Microscopic and infrared spectroscopic comparison of the underwater adhesives produced by germlings of the brown seaweed species *Durvillaea antarctica* and *Hormosira banksii*

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Adhesives from marine organisms are often the source of inspiration for the development of glues able to create durable bonds in wet environments. In this work, we investigated the adhesive secretions produced by germlings of two large seaweed species from the South Pacific, *Durvillaea antarctica*, also named ‘the strongest kelp in the world’, and its close relative *Hormosira banksii*. The comparative analysis was based on optical and scanning electron microscopy imaging as well as Fourier transform infrared (FTIR) spectroscopy and principal component analysis (PCA). For both species, the egg surface presents peripheral vesicles which are released soon after fertilization to discharge a primary adhesive. This is characterized by peaks representative of carbohydrate molecules. A secondary protein-based adhesive is then secreted in the early developmental stages of the germlings. Energy dispersive X-ray, FTIR and PCA indicate that *D. antarctica* secretions also contain sulfated moieties, and become cross-linked with time, both conferring strong adhesive and cohesive properties. On the other hand, *H. banksii* secretions are complemented by the putative adhesive phlorotannins, and are characterized by a simple mechanism in which all constituents are released with the same rate and with no apparent cross-linking. It is also noted that the release of adhesive materials appears to be faster and more copious in *D. antarctica* than in *H. banksii*. Overall, this study highlights that both quantity and quality of the adhesives matter in explaining the superior attachment ability of *D. antarctica*.

1. Introduction

Adhesive formulations and designs inspired from Nature represent the new frontier for the production of smart, green, biocompatible and sustainable adhesives and sealants. Biological adhesives can be exploited in a vast range of industries including defence, naval, biomedical, biosensor and surgical applications [1]. For example, devices mimicking the attachment of geckos are now a reality in climbing robots [2], whereas biological adhesives derived from mussels are under investigation as surgical tissue adhesive [3]. For these reasons, research interest in biological adhesives is increasingly receiving attention from both the academic and the industrial worlds, in the search for both new ‘sticky’ organisms and new applications [4,5].

Nature offers an extensive range of biological species with adhesive capabilities that can be sourced to seek ideas and inspiration. For example, underwater adhesion is very elegantly achieved by a multitude of organisms such as mussels.

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Different methods have been used to determine the structural characteristics as well as the chemical composition of biological adhesives from seaweed species, including optical [22,23] and electron microscopy (scanning electron microscopy (SEM) [24–26], environmental SEM [27] and transmission electron microscopy [23,28,29]), atomic force microscopy [30–32], quartz crystal microbalance with dissipation [33,34], zeta potential [35], energy dispersive X-ray (EDX) [29,30], chemical extraction [36,37], enzymatic assays [38,39], staining and labelling techniques [23,24,40] and infrared spectroscopy [29]. The preparation protocols as well as the testing procedure of most methods require specific conditions often harmful to the biological sample tested and in most cases lead to the death of the specimen, hence the information obtained is usually a snapshot of the sample at a certain point in time. Among others, attenuated total reflection–Fourier transform infrared (ATR-FTIR) spectroscopy is an effective technique to study biological adhesives, probing only a thin layer of the sticky materials deposited on the optical ATR element. ATR-FTIR is particularly interesting as it is a non-invasive technique that permits the analysis of biological samples under conditions resembling their natural environment, making it possible to maintain the physiological requirements necessary for cell survival. The opportunity offered by ATR-FTIR to study the production of bioadhesives from complex biological species in real time, *in vivo* and *in situ*, has been clearly recognized by two recent reviews by Barlow & Wahl [41] and Petrone [42]. For example, ATR-FTIR has been successfully employed to identify the composition of adhesives from a range of biological species including barnacles [43], bacteria [44], diatoms [30], algae [29] and mussels [45].

In general, brown algal bioadhesives are a complex mixture of different organic compounds, including proteins, carbohydrates, glycoproteins, polyhydroxyphenols and metal ions, mutually interacting through cross-linked bonds as well as electrostatic forces and metal ion bridge complexes [30,46–50]. Most of the organic compounds listed above have been associated with the adhesive simply because they are secreted after fertilization, even though the same compounds probably have other important physiological and ecological roles, e.g. in the formation of the cell wall, prevention of polyspermy, detention of grazing predators, etc. For example, Bitton & Bianco-Peled [51] demonstrated that oxidation and cross-linking mediated by a haloperoxidase enzyme is not strictly required to achieve strong adhesion bonds, questioning the role of the enzyme in algal attachment.

In this work, we have studied and compared the adhesion of recently fertilized zygotes of the two intertidal seaweed species *H. banksii* and *D. antarctica*, with the objective of identifying the key features that make the latter the ‘strongest kelp in the world’. Microscopy was initially employed to clarify the mechanism of release of the glue ingredients in kelp zygotes as well as morphological changes in the adhesive pad. EDX spectroscopy was also considered as a tool to identify specific elements in the attaching holdfast. ATR-FTIR spectroscopy was then employed to characterize the chemical constituent present in the adhesive secretions. Principal component analysis (PCA) was finally applied to the spectroscopic results to find small sources of variance in the spectral features associated with the chemical components involved in the attachment process. PCA is a widely used technique in investigative spectroscopic analysis [52–55]; however, at this point in time it has been used minimally in the field of bioadhesives.

