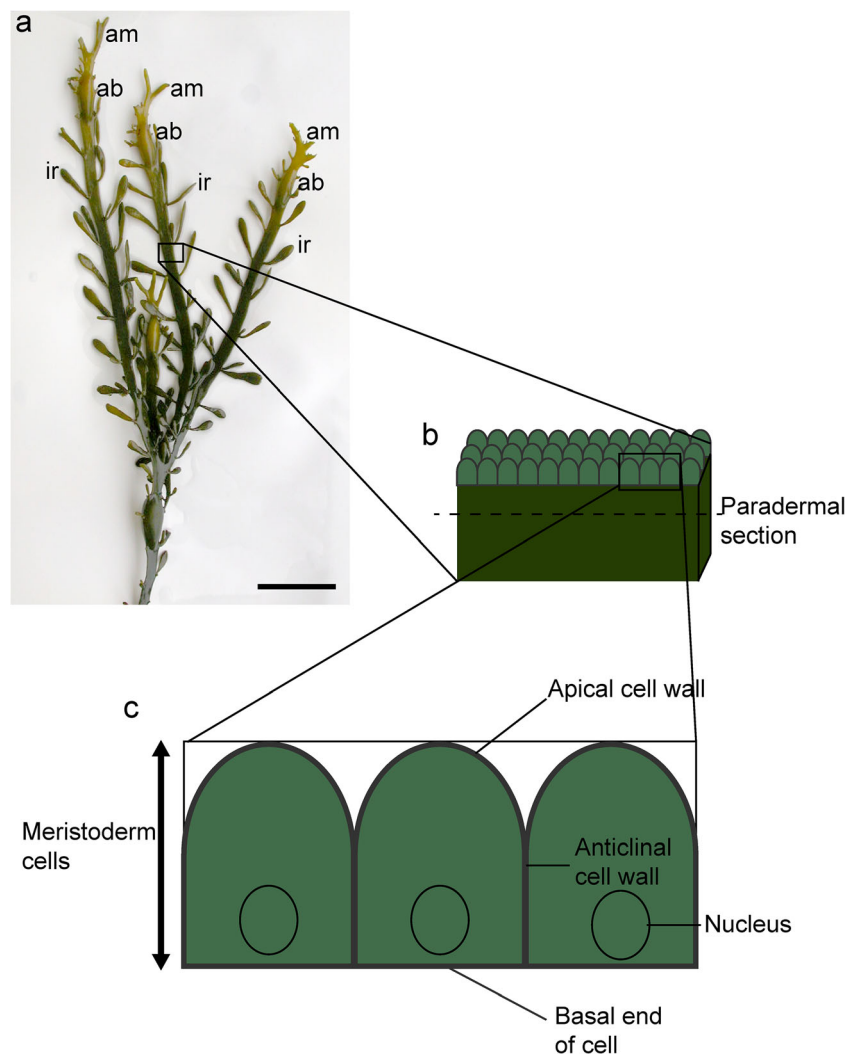
following year's bladder by an intervening period of apical growth (Fig. 1a). Lateral branches, either vegetative or reproductive, the latter bearing the gamete-producing receptacles, are formed at regular intervals along each branch as it grows. The epidermis consists of a thin layer of small columnar cells forming a tightly packed polygonal array (Fig. 1b, c). Anticlinal and periclinal divisions of these meristoderm cells contribute to frond growth. In *Ascophyllum*, epidermal shedding has been observed in plants collected throughout the year from both sides of the North Atlantic (Filion-Myklebust and Norton 1981; Sieburth and Tootle 1981; Stengel and Dring 2000). Weekly sampling of a local population revealed strikingly regular (roughly monthly) increases and decreases in shedding throughout the warmer months of the year (Halat et al. 2015). These features make *Ascophyllum* an ideal model plant in which to study shedding. Initial reports of *Ascophyllum* shedding indicated that whole cells were shed (Filion-Myklebust and Norton 1981; Sieburth and Tootle 1981), and this influenced later studies (Stengel and Dring 2000; Garbary et al. 2009). Here we augment traditional approaches to brown algae histology (e.g., McCully 1965, 1966) with confocal laser scanning microscopy of *Ascophyllum* meristoderm cell walls stained for β -glucans with Solophenyl Flavine 7GFE (Direct Yellow 96) and cytoplasm stained with the lipophilic dye Rhodamine B to conclusively demonstrate that under natural and laboratory growing conditions, *Ascophyllum* sheds patches of cell wall material, consistent with reports from other brown algae. While the shed material superficially resembled a detached interconnected layer of small cells, the shed layers form by a locally synchronized abscission-like process in which the fouled outer layer of the cell wall and part of the adjoining anticlinal cell walls of meristoderm cells separates and detaches. Before cell wall separation occurs, there is major change in cell wall structure: a new centrally located layer of non-fibrillar amorphous material forms within the fibrillar lamellae of the cell walls. This layer separates the rest of the wall into an outer layer to be shed, and an inner layer that will form the new outer surface of the meristoderm cells once shedding is completed. Taken together, the data reveal the development of *Ascophyllum* meristoderm cell walls through histochemically and morphologically distinct stages as epibionts accumulate to the point of removal by cell wall shedding. This provides baseline data for future investigations of the phenomenon and its regulation.

Materials and methods

Plant collections

Ascophyllum was collected from a channel constructed before 1925 that connects the small lagoon Captains Pond, to Antigonish Harbour in Nova Scotia, Canada (45.68°N, 61.87°W; see Halat et al. 2015 for additional details). Fronds

