



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

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

Citation for published version:

Dimartino, S, Savory, D, Fraser, S, Gordon, K, Gordon, K & McQuillan, J 2016, 'Microscopic and IR spectroscopic comparison of the underwater adhesives produced by germlings of the brown seaweed species *Durvillaea antarctica* and *Hormosira banksii*' *Journal of the Royal Society Interface*. DOI: 10.1098/rsif.2015.1083

Digital Object Identifier (DOI):

[10.1098/rsif.2015.1083](https://doi.org/10.1098/rsif.2015.1083)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Journal of the Royal Society Interface

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



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

3 Simone Dimartino^{1,2,*}, David M. Savory³, Sara J. Fraser-Miller^{3,4},
4 Keith C. Gordon^{3,4}, A. James McQuillan³

5
6 ¹ Department of Chemical and Process Engineering, University of Canterbury, Private Bag
7 4800, Christchurch 8140, New Zealand

8 ² Biomolecular Interaction Centre, University of Canterbury, Private Bag 4800, Christchurch
9 8140, New Zealand

10 ³ Department of Chemistry, University of Otago, P.O. Box 56, Dunedin 9054, New Zealand

11 ⁴ The Dodd-Walls Centre, University of Otago, Dunedin 9054, New Zealand

12 * Corresponding author: simone.dimartino@canterbury.ac.nz

13
14 **Abstract**

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

33
34 **Keywords**

35 Adhesive, brown algae, microscopy, ATR-FTIR, principal component analysis.

36

37 **1. Introduction**

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

46 Nature offers an extensive range of biological species with adhesive capabilities which can
47 be sourced to seek ideas and inspiration. For example, underwater adhesion is very elegantly
48 achieved by a multitude of organisms such as mussels [6], barnacles [7], sandcastle worms
49 [8], sea urchins [9] and starfishes [10] to name a few. All of these species are able to firmly
50 adhere in wet environments, either temporary or irreversibly, an achievement that humans still
51 struggle to successfully mimic.

52 The attachment achieved by brown algae is particularly fascinating. For example, adult
53 plants of the giant kelps *Macrocystis pyrifera* and *Nereocystis luetkaena* [11] and the fucoid
54 *Durvillaea antarctica* [12] can easily be longer than 10 m and weigh more than 50 kg. They
55 are able to produce a strong, underwater, irreversible and long-lasting attachment with a
56 surface, able to withstand the severe hydrodynamic drags associated with wave action and
57 tidal currents [13,14]. These features make brown algae especially interesting in the bio-
58 adhesion arena. In particular, *Durvillaea antarctica* and *Hormosira banksii* thrive in the
59 intertidal zone, i.e. in wave-exposed areas where the hydrodynamic drag is highest. These two
60 species are closely related, belonging to the most evolutionally developed species within the
61 class of the Phaeophyceae. They display characteristic dichotomous reproduction, i.e.
62 sexually distinct plants releasing separate male (sperm) and female (eggs) gametes, with
63 consequent production of a zygote upon mating of the two gametes [15]. However, the two
64 plants are distinguished, among other features, by their different location in the intertidal
65 habitat and in their size: while *H. banksii* is relatively small, with an average size of 40 cm,
66 and mostly present in relatively wave-sheltered locations [16,17], *D. antarctica* can be as long
67 as 10 m and thrives only in extremely wave-exposed areas [12,18]. This observation leads to
68 the postulate that the two species have different attachment mechanisms. Interestingly,

69 Stevens *et al.* suggested that *D. antarctica* might be “the strongest kelp in the world” because
70 of its particularly high attachment strength and the mechanical properties of its thallus [19].
71 Taylor *et al.* carried out basic adhesion studies on brown algae zygotes aimed to explain
72 ecological differences in gamete dispersal and zygote settlement, demonstrating that
73 attachment of *D. antarctica* zygotes is greater than that presented by *H. banksii* even in the
74 early stages of their life cycles [20].

75 The use of zygotes for the determination of the adhesion characteristics of seaweed is
76 particularly interesting. While adult seaweeds cling onto surfaces by a combination of
77 mechanical (interlocking with surface asperities in the mm scale) and chemical interactions,
78 zygotes must primarily rely on chemically-based adhesion. For example, Dimartino *et al.*
79 employed a laminar flow cell to estimate the adhesion strength of settled zygotes of *H. banksii*
80 while excluding the mechanical component of their attachment [21]. In addition, even though
81 gametes do not have adhesive abilities, the secretion of bio-adhesive components is
82 immediately triggered upon fertilization to maximize survival rates in the harsh natural
83 environment. For these reasons, zygotes represent an ideal system to further study the
84 attachment characteristics of the glue produced by large seaweed.

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

103 identify the composition of adhesives from a range of biological species including barnacles
104 [43], bacteria [44], diatoms [30], algae [29], and mussels [45].