2. Material and methods

2.1. Sample collection and preparation of gamete suspensions

The procedure employed to harvest suspensions of gametes from *H. banksii* and *D. antarctica* reflects the protocol described by Dimartino *et al.* [56]. Briefly, fertile fronds from mature plants were collected at Shag Point, New Zealand (−45°27′48″, 170°
2.2. Cultures of germlings

*D. antarctica* and *H. banksii* zygotes were produced by simple mixing of the two gamete suspensions for around 30 min. The zygotes were inoculated onto the desired substrate and settled under gravity. The resulting cultures were placed in a temperature-controlled chamber at 13°C with 12:12 h light–dark cycles. The light intensity during the light period was of 23 ± 6 µmol photons m⁻² s⁻¹ of photosynthetically active radiation as measured using a 2π quantum sensor coupled to a LI-250A light meter (LI-COR Biosciences, Lincoln, NE). Seawater was changed every 12 h to maintain the culture’s viability. This protocol was effective to maintain the cultures up to at least 140 h. All due care has been devoted during sample preparation to avoid bacterial contamination. However, owing to the wild nature of the samples, this could not be completely prevented. Yet, within the relevant time window of the experiments, the observed growth rate of the microorganisms was much less than the development of the zygotes/germlings, thus its influence on the experimental results was negligible.

In the following, the term zygote refers to fertilized eggs in the very early life stages, whereas germlings will be used to denote the zygotes after settling on the slide surfaces as well as at later developmental stages.

2.3. Optical, electron microscopy and energy dispersive X-ray spectroscopy

Cultures on glass and ZnSe slides (Thermo Fisher Scientific, Auckland, New Zealand, and Harrick Scientific, Horizon, Pleasantville, NY, respectively) were monitored at regular intervals up to 140 h after initial settlement under optical microscopy using a Zeiss Axiosstar Plus microscope equipped with a Canon Powershot A620 camera (Carl Zeiss, North Ryde, Australia). The slides were carefully removed to avoid perturbation that could potentially dislodge loosely adhered germlings. A layer of seawater at 13°C was kept on the slides above the germlings during microscopic observation to minimize sample drying and temperature increase. A total of four replicate slides was observed at each time interval. Once imaged, the slides were dismissed to preclude the influence on successive germling development of environmental disturbances introduced during imaging.

Cultures on Thermax™ coverslips (Thermo Fisher Scientific) were employed for SEM. Four replicate slides of settled eggs as well as of germlings at 1 and 24 h following fertilization were imaged. The samples were fixed in 2.5% glutaraldehyde in seawater, buffered in 0.1% cacodylate and postfixed in 1% osmium tetroxide (Sigma Aldrich, Castle Hill, Australia). The slides were mounted on aluminium stubs, sputter coated with gold/palladium (Emitech K575X, EM Technologies Ltd, Ashford, UK) and imaged under a JEOL 6700 SEM (JEOL Ltd, Tokyo, Japan) using an accelerating voltage of 3 kV. Preliminary information on the elemental composition of the adhesive pad was obtained using the EDX system fitted in the SEM (JEOL 2300F EDS; JEOL Ltd) at an accelerating voltage of 10 kV.

Different sample preparation methods were employed, including freeze-drying, cryo-SEM (Gatan Alto 2500 cryo stage; Gatan Inc., Pleasanton, CA) and critical point drying (CPD; Bal-Tec CPD-030; Bal-Tec AG, Balzers, Liechtenstein). While all preparation methods consistently produced similar morphological features of the samples imaged, freeze-drying often caused cell breakage and a barely visible adhesive footprint, cryo-SEM resulted in germlings detaching from the SEM stubs, whereas CPD preserved the structure of the attached cells and their adhesive secretions. Accordingly, unless otherwise stated, the images presented in the manuscript have been obtained through CPD.

Features in the microscopy images were measured using ToupVIEW software (v. 3.5.563; ToupTek Photonics, Zhejiang, P.R. China). In the Results and discussion section, the average size of objects is reported ± standard deviation (s.d.).

2.4. Attenuated total reflection—Fourier transform infrared spectroscopy

Spectroscopic measurements were carried out with a Digilab ITS-4000 FTIR spectrometer (Digilab LCC, Randolph, MA) equipped with a KBr beamsplitter and DTGS detector and controlled by Digilab Resolutions software (v. 4.0). A horizon ATR-FTIR optical accessory with a ZnSe 13-reflection 50 × 10 × 2 mm (45°) prism (Harrick Scientific, Pleasantville, NY) was mounted in a custom-built flow cell fitted in a temperature-controlled jacket at 13°C in a similar set-up to that depicted previously [45,57]. The penetration depth of the exponentially decaying evanescent wave into the sample is about 0.7 µm at 1500 cm⁻¹ for the ZnSe/seawater interface. All spectra were obtained from 64 co-added scans at 4 cm⁻¹ resolution measured at regular time intervals.