Fig. 1 *Ascophyllum nodosum* plant anatomy and cell terminology. **a** Portion of branched frond of *Ascophyllum nodosum* collected in early summer. Each of the three long branches bears many immature lateral receptacles (ir), and each terminates in an apical air bladder (ab) surmounted by a growing apical meristem (am) able to dichotomize or produce additional lateral branches in subsequent years. **b** For light microscopy and histochemical staining, paradermal hand sections were cut parallel to the meristoderm surface as indicated by the dashed line in diagram. **c** Transverse section detail of meristoderm cells with nomenclature used to describe the cell walls. Scale bar in **a** 3 cm



were collected weekly within 2 h of low tide from May 2013 to August 2014 and either kept in seawater in a controlled environment chamber at 15 °C with a light level of 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ using a combination of daylight fluorescent lights and incandescent bulbs on a 16 h light/8 h dark cycle, or they were placed in a circulating seawater tank at 15 °C under continuous illumination of approximately 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation, which was sufficient to maintain test plants for at least 3 months (June–August 2014). Epidermal tissue was harvested from these fronds within 2–4 days of collection. The tissue samples were either used unfixed for light microscopy and confocal laser scanning microscopy (CLSM), or the samples were fixed for light and electron microscopy. Epidermal tissue was always harvested from apical portions of fronds produced within the previous 2 years, that is, between the growing apices and the top of the second air bladder formed 2 years previously. Shedding is more readily detected in these younger apical regions of fronds, which have fewer adhering shellfish and

large epiphytes. The absence of large epibionts facilitates tissue sampling and observation, while removing the complication that large adhering organisms could affect shedding mechanically or chemically. Younger regions are also more lightly pigmented than older regions, making variations in the structure and fouling of the frond surfaces easier to observe.

Some whole plants were collected and kept in circulating seawater for several months to enable the meristoderm to grow and develop in the absence of strong tidal currents, which results in the retention of partially separated shed material at the surfaces of the fronds as previously reported by Filion-Myklebust and Norton (1981).

Light microscopy of toluidine blue-stained epidermis

To identify major changes in the meristoderm cells associated with epibiont accumulation and shedding by light microscopy, sections of both unfixed and fixed epidermal tissue were stained with 0.05% toluidine blue (TB) dye for light

microscopy following McCully (1966). Fresh, unfixed tissue was embedded into 10% agar, from which 50 μm transverse sections were cut into a phosphate-buffered saline solution at pH 7.4 using a razor-blade equipped Vibratome 1000 (Ted Pella Inc., Redding, CA, USA), before staining in TB. For fixation, 2–4 mm pieces of epidermal tissue were fixed for 24 h in 5% glutaraldehyde in a modified Von Stosch medium (Guiry and Cunningham 1984) at 4 °C. After washing, the tissue was divided into smaller pieces and post-fixed in 1% osmium tetroxide for 1 h, rinsed in distilled water, and stained in 2% aqueous uranyl acetate for 1 h, before dehydration in ethanol solutions of increasing concentration, transfer to propylene oxide and embedding in Epon. One-micrometer thick sections were stained with 0.05% TB dye at pH 10. All sections were viewed with a Nikon Eclipse E800 microscope, and TIFF files were recorded with a DS-Fi1 Digital Sight camera using NIS-Elements software.

Staining of meristoderm cell walls for epifluorescence microscopy and CLSM

For epifluorescence microscopy of cell walls, shed material and unfixed hand sections were stained in a fluorescent brightener, FB28 (Sigma F-3543; Calcofluor White M2R). For CLSM, the blue-light excited, β -glucan-staining cell wall dye Solophenyl Flavine 7GFE (SF), also known as Direct Yellow 96 (Sigma-Aldrich S472409), was used (Hoch et al. 2005; Anderson et al. 2010). Unfixed paradermal hand sections were stained in 0.02% SF in artificial seawater (ASW; Cold Spring Harbor Protocol 2012: 2 doi:<https://doi.org/10.1101/pdb.rec068270>) at pH 7.8 for 30 min, then washed. Based on the results of Anderson et al. (2010), SF is expected to fluoresce most intensely upon binding cellulose and to a lesser extent upon binding β -1,3-linked glucans and mixed-link glucans; all three polysaccharides are minor components in the cell walls of fucallean brown algae (Deniaud-Bouët et al. 2014; Salmeán et al. 2017). SF staining of many epibionts was far more intense than SF staining of *Ascophyllum* cells, and *Ascophyllum* meristoderm apical cell walls stained more intensely than the basal or anticlinal walls or the walls of underlying cortical cells. Thus to optimize imaging of apical meristoderm cell walls, it was necessary to underexpose other *Ascophyllum* cell walls.

Staining of meristoderm cytoplasm and nuclei for epifluorescence microscopy and CLSM

To stain meristoderm cell cytoplasm, fresh unfixed hand sections were stained with a 5- μM solution of Rhodamine B (RB) in ASW for 30 min followed by washing. RB (R6626, Sigma-Aldrich, St. Louis, MO, USA) is a moderately lipophilic water soluble green light excited dye that has seen some use as a vital stain for marine algae (Tornbom and Oliveira 1993;

Knoblauch et al. 2016) as well as some flowering plants (Liu 2004; Guan et al. 2013). RB stained *Ascophyllum* cytoplasm including plastids and also epibionts. In some experiments, RB was replaced by the green-light excited, DNA-binding dye propidium iodide (PI) to confirm the absence of nuclei in shed material and to check the basal location of nuclei within meristoderm cells. After cell wall staining, PI was applied as a 0.1-mg/mL solution in water or buffer. However, to ensure dye penetration and consistent staining of all nuclei in hand sections, it was necessary to freeze-thaw the hand sections before PI staining.

Epifluorescence microscopy and CLSM

Standard epifluorescence (Nikon E800 and Olympus BX50WI) and CLSM (Olympus FV300 attached to upright Olympus BSX50WI) were both used to view (a) patches of shed material and (b) freshly prepared paradermal or transverse hand sections of *Ascophyllum* fronds labeled with either one or two of the fluorescent dyes described above. Stained and washed samples were mounted on slides in Citifluor AF3 (Electron Microscopy Sciences, Hatfield, PA, USA). Paradermal sections were oriented with the epidermal surface facing the incident light before viewing. Epifluorescence microscopy employed standard filter sets required to view blue light excitation of green fluorescence (e.g., FITC) or green light excitation of red fluorescence. For CLSM of cell walls in SF-stained sections, a 488-nm laser (close to the upper limit of the dye's absorption spectrum) was used, and fluorescence was recorded between 510 and 530 nm. RB-stained cytoplasm and PI-stained nuclei were visualized using a 543-nm laser, and fluorescence over 605 nm was recorded. Inspection of both stained and unstained sections excited with these lasers revealed little autofluorescence or spectral bleed-through. In unstained sections exposed to 488-nm laser light, faint cell wall and plastid fluorescence was detected between 510 and 530 nm as well as at wavelengths over 605 nm. With 543-nm laser light, faint plastid fluorescence was detected in unstained sections between 510 and 530 nm. However, section autofluorescence was undetectable in stained sections with settings adjusted for optimum image quality. To determine the degree of fouling in relation to the epidermal shedding process, XY, XZ, and XYZ scans of SF and RB double-stained paradermal hand sections were recorded using a 60 X water immersion lens. Basally located RB-stained plastids (or PI-stained nuclei) served as convenient markers for the basal ends of meristoderm cells. Laser and image acquisition settings were adjusted for optimum image quality in the apical regions of meristoderm cells. Note that intense staining of many epibionts by both SF and RB relative to the *Ascophyllum* meristoderm cells makes the latter appear less well-stained in XZ and XYZ images that include epibionts. TIFF or multi-

TIFF image files were recorded with FluoView FV300 software.