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

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

127

128 **2. Materials and Methods**

129

130 **2.1. Sample collection and preparation of gamete suspensions**

131 The procedure employed to harvest suspensions of gametes from *H. banksii* and *D.*
132 *antarctica* reflects the protocol described by Dimartino *et al.* [56]. Briefly, fertile fronds from
133 mature plants were collected at Shag Point (-45° 27' 48" , 170 ° 49' 20") in August 2013,
134 transported in a chilly bin and thoroughly washed with 2 µm filtered and UV-treated seawater.
135 Each frond was stored in a separate plastic bag and kept in the dark at 4 °C for a period of
136 between 12 and 48 h, followed by thermal and light shock treatment using 2 halogen portable

137 floodlights (500 W each) to stimulate gamete release. Sex of the gametes released is
138 determined by visual inspection of the exudates, i.e. white and orange for male specimen of
139 *D. antarctica* and *H. banksii*, respectively, and olive brown for female gametes of both
140 species. The various gametes were harvested by washing the blades in separate reservoirs
141 containing sterile seawater at 13 °C. The suspensions obtained were filtered through plankton
142 nets (mesh size was 105 and 25 µm for eggs and sperm, respectively) and further clarified by
143 three subsequent sedimentation cycles under gravity. At all times, fronds, labware and
144 suspensions were carefully manipulated to avoid anticipated contact of eggs and sperm
145 leading to uncontrolled fertilization.

146

147 **2.2. Cultures of germlings**

148 *D. antarctica* and *H. banksii* zygotes were produced by simple mixing of the two gamete
149 suspensions for around 30 min. The zygotes were inoculated onto the desired substrate and
150 settled under gravity. The resulting cultures were placed in a temperature controlled chamber
151 at 13 °C with 12 h:12 h light dark cycles. The light intensity during the light period was of 23
152 ± 6 µmol photons m⁻² s⁻¹ of photosynthetically active radiation as measured using a 2π
153 quantum sensor coupled to a LI-250A light meter (LI-COR, Lincoln, NE, USA). Seawater
154 was changed every 12 h to maintain the culture's viability. This protocol was effective to
155 maintain the cultures up to at least 140 h. All due care has been devoted during sample
156 preparation to minimize bacterial contamination. However due to the wild nature of the
157 samples this could not be completely prevented. Yet, within the relevant time window of the
158 experiments, the observed growth rate of the microorganisms was much less than the
159 development of the zygotes/germlings, thus its influence on the experimental results was
160 negligible.

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

164

165 **2.3. Optical, electron microscopy and energy dispersive X-ray spectroscopy**

166 Cultures on glass and ZnSe slides (Thermo Fisher Scientific, Auckland, New Zealand,
167 and Harrick Scientific, Horizon, Pleasantville, NY, USA, respectively) were monitored at
168 regular intervals up to 140 h after initial settlement under optical microscopy using a Zeiss
169 Axiostar Plus microscope equipped with a Canon Powershot A620 camera (Carl Zeiss, North
170 Ryde, Australia). The slides were carefully removed from the seawater bath to avoid

171 turbulence that could potentially dislodge loosely adhered germlings. A layer of seawater at
172 13 °C was kept on the slides above the germlings during microscopic observation to minimize
173 sample drying and temperature increase. A total of 4 replicate slides were observed at each
174 time interval. Once imaged, the slides were dismissed to preclude the influence on successive
175 germling development of environmental disturbances introduced during imaging.

176 Cultures on Thermanox™ coverslips (Thermo Fisher Scientific, Auckland, New Zealand)
177 were employed for scanning electron microscopy (SEM). Four replicate slides of settled eggs
178 as well as of germlings at 1 and 24 h following fertilization were imaged. The samples were
179 fixed in 2.5 % glutaraldehyde in seawater, buffered in 0.1 % cacodylate and postfixed in 1 %
180 osmium tetroxide (Sigma Aldrich, Castle Hill, Australia). The slides were mounted on
181 aluminum stubs, sputter coated with gold/palladium (Emitech K575X, EM Technologies Ltd,
182 Kent, UK) and imaged under a JEOL 6700 SEM (JEOL Ltd, Tokyo, Japan) using an
183 accelerating voltage of 3 kV. Preliminary information on the elemental composition of the
184 adhesive pad were obtained using the EDX system fitted in the SEM (JEOL 2300F EDS,
185 JEOL Ltd, Tokyo, Japan) at an accelerating voltage of 10kV.

186 Different sample preparation methods were employed, including freeze-drying, cryo-SEM
187 (Gatan Alto 2500 cryo stage, Gatan Inc, Pleasanton, CA, USA) and critical point drying
188 (CPD, Bal-Tec CPD-030, Bal-Tec AG, Balzers, Liechtenstein). While all preparation
189 methods consistently produced similar morphological features of the samples imaged, freeze
190 drying often caused cell breakage and a barely visible adhesive footprint, cryo-SEM resulted
191 in germling detaching from the SEM stubs, while CPD preserved the structure of the attached
192 cells and their adhesive secretions. Accordingly, unless otherwise stated, the images presented
193 in the manuscript will be as obtained through CPD.