The solutions were fed to the ATR flow cell at a flow rate of 2 ml min⁻¹ using a peristaltic pump placed upstream of the ATR measuring chamber. A spectral background was acquired in an initial equilibration step with flowing seawater at 13°C for 30 min. In the presented absorbance spectra, which may be regarded as difference spectra, the seawater absorptions in both sample and background spectra are effectively removed. After equilibration, 5 ml of the egg suspension was introduced into the flow chamber, where the female gametes settled on the ZnSe surface by gravity forming a relatively uniform monolayer of eggs. Seawater was then flowed for 1 h to allow stable settlement of the eggs on the prism surface, followed by introduction of the sperm suspension for 30 min. Finally, seawater was fed again to wash out excess of sperm, and the first 24 h of germling development was spectrally monitored. It is worth noting here that fertilization is a fast process which occurs in around 10–15 min with high success rates (more than 95%) [58,59]; therefore, it was assumed all viable eggs settled on the prism surface were successfully fertilized.

The potentially noxious effect of ZnSe solutes on the cultures was evaluated using control cultures on glass slides. The cultures were monitored for up to 140 h. Cell division and germling proliferation occurred similarly on both substrates, indicating no apparent toxic effects arising from ZnSe in the early life stages of the germlings.

2.5. Principal component analysis

Principal component analysis (PCA) was carried out on the ATR-FTIR spectra to identify small sources of variance in the spectra. The spectra were first preprocessed using standard normal variate normalization over the spectral region of interest, which was 900–1800 cm⁻¹. PCA was carried out on these normalized spectra.
3. Results and discussion

3.1. Microscopy

The development of *H. banksii* and *D. antarctica* germlings was compared under optical microscopy (figure 1) and SEM (figures 2 and 3) at different times.

Eggs, zygotes and cultured germlings from *D. antarctica* are smaller in size than those of *H. banksii*. In particular, the zygotes have an average diameter of $37 \pm 3 \mu m$ and $66 \pm 1 \mu m$ for *D. antarctica* and *H. banksii*, respectively. This observation offers a first morphological clue for the stronger attachment (i.e. higher survival) of *D. antarctica* germlings in wave-exposed environments. In fact, according to Boullene *et al.* [60], the hydrodynamic forces experienced by a submerged spherical body scale with the cube of its radius. Thus, *H. banksii* germlings will experience hydrodynamic stresses one order of magnitude greater than those acting on the comparatively smaller *D. antarctica* germlings.

Germlings from both species undergo similar growth phases; however, *D. antarctica* seems to develop faster than its close relative *H. banksii*. In particular, 6 h old germlings of *D. antarctica* present apparent polarization and the formation of a rhizoidal tip (figure 1a), whereas the first cellular division occurs between 24 and 36 h after fertilization (figure 1b), with the differentiation of a rhizoidal mother cell and an apical mother cell. After 140 h (6 days), *D. antarctica* germlings have a prolonged body of variable dimensions approximately 90–360 \( \mu m \) long, i.e. 3–10 times the size of the original zygotes. In a few instances, the thallus detaches from the surface, allowing the germlings to stand upright, with attachment to the substrate secured through the rhizoid only (arrows in figure 1c). On the other hand, *H. banksii* germlings grow to a lesser extent and show the first signs of polarization only after around 36 h (figure 1e). Cultures 140 h old display germlings still entirely anchored to the substrate, suggesting a less mature rhizoid not able to guarantee a solid attachment to secure the germling. Morphologically, these are characterized by a bulky apical cell of similar dimensions to the zygote (diameter of head $71 \pm 4 \mu m$) and a thin rhizoidal prolongation $317 \pm 39 \mu m$ long (figure 1f). Accordingly, faster physiological development of *D. antarctica* germlings can be reasonably associated with greater production of adhesive components with respect to *H. banksii* germlings, a second clue to explain the stronger adhesion of the former species over the latter. This hypothesis will be tested in §3.3.

Careful inspection of microscopic images at higher magnification reveals the presence of a mucilaginous coat surrounding the germlings (electronic supplementary material, figure S1). This film fully develops in the first 24 h following fertilization, reaching a maximum observed thickness of the order of $10 \mu m$. The coat appears more abundant on the rhizoidal tip, especially at later times during germling development. This layer has been described as a water-rich hydrogel composed of a range of broadly defined mucopolysaccharides and polyphenolic material responsible for the attachment of the germlings [61]. The characteristics of such an adhesive hydrogel gradually secreted by the germling will be further discussed in §3.3.