Quantifying cell wall size changes from CLSM images

CLSM scans of *Ascophyllum* paradermal hand sections were sorted into three types: (a) thin outer cell walls, few epibionts; (b) thicker outer cell walls with epibionts; and (c) thickest outer cell walls with thick layer of epibionts. XYZ scans of at least three examples of each section type were viewed using the FluoView 300 “view multi-plane form” tool. Sixty different meristoderm cells were selected from each specimen, and for each cell, cell height and cell wall thickness were measured in the XZ plane with the line measurement tool supplied for image annotation in the FluoView software. This method enables relative size differences to be identified, but does not take into account artifacts such as spherical aberration that distort actual dimensions. Patches of shed material were obtained from seawater in which *Ascophyllum* fronds were collected or as they detached from fronds, and the height of the cell walls in SF-stained patches were measured. Statistical comparisons of cell wall thickness throughout different stages of shedding were performed with a Kruskal-Wallis test and Dunn’s post hoc test in R (R Core Team 2017).

Scanning electron microscopy of *Ascophyllum* frond epibiont cover and epidermal shedding

Each week between May 2013 and June 2014, frond pieces collected for a survey of epibiont coverage on *Ascophyllum* (Halat et al. 2015) were stored frozen. For scanning electron microscopy (SEM), frond segments from five dates for which extensive epibiont coverage was recorded were thawed and rehydrated in ASW and prepared for SEM (Halat et al. 2015). In brief, 2 mm × 2 mm pieces taken from the center and both ends of each rehydrated segment were fixed for 24 h in a solution of 2.5% glutaraldehyde and 1 mM HEPES buffer in ASW at pH 7.8. Fixed pieces were then washed in ASW followed by distilled water and then gradually dehydrated in ethanol over an 8-h period, followed by critical point drying and gold coating before mounting on aluminum stubs and observing with a JEOL JSM-5300 SEM. To determine if freezing or SEM preparation affected tissue morphology, some of the fixed and washed pieces, as well as small pieces of living frond tissue, were placed on adhesive carbon tabs on aluminum stubs and examined in an environmental scanning microscope (ESEM; JEOL JSM-6010LA Analytical Scanning Electron Microscope) operating under low vacuum (50 Pa); comparable results were obtained but not shown due to the poorer quality of the resulting images.

Freeze substitution for light microscopy and transmission electron microscopy

For optimum preservation of normal organelle distribution, freshly prepared paradermal or transverse sections were placed on small squares of standard food wrap-grade aluminum foil, plunge-frozen in liquid propane, then freeze-substituted in a solution of 2% osmium tetroxide and 0.4% uranyl acetate in acetone followed by dehydration and embedding in with a 1:1 mixture of Spurr’s and Epon 812, before sectioning, staining, and examining using a Philips EM 410 microscope (Xu et al. 2008). These specimens were supplemented with thin sections cut from tissue conventionally fixed in glutaraldehyde and embedded in Epon as described previously for light microscopy. *Ascophyllum* epidermis preserved by both methods was examined by light and electron microscopy for evidence of shedding.

Software

Figures were assembled in Adobe Illustrator v15.1.0.

Results

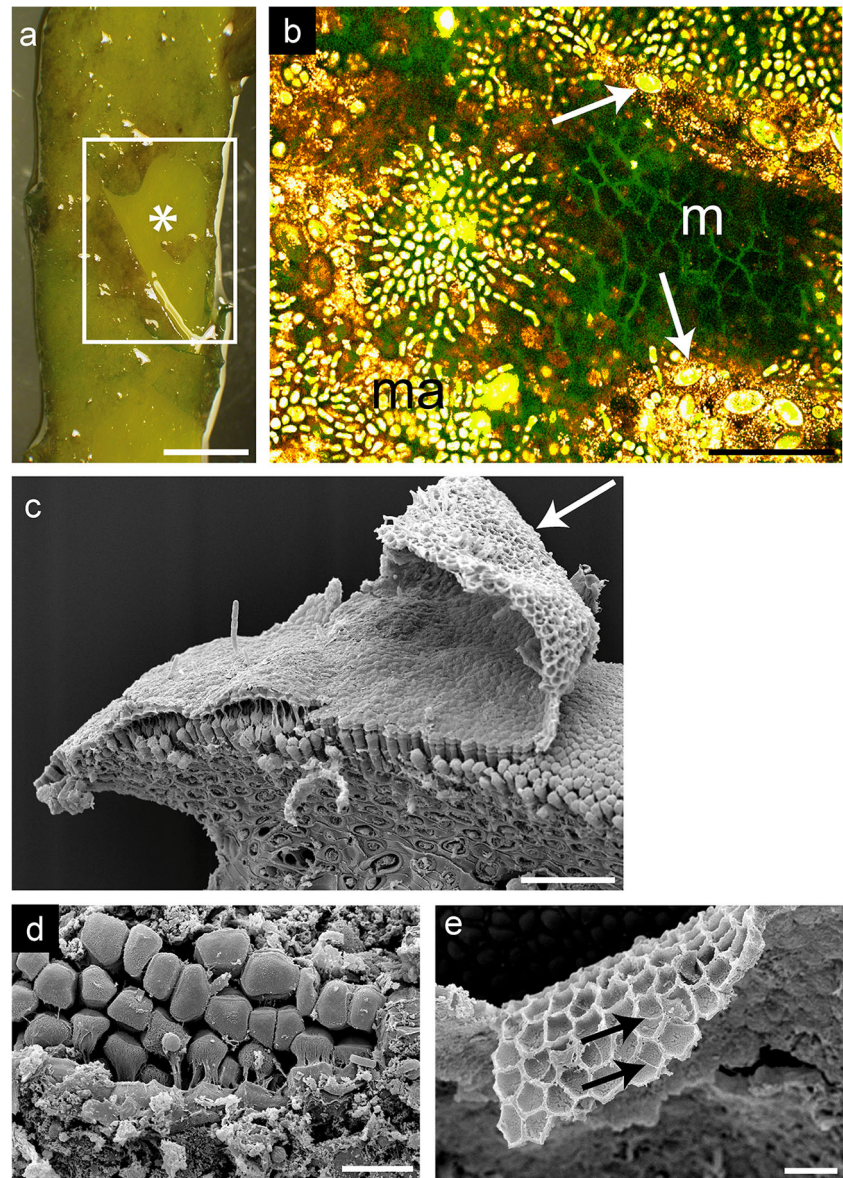
Epidermal shedding releases fouled cell wall patches