194 Features in the microscopy images were measured using ToupView software (v 3.5.563,
195 ToupTek Photonics, Zhejiang, P.R.China). In the Results and Discussion section the average
196 size of objects is reported \pm standard deviation (SD).

197

198 **2.4. ATR-FTIR spectroscopy**

199 Spectroscopic measurements were carried out with a Digilab FTS-4000 FTIR spectrometer
200 (Digilab, Marlborough, MA, USA) equipped with a KBr beamsplitter and DTGS detector and
201 controlled by Digilab Resolutions Software (v 4.0). A Horizon ATR-FTIR optical accessory
202 with a ZnSe 13-reflection 50 x 10 x 2 mm (45°) prism (Harrick Scientific, Pleasantville, NY,
203 USA) was mounted in a custom built flow cell fitted in a temperature controlled jacket at 13
204 °C in a similar setup as depicted previously [45,57]. The penetration depth of the

Deleted: d

Deleted: helped

207 exponentially decaying evanescent wave into the sample is about 0.7 μm at 1500 cm^{-1} for the
208 ZnSe/seawater interface. All spectra were obtained from 64 co-added scans at 4 cm^{-1}
209 resolution measured at regular time intervals.

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

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

227

228 **2.5. Principal component analysis**

229 PCA was carried out on the ATR-IR spectra to identify small sources of variance in the
230 spectra. The spectra were first preprocessed using standard normal variate (SNV)
231 normalization over the spectral region of interest which was 900 to 1800 cm^{-1} . PCA was
232 carried out on these normalized spectra with full cross validation for outlier identification.
233 The two species were analysed both separately and together to identify variance within and
234 between the two species. The preprocessing and PCA analysis were carried out using the
235 Unscrambler X V13 (Camo, Norway).

236

237 **3. Results and discussion**

238

239 **3.1 Microscopy**

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

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

250 Germlings from both species undergo similar growth phases, however *D. antarctica* seems
251 to develop faster than its close relative *H. banksii*. In particular, 6 h old germlings of *D.*
252 *antarctica* present apparent polarization and the formation of a rhizoidal tip (Figure 1A),
253 while the first cellular division occurs between 24 and 36 h after fertilization (Figure 1B),
254 with the differentiation of a rhizoidal mother cell and an apical mother cell. After 140 h (6
255 days), *D. antarctica* germlings have a prolonged body of variable dimensions approximately
256 90–360 μm long, i.e. 3 to 10 times the size of the original zygotes. In a few instances the
257 thallus detaches from the surface allowing the germlings to stand upright, with attachment to
258 the substrate secured through the rhizoid only (arrows in Figure 1C). On the other hand, *H.*
259 *banksii* germlings grow to a lesser extent and show first signs of polarization only after
260 around 36 h (Figure 1E). Cultures 140 h old display germlings still entirely anchored to the
261 substrate, suggesting a less mature rhizoid not able to guarantee a solid attachment to secure
262 the germling. Morphologically, these are characterized by a bulky apical cell of similar
263 dimensions as the zygote (diameter of head $71 \pm 4 \mu\text{m}$) and a thin rhizoidal prolongation 317
264 $\pm 39 \mu\text{m}$ long (Figure 1F). Accordingly, faster physiological development of *D. antarctica*
265 germlings can be reasonably associated with greater production of adhesive components with
266 respect to *H. banksii* germlings, a second clue to explain the stronger adhesion of the former
267 species over the latter. This hypothesis will be tested in the IR section.

268 Careful inspection of microscopic images at higher magnification reveals the presence of a
269 mucilaginous coat surrounding the germlings (Fig. S1). This film fully develops in the first 24
270 h following fertilization, reaching a maximum observed thickness in the order of 10 μm . The
271 coat appears more abundant on the rhizoidal tip, especially at later times during germling
272 development. This layer has been described as a water-rich hydrogel composed of a range of
273 broadly defined mucopolysaccharides and polyphenolic material responsible for the

274 germlings attachment [61]. The characteristics of such adhesive hydrogel gradually secreted
275 by the germling will be further discussed in the IR section.

276 The outer surface of *H. banksii* eggs (Figure 2 A and B) presents a number of polydisperse
277 spherical vesicles (diameter $2.4 \pm 1.4 \mu\text{m}$). The eggs are not surrounded by mucilage, mainly
278 to facilitate interaction with and fertilization by sperms [26], therefore the protruding vesicles
279 are unprotected in the outermost layer of the eggs.

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

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

308 As early as 1 h after fertilization, the surface of *D. antarctica* germlings displays a copious
309 number of sub-micrometric vesicles (diameter $0.26 \pm 0.03 \mu\text{m}$, Figure 3B and C). Germlings
310 observed 96 h after fertilization still exhibit such vesicles. These vesicles are monodisperse in
311 size and uniformly present over the entire germlings' surface, possibly containing precursors
312 of the mucilaginous coat. *D. antarctica* germlings could have developed a strategy where
313 adhesive vesicles are released in all directions, thus maximizing the adhesion points with the
314 asperities of the natural substrate [66]. In contrast, *H. banksii* did not display such formations,
315 possible indication that the production of the external mucilage is slower, or that the hydrogel
316 is weakly bound and washed away during sample preparation. Given the prominent role of the
317 outer mucilage in surface attachment, early adhesion of *D. antarctica* germlings seems more
318 favored than those of *H. banksii*.