The outer surface of *H. banksii* eggs (figure 2a,b) presents a number of polydisperse spherical vesicles (diameter $2.4 \pm 1.4 \mu m$). The eggs are not surrounded by mucilage, mainly to facilitate interaction with and fertilization by sperms [26]; therefore, the protruding vesicles are unprotected in the outermost layer of the eggs.

Immediately after fertilization, all vesicles must be released and a smooth cell wall is formed [62] (figure 2c). It is possible to observe that both germlings in the image achieve a smooth surface, with one partly covered by remnants of sperm and antheridia following the fertilization step. The release of the peripheral vesicles and formation of a smooth cell wall is a distinctive characteristic in the reproduction of brown algae [63,64]. Tilted SEM images 1 h following fertilization do not
reveal signs of adhesive materials linking the germlings to the substrate (micrograph not shown). However, 24 h after fertilization, some isolated connecting threads as well as residual material on the substrate become visible (figure 2d). The threads could be remnants of the adhering hydrogel following the evacuation procedure, possibly indicating a fibrillar structure. Overall, the cell wall remains smooth. These observations suggest that the mucilage formed after fertilization does not leave visible residues after evacuation, indicating that it is a hydrogel extremely rich in water whose spare components collapse uniformly over the germling surface, without altering the smooth appearance of the cell wall.

SEM images of D. antarctica show the same basic features observed on H. banksii. For example, D. antarctica eggs display an irregular surface (figure 3a) which becomes smoother soon after fertilization owing to the formation of a cell wall (figure 3b) [63]. However, distinctive morphological differences are also apparent. In fact, the roughness of D. antarctica eggs is not associated with protruding vesicles; rather, it is associated with peculiar crater-like structures. These may correspond to erupted vesicles as a result of sample preparation. Other SEM images for the eggs show vesicles buried under this external surface, which in this case would represent an outer layer containing the vesicles (electronic supplementary material, figure S2). It is interesting to note that D. antarctica eggs are able to develop a somewhat firm adhesion to substrates even prior to fertilization [65]. Two different hypotheses can be formulated to combine the SEM observations with the preliminary stickiness of the eggs: (i) the vesicles are prone to eruption and release of primary adhesive components or (ii) the outer layer is made of an adhesive substance, and the windows in the shell act as passageways for incoming sperm as well as other biochemical components secreted soon after fertilization.

As early as 1 h after fertilization, the surface of D. antarctica germlings displays a copious number of submicrometric vesicles (diameter 0.26 ± 0.03 μm; figure 3b,c). Germlings observed 96 h after fertilization still exhibit such vesicles. These vesicles are monodisperse in size and uniformly present over the entire germlings' surface, possibly containing precursors of the mucilaginous coat. D. antarctica germlings could have developed a strategy where adhesive vesicles are released in all directions, thus maximizing the adhesion points with the asperities of the natural substrate [66]. In contrast, H. banksii did not display such formations, possibly indicating that the production of the external mucilage is slower or that the hydrogel is weakly bound and washed away during sample preparation. Given the prominent role of the outer mucilage in surface attachment, early adhesion of D. antarctica germlings seems more favoured than those of H. banksii.

A clear adhesive footprint is already visible in the first hours after fertilization (figure 3b). The footprint is constituted by an array of adhesive pads, which are possibly remnants of connective material that changed appearance as a consequence
of sample preparation for SEM imaging. Abundant discharge of adhesive materials continues as the germlings develop. In particular, 24 h after fertilization a layer of fibrils completely covers the rhizoidal tip and connects to the substrate, functioning as a clamp to hold the germling in place (figure 3c). Similar adhesive strands are also present below the apical cell, possibly reflecting analogous chemistry and secretion mechanisms of the adhesive materials over the entire surface of the germling (figure 3d). This observation has also been made about 96 h old germlings (SEM not reported). Structurally, the fibrils extend radially in all directions, covering a considerable distance on the surface away from the germlings (5.9 ± 0.6 μm), with a length of the same order of magnitude as the width of the rhizoidal tip (8.9 ± 1.0 μm). Multiple secondary threads interconnect proximal radial strands, both sideways and vertically, cross-linking the main fibrils. Notable enlargements are present on the nodes between the primary and secondary threads, as well as on the points where the radial fibres adhere to the substrate. This characteristic helps the network to sustain loads without breakage of the internal and external connections. Small traces of adhesive materials are present further away from the main fibrous pad, similar to the adhesive remnants imaged 1 h after fertilization, suggesting a progressive expansion of the attachment pad. The overall morphology of the fibrous network resembles the structure present in spider-silk anchors, with a heterogeneous meshwork of threads rather than a homogeneous adhesive substance [67]. Such a complex structure is essential to dissipate the energy associated with detachment forces and to reduce the risk of crack propagation.