Frond surfaces are a mosaic of unfouled (clean), lightly fouled, and heavily fouled regions (Fig. 2a, b) due to the continual separation and shedding of thin, centimeter-sized pieces of material from the epidermis, each one approximately 10 μm thick (Halat et al. 2015). In less fouled surface regions, a polygonal array of tightly packed meristoderm cells is clearly visible (Fig. 2b, d). Partially detached pieces of shed material with flipped-up edges (Fig. 2c, e) show that each patch consists of a layer of epibiont-fouled surface and, on the other side, a honeycomb-like polygonal array of open-ended caps, due to the detachment of the fouled outer cell walls and upper parts of the anticlinal cell walls from multiple meristoderm cells.

Meristoderm apical cell walls thicken as epibionts accumulate

Since shedding removes epibionts from the frond and exposes a new unfouled surface for epibiont colonization, the presence or absence of epibionts are indicators of approaching or recent shedding events. Sampling many plants within the local population revealed changes in the meristoderm cell walls correlated with changes in epibiont fouling and epidermal shedding (Figs. 3, 4, and 5) which enabled the sequence of developmental changes leading to shedding to be established (Fig. 6). Only the structure and organization of the apical cell wall

Fig. 2 *Ascophyllum* fronds shed patches of fouled epidermal material. **a** Thin layer of fouled material has begun to detach, and edges have rolled back to expose light green unfouled surface below (outlined by white rectangle, with asterisk marking the unfouled region). **b** Wavy fouled surface of hand section stained for cell walls with SF enables a CLSM XY surface scan to show anticlinal walls of meristoderm cells (m), otherwise covered with microscopic algae (ma), including diatoms (arrows), and other epibionts. **c** Low magnification SEM of paradermal hand section showing partially detached outer layer of epidermis folded back to reveal honeycomb-like underside (arrow) that results from detachment of this layer from the remaining apical cell walls and apical portions of anticlinal cell walls belonging to multiple meristoderm cells. **d** Separation of fouled epidermal material reveals epibiont-free apical surfaces of underlying meristoderm cells, as in **b**, above. **e** Higher magnification detail of underside of shed layer, which is composed of an open-ended array of polygonal cell wall caps detached from multiple meristoderm cells. Arrows indicate very thin new cross walls arising from anticlinal cell divisions that occurred in some meristoderm cells before shedding. Scale bars **a** 4 mm, **b** 0.1 mm, **c** 100 μ m, **d** 20 μ m, **e** 20 μ m



changed in relation to fouling, as described below. The time between successive shedding events in the summer is estimated to be about a month (Halat et al. 2015), but this has yet to be assessed in individual plants. Meristoderm cells from recently shed, epibiont-free regions of *Ascophyllum* fronds had thin apical cell walls (Fig. 3a) that were well-stained with SF, indicating the presence of β -glucans (Fig. 3b). In interphase, meristoderm cells always exhibit a polarized apical-basal distribution of organelles (previously described by Xu et al. 2008) regardless of epibiont load. Nuclei and plastids are located at the basal ends of meristoderm cells, while mitochondria, vacuoles, and phlorotannin-containing physodes are apically abundant (Fig. 3c). A striking feature is the extreme thinness of the apical ends of anticlinal cell walls between meristoderm cells, also depicted in Xu et al. (2008). Increased epibiont coverage was associated with increased

apical cell wall thickness (Fig. 3d). The thicker walls are composed of 5 or 6 distinct lamellae indicative of distinct changes in cell wall structure as the walls thicken over time (Fig. 3e, f).

Changes in meristoderm apical cell wall β -glucan staining precede cell wall separation

Among heavily fouled meristoderm cells with the thickest apical (outer) cell walls was a subset that exhibited differential staining of β -glucans by SF. In these cells, SF stains the outer and inner layers of the cell wall, but no longer stains the middle of the cell wall, producing a double-walled appearance (Fig. 4a). No autofluorescence or RB staining of this cell wall central layer was detected. The poorly stained central layer terminates where the well-stained outer and inner cell wall layers merge with the anticlinal cell walls of the meristoderm