319 A clear adhesive footprint is already visible in the first hours after fertilization (Figure 3B).
320 The footprint is constituted by an array of adhesive pads, possible remnants of connective
321 material that changed appearance as a consequence of sample preparation for SEM imaging.
322 Abundant discharge of adhesive materials continues as the germlings develop. In particular,
323 24 h after fertilization a layer of fibrils completely covers the rhizoidal tip and connects to the
324 substrate, functioning as a clamp to hold the germling in place (Figure 3C and D). Similar
325 adhesive strands are also present below the apical cell, possibly reflecting analogous
326 chemistry and secretion mechanisms of the adhesive materials over the entire surface of the
327 germling. This observation has also been made about 96 h old germlings (SEM not reported).
328 Structurally, the fibrils extend radially in all directions, covering a considerable distance on
329 the surface away from the germlings ($5.9 \pm 0.6 \mu\text{m}$), with a length on the same order of
330 magnitude as the width of the rhizoidal tip ($8.9 \pm 1.0 \mu\text{m}$). Multiple secondary threads
331 interconnect proximal radial strands, both sideways and vertically, crosslinking the main
332 fibrils. Noticeable enlargements are present on the nodes between the primary and secondary
333 threads, as well as on the points where the radial fibers adhere to the substrate. This
334 characteristic helps the network sustaining loads without breakage of the internal and external
335 connections. Small traces of adhesive materials are present further away from the main
336 fibrous pad, similar to the adhesive remnants imaged 1 h after fertilization, suggesting a
337 progressive expansion of the attachment pad. The overall morphology of the fibrous network
338 resembles the structure present in spider-silk anchors, with a heterogeneous meshwork of
339 threads rather than a homogeneous adhesive substance [67]. Such complex structure is
340 essential to dissipate the energy associated with detachment forces and to reduce risks of
341 crack propagation.

342

343 **3.2 Energy dispersive X-ray spectroscopy**

344 EDX was also performed on the rhizoidal tip of 24 h old germlings of *D. antarctica* (Fig.
345 S3). Traces of K, C, O, Na, S, and Ca were detected in the attachment pad. The counts in the
346 C and O peaks contain a strong contribution from the polymeric thermanox slide used to
347 support the specimens, therefore quantitative conclusions on the relative amounts of the
348 various elements identified cannot be put forth. Yet, the presence of K, Na, S and Ca is
349 characteristic of the adhesive holdfast. Sulfated polysaccharides are abundantly present in
350 carbohydrates secreted by large seaweed such as carrageenan and fucoidan [68], thus
351 explaining the abundant presence of this element in the EDX spectrum. The presence of
352 sulfated moieties will be further evidenced in the IR section. On the other hand, positive metal
353 ions contained in seawater readily form electrostatic complexes with negatively charged
354 polysaccharides and polyphenolics present in the adhesive secretions [46]. In particular, Ca
355 ions are able to form divalent coordination complexes, thus mediating both adhesion and
356 cohesion, e.g. by bridging the negatively charged adhesive secretions with a negatively
357 charged surface and by favoring gelling of the carbohydrate network [69]. Ca ions have been
358 extensively reported as key components in adhesive secretions in marine organisms, including
359 bacteria [70] and sandcastle worms [71] and will probably play a key role also in brown algal
360 attachment. The absence of a suitably developed adhesive pad in *H. banksii* germlings
361 prevented to employ EDX on this species and complete a full comparison between the two
362 species. Future work will be targeted to the analysis of the footprints of the germlings, so to
363 clarify the elemental composition of the adhesive pads and the putative role of the various
364 metal ions present.

365

366 **3.3 ATR-FTIR analysis**

367

368 **3.3.1 IR spectra from eggs before fertilization**

369 Prior to study of the adhesive produced by the settled germlings, preliminary IR
370 information on the content of the peripheral structures initially present around the eggs are
371 required. The spectra obtained from settled eggs of the two seaweed species in seawater are
372 reported in Figure 4. The absorbances are of the same order of magnitude for both spectra,
373 confirming that a relatively uniform and densely packed monolayer of eggs was produced,
374 also consistent with visual observation of the settled eggs on the ATR prism. Accordingly, the
375 amount of material sampled by the IR evanescent wave will be similar for both species, and

376 spectral differences are predominantly associated with the chemical composition of the egg
377 surface layers. These spectra can be compared qualitatively. Note that cell size may have a
378 minor influence on the sample of cellular material probed, but this effect was considered
379 negligible for the experimental system investigated.