3.2. Energy dispersive X-ray spectroscopy
EDX was also performed on the rhizoidal tip of 24 h old germlings of D. antarctica (electronic supplementary material, figure S3). Traces of K, C, O, Na, S and Ca were detected in the attachment pad. The counts in the C and O peaks contain a strong contribution from the polymeric Thermonox slide used to support the specimens; therefore, quantitative conclusions on the relative amounts of the various elements identified cannot be put forth. Yet, the presence of K, Na, S and Ca is characteristic of the adhesive holdfast. Sulfated polysaccharides are abundantly present in carbohydrates secreted by large seaweed such as carrageenan and fucoidan [68], thus explaining the abundant presence of this element in the EDX spectrum. The presence of sulfated moieties will be further evidenced in §3.3. On the other hand, positive metal ions contained in seawater readily form electrostatic complexes with negatively charged polysaccharides and polyphenolics present in the adhesive secretions [46]. In particular, Ca ions mediate both adhesion and cohesion, e.g. by bridging the negatively charged adhesive secretions with a negatively charged
Figure 4. ATR-FTIR spectra from settled eggs of D. antarctica (bottom line) and H. banksii (top line). Background from seawater.

surface and by favouring gelling of the carbohydrate network [69]. Ca ions have been extensively reported as key components in adhesive secretions in marine organisms, including bacteria [70] and sandcastle worms [71], and will probably play a key role also in brown algal attachment. The absence of a suitably developed adhesive pad in H. banksii germlings prevented EDX being used on this species to complete a full comparison between the two species. Future work will be targeted to the analysis of the footprints of the germlings to clarify the elemental composition of the adhesive pads and the putative role of the various metal ions present.

3.3. Attenuated total reflection—Fourier transform infrared analysis

3.3.1. Infrared spectra from eggs before fertilization

Prior to studying the adhesive produced by the settled germlings, preliminary IR information on the content of the peripheral structures initially present around the eggs is required. The spectra obtained from settled eggs of the two seaweed species in seawater are reported in figure 4. The absorbances are of the same order of magnitude for both species, and spectral differences are predominant. The presence of a peripheral layer of cells in the formation of the adhesive bond mediating cross-linking reactions [46,49] or simply is excreted as a result of other cellular processes.

Figure 5 shows the changes in IR spectra from H. banksii eggs to D. antarctica eggs, with strong bands at 1651 and 1547 cm\(^{-1}\), related to the amide I and II modes of proteins in the \(\alpha\)-helix conformation [78].

In conclusion, the two seaweed species share only IR signatures of carbohydrate-related compounds. In addition, while H. banksii eggs contain phlorotannin-bound constituents in its periphery, the D. antarctica surface layer contains protein molecules and sulfated carbohydrates. Phlorotannins have been reported to be constituents of algal adhesives [79], but need to be cross-linked to achieve the optimal adhesive properties [46,80]. These are likely to be stored in the peripheral vesicles of H. banksii eggs, ready to be released in the extracellular environment upon fertilization [48,58]. Vesicles containing phlorotannins have also been reported for D. antarctica [81,82], but they seem to be contained within the eggs thus not probed by the IR evanescent wave [64]. Interestingly, ionic moieties present in the carbohydrate chains have a strong tendency to form stable complexes with metals and metal ions [83]. In addition, negatively charged polysaccharides are highly hygroscopic and form stable hydrogels in the presence of divalent metal ions such as Ca\(^{2+}\) and Mg\(^{2+}\) present in seawater [84]. This mechanism is consistent with the EDX results previously presented. Accordingly, the observed tendency of D. antarctica eggs to bind to substrates prior to fertilization might be explained by the presence of an adhesive layer composed of a fraction of partially secreted sulfated polysaccharides. The high protein component present in D. antarctica may be involved in the formation of the adhesive layer mediating cross-linking reactions [46,49] or simply is excreted as a result of other cellular processes.

3.3.2. Infrared spectra from Hormosira banksii germlings

Our previous microscopic observations, as well as previous studies on the formation of the adhesive mucilage [61], indicate that a connective layer of the order of a couple of micrometres thick is being produced between the germling and a substrate following fertilization. Accordingly, we hypothesize that the substances interacting with the surface, i.e. those probed by the IR beam, are the ones mostly involved in the adhesion process.

Figure 5 shows the changes in IR spectra from H. banksii germlings in the 24 h following fertilization. These results are
reported as difference spectra obtained by subtracting the initial ATR-FTIR spectrum recorded at the end of the fertilization stage. Accordingly, any spectral contribution from sperm suspension is removed in the subtraction and the spectral evolution considered is representative of germinating development only. The spectra are very similar to spectra of bacterial species, with distinct bands at 1651 (amide I), 1544 (amide II), 1454 (C–H deformation), 1400 (symmetric carboxylate stretch) and 1245 cm\(^{-1}\) (amide III, P=O and C–O–C stretch) [85]. Some spectral changes are also visible in the 1100–1000 cm\(^{-1}\) region characteristic of polysaccharides (C–O stretch, C–C stretch, C–O–H stretch and bend, and ring vibrations). It is important to note that these changes are only a direct consequence of the fertilization, as the spectra of the eggs before fertilization did not change with time.