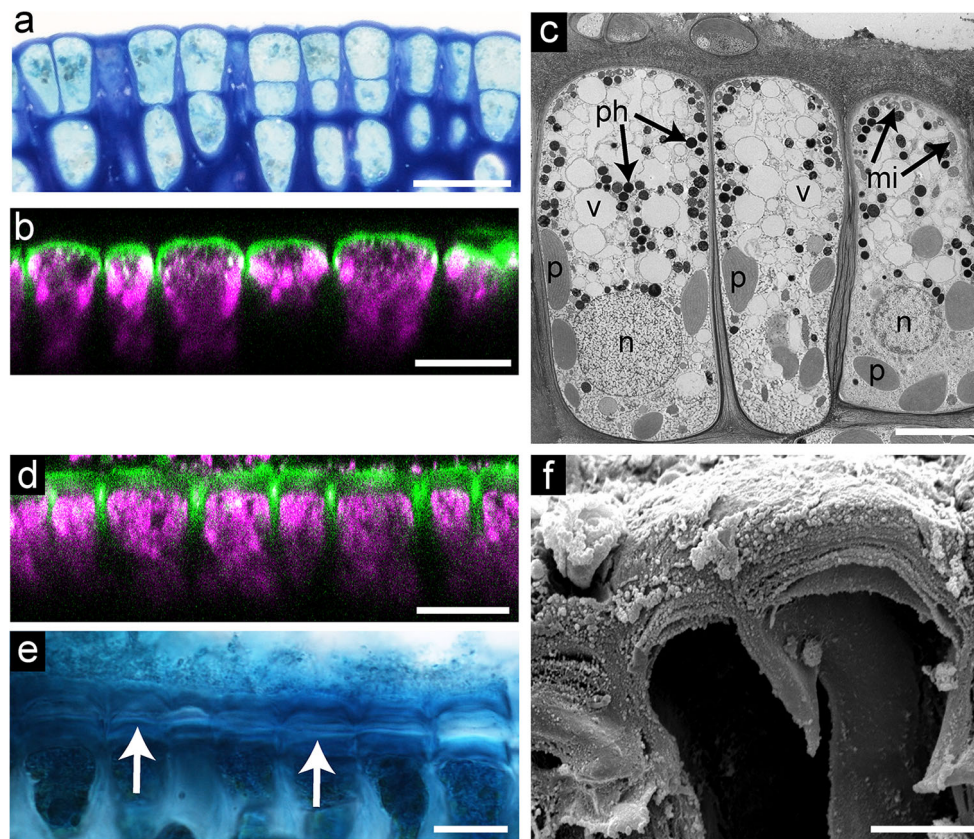


Fig. 3 Unfouled meristoderm cells have thin apical walls that thicken as epibionts accumulate. **a** TB-stained section of unfouled meristoderm from chemically fixed and resin-embedded tissue with thin convex apical cell walls; note that some recent anticlinal and periclinal divisions have produced pairs of smaller daughter cells separated by thin new cell walls. **b** Transverse CLSM scan of a hand section from an unfouled region of epidermis exhibiting SF-stained thin apical cell walls (green) and lipophilic RB-stained cytoplasm (magenta). **c** TEM of lightly fouled meristoderm cells with thicker apical cell walls and typical apical-basal polarized distribution of organelles (preserved by freeze substitution), nucleus (n), plastid (p), mitochondria (mi). Note apical distribution of darkly stained tannin-containing physodes (ph) and vacuoles (v), and

the thinner apical ends of the anticlinal walls between cells; ice crystallization damage is evident in nucleus (n) of cell on left. **d** Transverse CLSM scan of hand section from a fouled region of epidermis exhibiting SF-stained apical cell walls (green) that are thicker than those depicted in **b**; some epibiont cell walls (green) and cytoplasm (magenta) are visible at outer surface of apical cell walls at top of frame. **e** Transverse vibratome section of TB-stained meristoderm cells reveals multiple lamellae within thickened apical cell walls (arrows). **f** Five or six cell wall lamellae are visible in an SEM of hand sectioned, thick-walled, and fouled meristoderm cells similar to those in **e**; note that cell contents were lost during cutting and processing for SEM. Scale bars **a** 40 μm , **b** 20 μm , **c** 5 μm , **d** 20 μm , **e** 20 μm , **f** 5 μm

cells (Fig. 4a). The outer and inner cell wall layers have a fibrillar structure, but the central cell wall layer consists of amorphous, poorly stained, and poorly infiltrated cell wall material (Fig. 4b, c; see also comparable light microscopy thick section in Fig. 5d). A mottled region of heterogeneous staining defines the border between the central layer and the clearly fibrillar outer and inner cell wall layers (Fig. 4b, c).

Meristoderm apical cell wall layer separation and properties of the cell wall separation layer

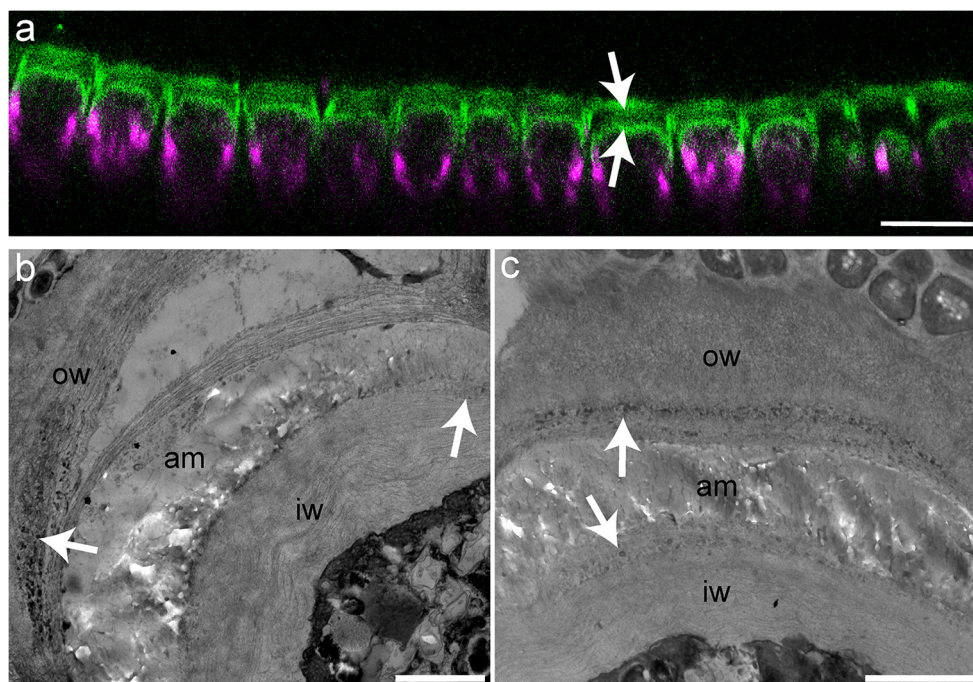
In meristoderm cells undergoing epidermal shedding, the cell wall layers separate at the unstained amorphous central layers (Fig. 5a–d) that developed within the thick fouled walls (Fig. 4a–c). During separation, strands of deformable material extend between the separating cell wall layers (Fig. 5b, c) and coat the meristoderm cell surfaces after separation (Fig. 5c).