380 Peaks in the 1000-1100 cm^{-1} region are in common for the two species, with characteristic
381 signature for polysaccharides ($\nu\text{C-O}$, and ring vibrations at 1086 cm^{-1} , $\nu\text{C-O-C}$ and $\nu\text{C-C}$ at
382 1063 cm^{-1} , and $\nu\text{C-O-C}$ and $\nu\text{C-C}$ at 1038 cm^{-1} , and $\nu\text{C-O-H}$ at 1005 cm^{-1}). From the
383 intensity of the two spectra, it appears that the amount of polysaccharide compounds probed
384 by the IR is higher in eggs from *D. antarctica* than those from *H. banksii*.

385 Strong differences are remarkable at higher wavenumber regions. In particular, bands
386 characteristic for phlorotannins are apparent in the spectra from *H. banksii* eggs, with peaks at
387 1610 and 1546 cm^{-1} (C=C aromatic ring vibrations), 1208 cm^{-1} (C-O-C stretch in aryl-aryl
388 ethers) and 1154 cm^{-1} (C-O stretch in phenolic compounds) [72]. It is worth noticing that the
389 phlorotannin molecules probed in solution display a significantly different spectra than those
390 reported for solid phlorotannin extracts [73,74]. In particular, the bands comprised between
391 1450 and 1200 cm^{-1} , strong in the solid state, will have a dampened intensity in aqueous
392 environment. The difference is probably due to the formation of hydrogen bonds with water
393 molecules, thus altering the vibrational states of the polyphenols (see Fig. S4 in the
394 Supporting Information for spectra of phloroglucinol in solid state vs in aqueous solution). On
395 the other hand, *D. antarctica* eggs present a broad band in the 1199-1269 cm^{-1} range (centred
396 at around 1240 cm^{-1}), likely related to the SO_2^- asymmetric stretch in complex sulphated
397 polysaccharides such as carrageenan, porphyran and fucoidan, constituents usually
398 abundantly present in seaweed extracts from brown algae with broad absorption in the 1195-
399 1280 cm^{-1} region [30,75-77]. The width of this peak could be affected by the specific
400 configuration of the charged moiety in the polysaccharide, e.g. its position around the
401 pyranose ring [68]. Proteins are another major constituent of the outer layer of *D. antarctica*
402 eggs, with strong bands at 1651 and 1547 cm^{-1} , related to the amide I and II modes of proteins
403 in α -helix conformation [78].

404 In conclusion, the two seaweed species share only IR signatures of carbohydrate-related
405 compounds. In addition, while *H. banksii* eggs contain phlorotannin based constituents in its
406 periphery, the *D. antarctica* surface layer contains protein molecules and sulphate
407 carbohydrates. Phlorotannins have been reported to be constituents of algal adhesives [79],
408 but need to be cross-linked to achieve the optimal adhesive properties [46,80]. These are
409 likely stored in the peripheral vesicles of *H. banksii* eggs, ready to be released in the

410 extracellular environment upon fertilization [48,58]. Vesicles containing phlorotannins have
411 also been reported for *D. antarctica* [81,82], but they seem to be contained within the eggs
412 thus not probed by the IR evanescent wave [64]. Interestingly, ionic moieties present in the
413 carbohydrate chains have a strong tendency to form stable complexes with metals and metal
414 ions [83]. In addition, negatively charged polysaccharides are highly hygroscopic and form
415 stable hydrogels in the presence of divalent metal ions such as Ca^{2+} and Mg^{2+} as present in
416 seawater [84]. This mechanism is consistent with the EDX results previously presented.
417 Accordingly, the observed tendency of *D. antarctica* eggs to bind to substrates prior to
418 fertilization might be explained by presence of an adhesive layer composed of a fraction of
419 partially secreted sulphated polysaccharides. The high protein component present in *D.*
420 *antarctica* may be involved in the formation of the adhesive bond mediating cross-linking
421 reactions [46,49] or simply is excreted as a result of other cellular processes.

422

423 **3.3.2 IR spectra from *H. banksii* germlings**

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

429 In Figure 5 are presented the changes in IR spectra from *H. banksii* germlings in the 24 h
430 following fertilization. These results are reported as difference spectra obtained by subtracting
431 the initial ATR-IR spectrum recorded at the end of the fertilization stage. Accordingly, any
432 spectral contribution from sperm suspension is removed in the subtraction and the spectral
433 evolution considered is representative of germling development only. The spectra are very
434 similar to spectra of bacterial species, with distinct bands at 1651 cm^{-1} (amide I), 1544 cm^{-1}
435 (amide II), 1454 cm^{-1} (C–H deformation), 1400 cm^{-1} (symmetric carboxylate stretch), 1245
436 cm^{-1} (amide III, P=O and C–O–C stretch) [85]. Some spectral changes are also visible in the
437 $1100\text{--}1000\text{ cm}^{-1}$ region characteristic of polysaccharides (C–O stretch, C–C stretch, C–O–H
438 stretch and bend, and ring vibrations). It is important to note that these changes are only a
439 direct consequence of the fertilization, as the spectra of the eggs before fertilization didn't
440 change with time.