PCA was carried out on the spectra of \(H.\) banksii germlings to identify the major spectral changes in the first 24 h after fertilization. The first principal component (PC1) shows a trend of sample evolution over time with early time points in negative PC1 space through to later time points in positive PC1 space (figure 6a). This principal component is describing 97% of the spectral variance in the sample and is the most important for looking at changes in the spectra. The spectral features associated with this separation are shown in the loadings plots (figure 9a). The absorbance maxima were normalized to 1 to facilitate the comparison with PC1 (figure 7). All bands follow a similar trend as the evolution of PC1, indicating that adhesion of \(H.\) banksii germlings follows a relatively simple process where all components are released with the same rate. This result also confirms that the univariate and multivariate approaches employed result in the same observations. Interestingly, the trends follow a sigmoidal shape, with a slow release of adhesive components in the first few hours, followed by a rapid increase between approximately 5 and 15 h post fertilization, and concluded by a final slow release reaching a plateau at the end of the period probed. Adhesion experiments performed by Taylor et al. [65] and Dimartino et al. [21] on \(H.\) banksii zygotes demonstrated that little to no adhesion was achieved in the first 6 h after fertilization, whereas higher adhesion strength is attained after approximately 12 h after fertilization. Our FTIR results corroborate these findings from a chemical perspective. In fact, out of the total adhesive components released in the first 24 h, only 20% are secreted in the first 6 h, whereas more than 50–60% are released at approximately 12 h after fertilization.

In general, the production of proteinaceous material is prevalent over other biological constituents, with bands at around 1650 and 1530 cm\(^{-1}\) typical of protein in the \(a\)-helix conformation. Protein-based adheres are often reported as key components in the adhesive secretions of a number of underwater adhesive organisms such as mussels [87], barnacles [88] or arthropods [6]. The increase of the 1245 cm\(^{-1}\) band is mainly related to amide III absorption, with a possible contribution from post-translational modified amino acids with sulfated and phosphorylated moieties. Similar post-translational modifications are in fact typical of marine adhesive proteins [89,90].

Finally, after about 18 h, the system seems to have achieved a relatively stable steady state, with a considerably slower production of all adhesive materials.

3.3.3. Infrared spectra from Durvillaea antarctica germlings

Figure 8 presents the spectral evolution of \(D.\) antarctica germlings in the 24 h following fertilization. As observed in \(H.\) banksii germlings, the spectra for \(D.\) antarctica also present features typical of biological species, with distinct bands at 1651 (amide I), 1544 (amide II), 1454 (C–H deformation), 1400 (symmetric carboxylate stretch) and 1245 cm\(^{-1}\) (amide III, P=O and C–O–C stretch). In this case, the spectral changes in the 1100–1000 cm\(^{-1}\) region, characteristic of polysaccharides (C–O stretch, C–C stretch, C–O–H stretch and bend, and ring vibrations), are much more apparent.

PCA was carried out on the spectra from \(D.\) antarctica germlings to identify the major spectral changes occurring in the first 24 h after fertilization. As with the PCA of \(H.\) banksii, PC1 shows a trend of sample evolution over time with early time points in negative PC1 space through to later time points in positive PC1 space (figure 9a). This principal component describes 84% of the spectral variance in the sample and is the most important for looking at changes in the spectra. The spectral features associated with this separation are shown in the loadings plots (figure 9b). The negative PC1 features at 999, 1032, 1069 (broad, shoulder) and a broad envelope between 1170 and 1290 cm\(^{-1}\) are stronger features in the spectra collected from earlier time points. These bands are associated with vibrational modes of}
polysaccharides and sulfated compounds present on the eggs and prominent during the early stages after fertilization. The positive PC1 features at 1541, 1558 and 1653 cm$^{-1}$ are more dominant in the spectra collected from later time points and are consistent with amide II and amide I features, respectively, indicative of protein-based secretions in the adhesive.

PC2 accounts for a further 10% of explained spectral variance and separates early-stage zygote growth (negative PC2) from the middle time points (positive PC2), with later time points in neutral PC2 space. The spectral features associated with separation in positive PC2 space include features at 1015, 1051, 1094 (polysaccharides), 1225 ($\nu_3$SO$_2$), 1250 (amide III), 1360 and 1653 (amide I) cm$^{-1}$. The negative separating features are observed at 988, 1072, 1119 and 1184 cm$^{-1}$. It is clear from the loadings plot that baseline variation is also described by this principal component. PC2 shows temporal behaviour that suggests an increase or change in band intensities in the amide III and polysaccharide region. At later times, these changes are less distinct with evidence of band broadening particularly in the polysaccharide region. This latter finding is not inconsistent with cross-linking, which creates a variety of environments (and thus wavenumbers) for these vibrational modes.