TB stains this material a pale pink (Fig. 5d). The shed layers consist of fibrillar outer cell wall caps and adhering epibionts (Fig. 5e). PI staining and examination by CLSM and fluorescence microscopy revealed no nuclei (not shown).

Summary of cell wall changes related to epidermal shedding

To evaluate changes in cell wall thickness during a typical shedding cycle, the sequence of epidermal shedding in *Ascophyllum* was divided into four stages. Changes in the relative thickness of the outer (apical) cell wall during these stages were quantified using fluorescently stained samples viewed with CLSM (Fig. 6). The mean cell wall thickness of unfouled, recently shed meristoderm cells was $3.17 \pm 0.15 \mu\text{m}$, which increased significantly (by about 4.0 μm) to $7.14 \pm 0.45 \mu\text{m}$ for cells with thickened, epibiont-covered cell

Fig. 4 Formation and structure of central cell wall separation layer prior to shedding. **a** Some thick and heavily fouled meristoderm apical cell walls had central regions (between arrows) that did not stain with SF for β -glucans (green) or RB for lipophilic components (magenta). **b, c** TEM micrographs of the central layer of amorphous material (am; poorly infiltrated) enclosed between fibrillar inner wall (iw) and outer wall (ow) layers within thick apical cell walls of fouled meristoderm cells from chemically fixed and embedded tissue; area in **b** is near the junction of periclinal and anticlinal walls, while that in **c** is from midsection of adjacent cell; in both, heterogeneously stained mottled areas flank central layer (arrows). Scale bars **a** 20 μ m, **b** 1 μ m, **c** 2 μ m



walls. In SF-stained cells in which a “double” cell wall was detected, the top or outer cell wall thickness averaged $3.12 \pm 0.11 \mu\text{m}$, with a slightly thinner average thickness for the inner or bottom cell wall, at $2.72 \pm 0.11 \mu\text{m}$. The average height of the unstained central layer between these top and bottom cell walls was just $1.48 \pm 0.18 \mu\text{m}$. Measurements were also obtained from polygonal or honeycomb-like patches of shed cell wall material, which consist of a series of caps formed by the detachment of meristoderm outer apical wall layers and the upper parts of the anticlinal walls. The mean height from the fouled side of the apical wall to the detached ends of the anticlinal walls was $11.2 \mu\text{m}$, which may explain the initial misinterpretation of the shed patches as cells as described in the introduction. The average apical cell wall thickness within the shed caps was $3.70 \pm 0.15 \mu\text{m}$.

Discussion

Epidermal shedding is an infrequently reported antifouling defense, and the mechanisms remain essentially unexplored. In *Ascophyllum*, patches of shed material were originally thought to contain whole meristoderm cells (Filion-Myklebust and Norton 1981; Sieburth and Tootle 1981). This model was inconsistent with cell size measurements, leading to the proposal that the shed patches contained smaller cells derived from the apical ends of meristoderm cells (Garbary et al. 2009). Interpretation was complicated by the small size of the meristoderm cells and the limited methods first used (SEM and light microscopy) as well as by two key features of shedding in this species: the formation of a distinct

cell wall separation layer (described here for the first time) and the presence of the upper parts of anticlinal cell walls in the shed patches. The latter results in an array of very cell-like, thick cell wall caps. Expanding the repertoire of methods to include CLSM and TEM of chemically fixed and freeze-fixed, freeze-substituted tissue has conclusively demonstrated that epidermal shedding in *Ascophyllum* results from formation of a specialized zone of cell wall separation, followed by detachment of the fouled outer portion, in a process closely resembling that observed by TEM in *Halidrys siliquosa* (Linnaeus) Lyngbye (Fucales, Sargassaceae; Moss 1982).

Cell wall composition and modifications during the shedding cycle

Brown algal cell walls are complex and dynamic networks of polysaccharides, proteins, polyphenolic compounds (phlorotannins), and halide ions, primarily iodide (Schoenwaelder and Wiencke 2000; Andrade et al. 2004; Charrier et al. 2019). These walls evolved independently of those in land plants and other algae (Popper et al. 2011). Alginates and fucose-containing sulfated glucans (fucoidans) are the main polysaccharide components (e.g., Mabeau and Kloareg 1987; Deniaud-Bouët et al. 2017), but minor components include cellulose, β -1,3-linked glucans (Raimundo et al. 2017) and mixed-link glucans (Salmeán et al. 2017), all of which would enable cell wall staining with the glucan-binding fluorescent dyes such as FB28 and SF. TEM studies of brown algae, particularly the Ectocarpales which includes the model species *Ectocarpus siliculosus*, have furnished a basic ultrastructural model of brown algal cell wall structure