441 PCA analysis was carried out on spectra of *H. banksii* germlings to identify the major
442 spectral changes in the first 24 hours after fertilization. The first principal component (PC1)
443 shows a trend of sample evolution over time with early timepoints in negative PC1 space

444 through to later timepoints in positive PC1 space (Figure 6a). This PC is describing 97 % of
445 the spectral variance in the sample and is the most important for looking at changes in the
446 spectra. The spectral features associated with this separation are shown in the loadings plots
447 (Figure 6b) with positive features being more dominant in samples plotting in positive PC1
448 space and negative features being more dominant in samples plotting in negative PC1 space.
449 The negative PC1 features at 1005, 1041 and 1205 cm^{-1} are stronger features in the spectra
450 collected from earlier timepoints and are consistent with the polysaccharides present on the
451 egg membrane and phenolic OH [86], respectively. The positive PC1 features at 1549 and
452 1655 cm^{-1} are more dominant in the spectra collected from later timepoints and are consistent
453 with amide II and amide I features for proteins, respectively, which are released during
454 germling development.

455 The second principal component (PC2) is only describing 1 % of the spectral variance
456 across the sample set and may not be giving valuable information based on chemical
457 differences but rather the strength of the signal and baseline shape so should not be used for
458 interpretation.

459 The features identified by PCA are further inspected with univariate analysis methods to
460 look at the kinetic profiles in comparison to PC1. In particular, the kinetic development of the
461 release of the adhesive was quantitatively tracked by measuring the relative peak absorbance
462 of bands for key adhesive constituents, i.e. amide modes, CH deformation and $\nu_{\text{as}}\text{SO}_2^-$. The
463 absorbance maxima were normalized to 1 to facilitate the comparison with PC1 (Figure 7).
464 All bands follow the same trend as the evolution of PC1, indicating that adhesion of *H.*
465 *banksii* germlings follows a relatively simple process where all components are released with
466 same rate. This result also confirms that the univariate and multivariate approaches employed
467 result in the same observations. Interestingly, the trends follow a sigmoidal shape, with a slow
468 release of adhesive components in the first few hours, followed by a rapid increase between
469 approximately 5 to 15 h post-fertilization, and concluded by a final slow release reaching a
470 plateau at the end of the period probed. Adhesion experiments performed by Taylor *et al.* and
471 Dimartino *et al.* on *H. banksii* zygotes demonstrate that little to no adhesion is achieved in the
472 first 6 h after fertilization, while higher adhesion strength is attained after approximately 12 h
473 post fertilization [21,65]. Our FTIR results corroborate these findings from a chemical
474 perspective. In fact, out of the total adhesive components released in the first 24 h, only 20%
475 are secreted in the first 6 h while more than 50 to 60% is released at approximately 12 h after
476 fertilization.

477 In general, production of proteinaceous material is prevalent over other biological
478 constituents, with bands at around 1650 and 1550 cm^{-1} typical of protein in α -helix
479 conformation. Protein-based adhesives are often reported as key components in the adhesive
480 secretions of a number of underwater adhesive organisms such as mussels [87], barnacles
481 [88], or arthropods [6]. The increase of the 1245 cm^{-1} band is mainly related to amide III
482 absorption, with a possible contribution from post-translational modified amino acids with
483 sulfated and phosphorylated moieties. Similar post-translational modifications are in fact
484 typical of marine adhesive proteins [89,90].

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

487

488 3.3.3 IR spectra of *D. antarctica* germlings

489 In Figure 8 is presented the spectral evolution of *D. antarctica* germlings in the 24 h
490 following fertilization. As observed in *H. banksii* germlings, the spectra for *D. antarctica* also
491 presents features typical of biological species, with distinct bands at 1651 cm^{-1} (amide I),
492 1544 cm^{-1} (amide II), 1454 cm^{-1} (C–H deformation), 1400 cm^{-1} (symmetric carboxylate
493 stretch), 1245 cm^{-1} (amide III, P=O and C–O–C stretch). In this case, the spectral changes in
494 the 1100–1000 cm^{-1} region, characteristic of polysaccharides (C–O stretch, C–C stretch, C–
495 O–H stretch and bend, and ring vibrations), are much more apparent.

496 PCA analysis was carried out on the *D. antarctica* germlings spectra to identify the major
497 spectral changes occurring in the first 24 hours after fertilization. Like with the PCA of *H.*
498 *banksii*, PC1 shows a trend of sample evolution over time with early timepoints in negative
499 PC1 space through to later timepoints in positive PC1 space (Figure 9a). This principal
500 component describes 84 % of the spectral variance in the sample and is the most important for
501 looking at changes in the spectra. The spectral features associated with this separation are
502 shown in the loadings plots (Figure 9b). The negative PC1 features at 999, 1032, 1069 (broad,
503 shoulder) and a broad envelope between 1170 and 1290 cm^{-1} are stronger features in the
504 spectra collected from earlier timepoints. These bands are associated with vibrational models
505 of polysaccharides and sulphated compounds present on the eggs and prominent during the
506 early stages after fertilization. The positive PC1 features at 1541, 1558, and 1653 cm^{-1} are
507 more dominant in the spectra collected from later timepoints and are consistent with amide II
508 and amide I features, respectively, indicative of protein-based secretions in the adhesive.