The normalized kinetics of PC1 together with the main bands for the adhesive secretions from $D.\text{antarctica}$ are reported in figure 10. The different trends followed by the kinetic profiles also suggest that $D.\text{antarctica}$ produces adhesive components in a stepwise fashion. In particular, carbohydrate-related molecules with absorption in the 1000–1100 cm$^{-1}$ range are released first and at a relatively higher rate. In addition, with respect to what we observed with $H.\text{banksii}$, in this case the area of the 1000–1100 cm$^{-1}$ envelope at the end of the 24 h period is approximately 10-fold higher, indicating that $D.\text{antarctica}$ secretes a much larger amount of polysaccharides. The band at 1245 cm$^{-1}$ develops with similar kinetics to that of the carbohydrate envelope, indicating that this band is directly related to the carbohydrate secretions and, therefore, is assigned to sulfated moieties in polysaccharides. Protein-based compounds with an $\alpha$-helix configuration (typical peaks for amide I and II at 1645 and 1546 cm$^{-1}$) are released in the second phase of the adhesion process. The amount of protein material secreted at the end of the 24 h absorbs with similar intensity to that measured on $H.\text{banksii}$ germlings. The kinetic evolution of the protein-related bands does not seem to be correlated to other developing bands, albeit some minor peaks are likely to be masked by the main spectral bands.

Accordingly, $D.\text{antarctica}$ germlings initially secrete negatively charged carbohydrates as the primary adhesive, and do
so in much larger absolute quantities than *H. banksii* germlings. Protein-related constituents are released at later stages, as secondary adhesive and possibly with cross-linking functions of the previously deposited carbohydrate network. This mechanism is consistent with the microscopy results and the release of two different surface vesicles with time. Proteins have been associated with the cross-linking of carbohydrates in the cell wall of higher plants [91] and algae [92], usually through esterification reactions between uronic acid residues and hydroxyl groups on a neighboring polysaccharide chain, but also through other more complex mechanisms [93]. The specific chemistry of the polysaccharides involved in the adhesion process, and in particular the abundance of side groups such as carboxyl or sulfate, opens up the opportunity for a new variety of ester-based cross-linking modes in the adhesive hydrogel. Interestingly, all bands display a slight reduction in the production rate at the end of the 24 h period monitored, but components were still abundantly secreted in the external environment and interacting with the prism surface, overall indicating a more copious and longer release of adhesive components in *D. antarctica* than previously observed in *H. banksii*.

### 3.3.4. Comparison of infrared spectra from germlings of both species

The spectra from both *H. banksii* and *D. antarctica* germlings were analysed with PCA simultaneously to find the major spectral and hence chemical differences between these two species (figure 11). Like the individual PCAs, the PC1 is the most important to describe the spectral variation of the samples over time (70% of spectral variance), with earlier spectra in negative PC1 space and later time points in positive PC1 space. The negative features are observed at 1001 (ν(C–O–H)), 1034, 1042 (ν(C–O–C) and ν(CC)), 1063 (ν(C–O–C) and ν(CC)), 1111 and a broad feature of approximately 1202 cm\(^{-1}\) (ν(C–O–C) aryl–aryl ether), which can be collectively attributed to polysaccharide content of the egg/zygote before secretion of the mucoadhesives. The positively separating features are observed at 1545 (broad) and 1653 cm\(^{-1}\), which are attributed to amide II and amide I, respectively, and believed to be the major component of the adhesive secretions. PC2 accounts for a further 24% of the spectral variance, and is paramount to highlight small variations in the spectra from these two species. This principal component separates *D. antarctica* in positive PC2 space and *H. banksii* in negative PC2 space. The positive PC2 features, associated with *D. antarctica*, were observed at 1001, 1030, 1107 and 1246 (broad) cm\(^{-1}\), and are characteristic for sulfated polysaccharides. The main negative features, associated with *H. banksii*, were observed at 1200, 1474 and 1618 cm\(^{-1}\), and are indicative of polyphenolic molecules. The variance in the amide I region is difficult to interpret owing to interference from water signals.

PCA suggests that the production of adhesive materials follows similar stages in both *H. banksii* and *D. antarctica*, with charged (mostly sulfated) polysaccharides initially present in the egg surface followed by a prominent production of proteins. This process indicates that the carbohydrate molecules are responsible for creating quick adhesive and cohesive bonds, followed by slower formation of stronger and more durable bonds through the protein secretions. The sulfated polysaccharides must be flexible and able to...

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**Figure 9.** PCA of the IR spectra collected during the development of *D. antarctica* germlings. Pale samples represent earlier time points and darker represent later time points. (a) The scores plot represents 94% of spectral variance. (b) The loadings plots describe the spectral features contributing to spectral variance in comparison with some representative spectra. (Online version in colour.)

**Figure 10.** Band kinetics of developing germlings of *D. antarctica* normalized to 1. (Online version in colour.)
penetrate the layer of biofouling molecules (mostly uronic acids) ubiquitously present in marine environments [94]. The sulfated polysaccharides could be related to adhesion to the substrate through adsorption and formation of stable interactions with the surface. Because of the high concentration of divalent metal ions in seawater, and as supported by the EDX results, it is reasonable to believe that the polysaccharide matrix initially bonds through cationic bridges which can induce gelation.