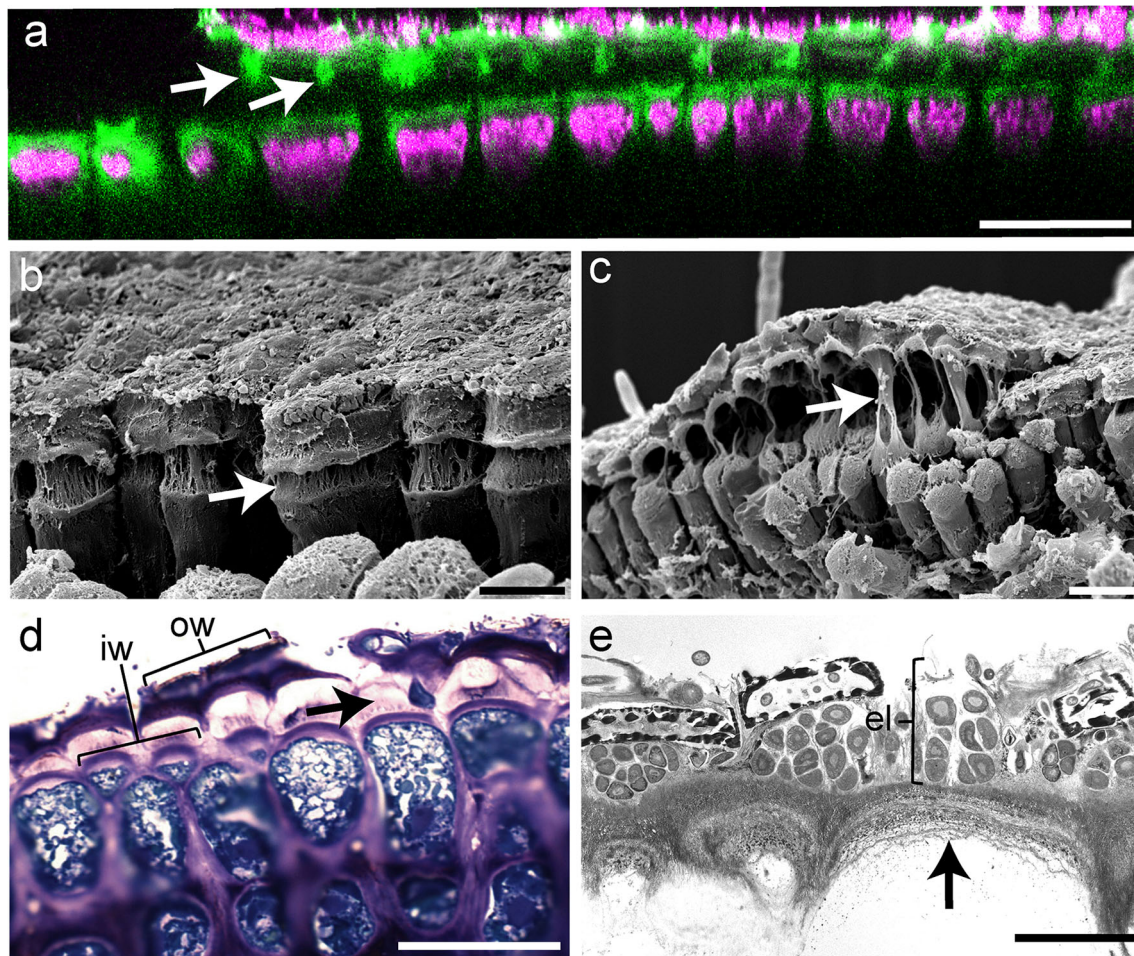


Fig. 5 In epidermal shedding of *Ascophyllum*, an outer layer of each meristoderm apical cell wall separates from an inner layer at a central cell wall separation layer. **a** Transverse CLSM scan of hand section from region of epidermal shedding in which outer apical cell wall layers and upper portions of adjoining anticlinal cell walls (arrows) are detaching from inner cell wall layers (SF, green fluorescence) at unstained central layer of the meristoderm apical cell walls; walls are SF-stained green and cytoplasm RB-stained magenta; the cytoplasm of some RB-stained epibionts is just visible at surface of detaching layer. **b**, **c** SEM of hand sections showing initial **b** and later **c** stages in cell wall separation; short strands (arrow) extending between separating inner and outer apical cell

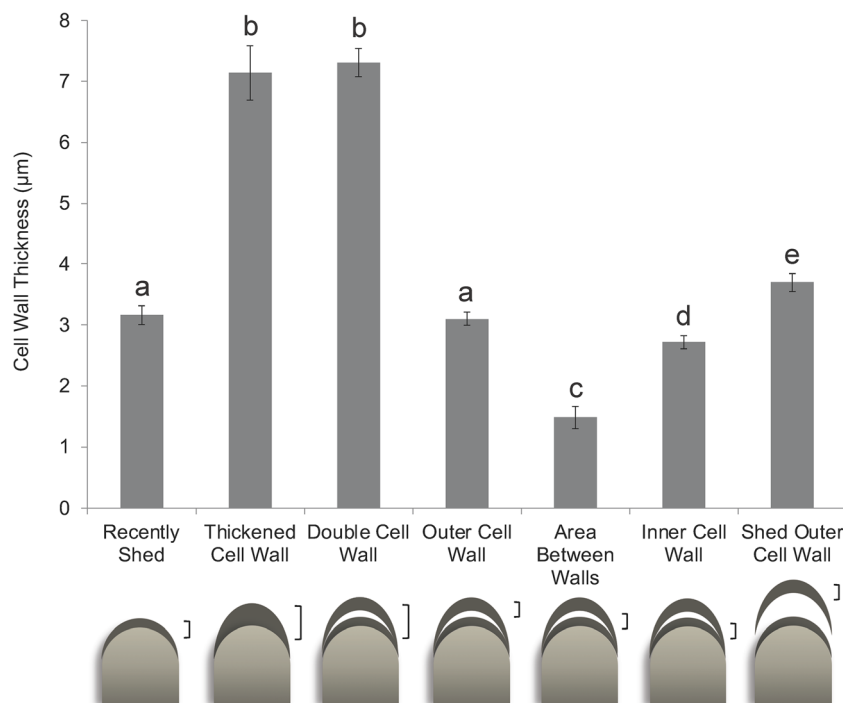
walls in **b** are succeeded by longer strands (arrow) in **c** as cell wall separation progresses, indicating that connecting strands or fibers are deformable. **d** In this TB-stained thick section, lightly stained pink material (arrow) occupies the region of cell separation between darkly stained outer apical cell wall layers (ow) and thinner, more lightly stained inner cell wall layers (iw) that will be exposed to become new frond surface. **e** TEM thin section of shed outer apical cell wall layer has a homogenous and darkly stained surface in contact with the epibionts layer (el), while surface that detached from the central cell wall separation layer has looser, fibrillar appearance (arrow). Scale bars **a** 40 μm , **b** 10 μm , **c** 20 μm , **d** 20 μm , **e** 50 μm

(e.g., Ponce et al. 2007, summarized by Terauchi et al. 2016) that is consistent with the structure of the *Ascophyllum* meristoderm cell apical walls as detailed by Xu et al. (2008). The latter distinguished four layers in thin and relatively unfouled walls. These layers are here called “lamellae” to distinguish them from the outer, inner, and cell wall separation layers identified in *Ascophyllum* through their function in epidermal shedding. Xu et al. (2008) identified an inner darkly stained lamella with closely packed fibrils oriented parallel to the plasma membrane. An outer lamella is weakly stained and described in *Ascophyllum* by Xu et al. (2008) and in *Ectocarpus siliculosus* (Dillwyn) Lyngbye (Terauchi et al. 2016) as “amorphous.” Sandwiched in between are two other lamellae distinguishable by staining or

fibril orientation and density (Xu et al. 2008). Terauchi et al. (2016) concluded that the fibrils observed by TEM in *E. siliculosus* cell walls (and presumably other brown algae) are composed of alginate chains. Since five or six lamellae were observed in thick, fouled apical cell walls that had not yet developed cell wall separation layers (Fig. 3e and f), it is likely that outer and inner fibrillar walls involved in shedding contain more than one lamella.