509 The second principal component (PC2) accounts for a further 10 % of explained spectral
510 variance and separates early stage zygote growth (negative PC2) from the middle timepoints

511 (positive PC2) with later timepoints in neutral PC2 space. The spectral features associated
512 with separation in positive PC2 space include features at 1015, 1051, 1094 (polysaccharides),
513 1225 ($\nu_{as}SO_2^-$), 1250 (amide III), 1360 and 1653 (amide I) cm^{-1} . The negative separating
514 features are observed at 988, 1072, 1119 and 1184 cm^{-1} . It is clear from the loadings plot that
515 baseline variation is also described by this PC. The second PC shows temporal behaviour that
516 suggests an increase or change in band intensities in the amide III and polysaccharide region.
517 At later times these changes are less distinct with evidence of band broadening particularly in
518 the polysaccharide region. This latter finding is not inconsistent with cross-linking which
519 creates a variety of environments (and thus wavenumbers) for these vibrational modes.

520 The normalized kinetics of PC1 together with the main bands for the adhesives secretions
521 from *D. antarctica* are reported in Figure 10. The different trends followed by the kinetic
522 profiles also suggest that *D. antarctica* produces adhesive components in a stepwise fashion.
523 In particular, carbohydrate-related molecules with absorption in the 1000-1100 cm^{-1} range are
524 released first and at a relatively higher rate. In addition, with respect to what observed with *H.*
525 *banksii*, in this case the area of the 1000-1100 cm^{-1} envelope at the end of 24 h period
526 observed is approximately tenfold higher, indicating that *D. antarctica* secretes a much larger
527 amount of polysaccharides. The band at 1245 cm^{-1} develops with similar kinetics as the
528 carbohydrate envelope, indicating this band is directly related to the carbohydrate secretions,
529 therefore assigned to sulphated moieties in polysaccharides. Protein based compounds with α -
530 helix configuration (typical peaks for amide I and II at 1645 and 1546 cm^{-1}) are released in the
531 second phase of the adhesion process. The amount of protein materials secreted at the end of
532 the 24 h absorbs with similar intensity as measured on *H. banksii* germlings. The kinetic
533 evolution of the protein-related bands does not seem to be correlated to other developing
534 bands, albeit some minor peaks are likely masked by the main spectral bands.

535 Accordingly, *D. antarctica* germlings initially secrete negatively charged carbohydrates as
536 primary adhesive, and do so in much larger absolute quantities than *H. banksii* germlings.
537 Protein-related constituents are released at later stages, as secondary adhesive and possibly
538 with cross-linking functions of the previously deposited carbohydrate network. This
539 mechanism is consistent with the microscopy results and the release of two different surface
540 vesicles with time. Proteins have been associated with the crosslinking of carbohydrates in the
541 cell wall of higher plants [91] and algae [92], usually through esterification reactions between
542 uronic acid residues and hydroxyl groups on a neighbouring polysaccharide chain, but also
543 through other more complex mechanisms [93]. The specific chemistry of the polysaccharides
544 involved in the adhesion process, and in particular the abundance of side groups such as

545 carboxyl or sulphate, opens the opportunity to a new variety of ester-based cross-linking
546 modes in the adhesive hydrogel. Interestingly, all bands display a slight reduction in the
547 production rate at the end of the 24 h period monitored, but components were still abundantly
548 secreted in the external environment and interacting with the prism surface, overall indicating
549 a more copious and longer release of adhesive components in *D. antarctica* than previously
550 observed in *H. banksii*.

551

552 3.3.4 Comparison of IR spectra

553 The spectra from both *H. banksii* and *D. antarctica* germlings were analyzed with PCA
554 simultaneously to find the major spectral and hence chemical differences between these two
555 species (Figure 11). Like the individual PCA analyses, the first PC is the most important to
556 describe the spectral variation of the samples over time (70 % of spectral variance), with
557 earlier spectra in negative PC1 space and later time points in positive PC1 space. The negative
558 features are observed at 1001 ($\nu(\text{C-O-H})$), 1034, 1042 ($\nu(\text{C-O-C})$ and $\nu(\text{CC})$), 1063 ($\nu(\text{C-O-C})$
559 and $\nu(\text{CC})$), 1111 and a broad feature $\sim 1202 \text{ cm}^{-1}$ ($\nu(\text{C-O-C})$ aryl-aryl ether) which can be
560 collectively attributed polysaccharide content of the egg/zygote before secretion of the
561 mucoadhesives. The positively separating features are observed at 1545 (broad) and 1653 cm^{-1} ,
562 attributed to amide II and amide I, respectively, and believed to be the major component of
563 the adhesive secretions. The second PC accounts for a further 24 % of the spectral variance,
564 and is paramount to highlight small variations in the spectra from these two species. This PC
565 separates *D. antarctica* in positive PC2 space and *H. banksii* in negative PC2 space. The
566 positive PC2 features, associated with *D. antarctica*, were observed at 1001, 1030, 1107 and
567 $1246 \text{ (broad) cm}^{-1}$, characteristic for sulfated polysaccharides. The main negative features,
568 associated with *H. banksii*, were observed at 1200, 1474 and 1618 cm^{-1} , indicative of
569 polyphenolic molecules. The variance in the amide I region is difficult to interpret due to
570 interference from water signals.