Still, significant differences are found in the production mechanisms of the adhesive molecules. For example, carbohydrates are produced in *H. banksii* at a slower rate than in *D. antarctica*. But the most striking difference is the amount of material produced, especially with regards to the polysaccharide constituents, which are 10 times more prominent in *D. antarctica* than in *H. banksii*. This observation is in line with the microscopy results, where the more rapid development of *D. antarctica* germlings is plausibly associated with a larger release of adhesive components. Accordingly, it is reasonable to state that the adhesive fibrils observed in the SEM are mostly composed of cross-linked non-soluble carbohydrates. On the other hand, the protein component may have an important role in the direct adhesion with the surface, as well as in the formation of cohesive bonds.

4. Conclusions

In this work, the adhesive secretion from germlings of *D. antarctica* and *H. banksii* has been investigated through microscopy and spectroscopy methods. It is worth noticing that the adhesive secretions were investigated in situ, in vivo and in real time, thus offering novel and interesting insights into bioadhesives as well as confirming the strong capabilities of the experimental methods employed. Overall, the two species display many similarities in the release mechanism and in the main biological constituents of the bioadhesive. Significant differences have also been highlighted, offering important clues for the understanding of the superior attachment performance of the adhesive from *D. antarctica* with respect to that from *H. banksii*.

In general, the adhesive components are initially stored within vesicles present in the outer surface of the eggs. Following fertilization, the germlings produce a secondary mucoadhesive through the release of new morphologically different vesicles. These are particularly abundant in *D. antarctica* but are not detected in *H. banksii*, probably because of a slower and less copious release. The adhesive pad has a characteristic fibrillar structure, composed of a heterogeneous meshwork of threads paramount to dissipate hydrodynamic and other detachment forces as well as minimize crack propagation. This structure is particularly complex and well established in *D. antarctica* germlings even after a few hours after fertilization, whereas it starts appearing in *H. banksii* only after at least 24 h. The difference in the amount and rate of the adhesive released is further confirmed by our ATR-FTIR results, and partly explains the improved adhesion of germlings from *D. antarctica* over those from *H. banksii*.

From a chemical standpoint, PCA reveals that the key compounds shared in the adhesive from the two species are polysaccharides, present in the primary egg vesicles and initially released, and proteins, secreted at later stages during germling development. However, the IR spectra of *D. antarctica* germlings indicate that their carbohydrates bear sulfated moieties, thought to mediate strong adhesive properties and cohesive characteristics. In addition, PCA suggests that cross-linking reactions, possibly facilitated by the proteins later secreted, cure the carbohydrate network, thus furthering the strength of the adhesive from *D. antarctica*. On the other hand, the IR spectra of *H. banksii* adhesive is specifically characterized by distinctive features for phlorotannins, a putative seaweed adhesive, but no other clues of rapid cross-linking reactions or other adhesive constituents are apparent.

Complex polysaccharides are abundant in seaweed and fulfill many of their biological and ecological functions [15]; therefore, it is reasonable that carbohydrate species may
have a strong role also in seaweed attachment. The particular role that sulfated polysaccharides may have in the adhesion process is consistent with the formation of stable interactions with a surface and within the carbohydrate network through electrostatic and metal complex bridges.

Currently, the prominent opinion for seaweed-based adhesives describes the proteins later secreted as enzymes mediating cross-linking of the polyphenolic molecules. However, our IR results indicate that polyphenols, often invoked in the adhesion of seaweed species [47,95], are not of primary importance in the adhesion processes, and are more likely to be involved in other physiological processes. Levi & Friedlander [36] have instead proposed that protein constituents have a similar structure to vitronectin, one of the glycoproteins responsible for cell–cell bonding in other adhesion systems. In this work, we speculate that specific enzymes cross-linking the carbohydrate network are also produced, leading to the cohesive strength required to secure the germlings to the substrate and fostering the performance of the adhesive.

This work helped to clarify the mechanism of release and the key biochemical features of the strong adhesives produced by kelps. The results presented will help the design and synthesis of biomimetic counterparts, with application in wet and complex environments such as in medicine. In the future, molecular biology methods will be employed to assess the amino acid composition and sequence of the adhesive and cross-linking proteins probed, thus clarifying their role in the adhesive formulation. Further investigation into the specific functions of the polyphenolic fraction and its possible interaction with the proteins is also of relevance and will constitute material for future research. These objectives coupled with the assessment of the attachment performances of adhesives isolated from the two brown seaweeds will further support the development and ideally real-word application of permanent biomimetic adhesives.

Authors’ contributions. S.D. designed and coordinated the study, carried out all experiments, participated in data analysis and wrote the manuscript; D.M.S. helped in the ATR-FTIR experiments and related data analysis; S.J.F.-M. and K.C.G. carried out the principal component analyses and helped draft the manuscript; A.J.M. helped in the data analysis and drafting the manuscript. All authors gave final approval for publication.

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