The transition between thick, uniformly SF-stained cell walls to walls containing the unstained cell wall separation layers may be rapid, as transitional stages were not identified. Since epidermal samples contained fewer examples of cell wall separation layers and separating cell walls than examples of epibiont accumulation and cell wall thickening, the process

Fig. 6 Mean cell wall thickness measurements for different developmental stages leading up to shedding. Schematic diagrams below graph illustrate each stage, and brackets indicate portion of cell wall that was measured from CLSM scans. Error bars represent 95% confidence intervals. $n = 60$ cells for each developmental stage. Different lower case letters indicate significant differences ($p < 0.05$) assessed using the Kruskal-Wallis and Dunn's post hoc test in R



of cell wall separation and shedding may occur soon after formation of the cell wall separation layer. Since there was no significant difference between thick, fouled cell walls before and after the formation of the central cell wall separation layers, formation of the separation layer can be attributed to chemical modification of the preexisting cell wall, perhaps analogous to fruit ripening or organ abscission in angiosperms. Whether the thin upper parts of the anticlinal cell walls are also modified to facilitate detachment before cell wall separation occurs remains to be determined.

The loss of SF staining in the cell wall separation layer indicates a change in cell wall composition. TEM and light microscopy showed that the layer is filled with a non-fibrillar amorphous material that stains lightly with TB, in contrast to the dense fibrillar layers on either side. This appearance is consistent with that of a structurally distinct and weaker region of cell wall, and this is reinforced by the appearance in SEM specimens of deformable strands extending across this region between the outer and inner cell wall layers as they separate. These strands suggest a soft gelatinous composition for the cell wall material in the separation layer. The pink color imparted by TB staining has been considered indicative of sulfated polysaccharides such as fucoidans (McCully 1965). Although alginates are considered the main gel-forming component in brown algal cell walls (Michel et al. 2010), fucoidans are abundant in many mucilages (Scriptsova 2015). To determine the composition of the amorphous material involved in cell wall separation in *Ascophyllum* and to better understand cell wall composition during shedding cycles, we will be taking advantage of recent advances in the

development of new antibodies and immunocytochemical staining techniques that have been developed to label brown algal cell wall epitopes (e.g., Torode et al. 2015, 2016; Raimundo et al. 2017).

The mechanism of cell wall separation

Using TEM images of chemically fixed tissue, Moss (1982) proposed that cell wall separation in *Halidrys siliquosa* involved the accumulation of extracellular vesicles in between the fibrillar inner and outer cell wall layers that pushed the layers apart after breaks occurred in the upper parts of the anticlinal cell walls in between cells. In *Ascophyllum*, vesicles have not been observed in the cell wall separation layer nor does it stain with the lipophilic dye RB. Rather, the development of a non-fibrillar amorphous cell wall layer in the middle of the wall combined with the thinner apical ends of the anticlinal cell walls may be sufficient for environmental factors such as wave action, tidal desiccation, and rehydration cycles to provide the mechanical forces needed to initiate cell wall separation, probably by first breaking the tops of some anticlinal cell walls. However, it cannot be excluded that swelling of the amorphous material as it is exposed to seawater could contribute to cell wall separation. Cell wall swelling is a means to generate force and cause movement in a variety of land plants and algae (Martone et al. 2010). For example, the hydration and rapid swelling of mucilage in some land plant seeds rupture the surrounding cell walls (Western 2012). In brown algae, cell wall swelling underlies gamete expulsion from the receptacles of fucoid algae (Speransky et al. 2001),

and a reversible cell wall swelling is proposed to be integral to normal sieve tube function and phloem transport in kelp (Knoblauch et al. 2016).

What signals regulate *Ascophyllum* epidermal shedding?

Triggers of shedding in *Ascophyllum* are currently unknown, but both external and internal stimuli are likely to be involved. External factors for future investigation are chemical signaling between the host and its colonizing epibionts, as well as reductions in light, nutrients, or gas exchange due to excessive surface fouling. As an example of the former, epibionts of *Ascophyllum* include polysaccharide-degrading bacteria (Martin et al. 2015) opening the possibility for microbe-generated cell wall fragments of *Ascophyllum* to act as elicitors of the shedding response, similar to the way that byproducts of alginate degradation elicit a defense response in kelp (Küpper et al. 2001; Küpper and Carrano 2019).

With respect to internal factors, the surfaces of the younger *Ascophyllum* fronds are a mosaic of areas at different stages of fouling. Epidermal shedding is continuous throughout the year, albeit with periodic increases and decreases, at least during the warmer months indicating some synchronization (Halat et al. 2015). In addition, the height of the individual caps in each patch of shed cell wall material was similar based on measurements of the distance from the top of outer cell walls to the bottom of attached anticlinal cell wall fragments. Therefore, cell wall changes leading to cell wall separation are likely synchronized within a local group of cells. One candidate for coordinating such a response are the reactive oxygen species involved in the oxidative “bursts” which are a key component of the defense response in algae, including the Phaeophyceae (Da Gama et al. 2014; Küpper and Carrano 2019). The epidermal shedding response to epibionts could well involve iodine metabolism and modifications of innate immune response signaling pathways similar to those proposed by Küpper and Carrano (2019) for the brown algae *Laminaria*.

In conclusion, this study clarifies the nature of epidermal shedding in *Ascophyllum* and demonstrates that it involves the shedding of cell wall layers, as reported in a variety of other brown algae. The continuous, year-round shedding of these cell wall patches by *Ascophyllum* makes it a suitable model plant in which to study this unusual phenomenon. By establishing a clear sequence of changes in meristoderm apical cell walls that precede shedding, this work provides a foundation for future investigation of this antifouling mechanism in the broader context of host-epibiont interactions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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