571 PCA suggests that the production of adhesive materials follows similar stages both in *H.*
572 *banksii* and *D. antarctica*, with charged (mostly sulphated) polysaccharides initially present in
573 the egg surface followed by a prominent production of proteins. This process indicates that
574 the carbohydrate molecules are responsible for creating quick adhesive and cohesive bonds,
575 followed by slower formation of stronger and more durable bonds through the protein
576 secretions. The sulphated polysaccharides must be flexible and able to penetrate the layer of
577 bio-fouling molecules (mostly uronic acids) ubiquitously present in marine environments
578 [94]. The sulphated polysaccharides could be related to adhesion to the substrate through

579 absorption and formation of stable interactions with the surface. Because of the high
580 concentration of divalent metal ions in seawater, and as supported by the EDX results, it is
581 reasonable to believe that the polysaccharide matrix is initially bond through metal
582 coordination bridges which can induce gelation.

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

593

594 **4. Conclusions**

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

604 In general, the adhesive components are initially stored within vesicles present in the outer
605 surface of the eggs. Following fertilization, the germlings produce a secondary mucilaginous
606 adhesive through the release of new morphologically different vesicles. These are particularly
607 abundant in *D. antarctica* but not detected in *H. banksii*, probably because of a slower and
608 less copious release. The adhesive pad has a characteristic fibrillar structure, composed of an
609 heterogeneous meshwork of threads paramount to dissipate hydrodynamic and other
610 detachment forces as well as minimize crack propagation. This structure is particularly
611 complex and well established in *D. antarctica* germling even after a few hours after
612 fertilization, while it only starts appearing in *H. banksii* after at least 24 h. The difference in

613 the amount and rate of the adhesive released is further confirmed by our ATR-FTIR results,
614 and partly explains the improved adhesion of germlings from *D. antarctica* over those from
615 *H. banksii*.

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

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

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

641 The present work helped clarify the mechanism of release and the key biochemical features
642 of the strong adhesives produced by kelps. The results presented will help the design and
643 synthesis of biomimetic counterparts, with application in wet and complex environments such
644 as in biomedicine. In the future, molecular biology methods will be employed to assess the
645 amino acid composition and sequence of the adhesive and cross-linking proteins probed, thus
646 clarifying their role in the adhesive formulation. Further investigation on the specific

647 functions of the polyphenolic fraction and its possible interaction with the proteins is also of
648 relevance and will constitute material for future research. These objectives coupled with the
649 assessment of the attachment performances of adhesives isolated from the two brown seaweed
650 will further support the development and ideally real-world application of permanent
651 biomimetic adhesives.

652

653 **Acknowledgements**

654 We acknowledge: the Department of Botany at UO and the research group of Dr. Catriona
655 Hurd for providing seawater as well as the use of the temperature controlled growth chambers
656 and the optical microscope; Liz Girvan in the Department of Anatomy for her support with
657 the SEM; the Marine Ecology Research Group at UC for their help with the release of
658 seaweed gametes; BIC at UC for funding. DS and SF would like to acknowledge the
659 University of Otago for doctoral scholarships.

660

661 **Funding**

662 SD was supported by a postdoctoral fellowship offered by the Biomolecular Interaction
663 Centre (BIC) at the University of Canterbury. DS and SF were supported by the postgraduate
664 scholarship from the University of Otago.

665

666 **Competing interests**

667 The authors have no competing interests.

668

669 **Author's contributions**

670 SD designed and coordinated the study, carried out all experiments, participated in data
671 analysis, and wrote the manuscript; DS helped in the ATR-FTIR experiments and related data
672 analysis; SF and KG carried out the principal component analyses and helped drafting the
673 manuscript; JM helped data analysis and drafting the manuscript. All authors gave final
674 approval for publication.

675

676

677 **References**

- 678 1. Lee H, Dellatore SM, Miller WM, Messersmith PB. 2007 Mussel-inspired surface
679 chemistry for multifunctional coatings. *Science*. **318**, 426-430.
680 (doi:10.1126/science.1147241)

- 681 2. Aksak B, Murphy MP, Sitti M. 2008 Gecko inspired micro-fibrillar adhesives for wall
682 climbing robots on micro/nanoscale rough surfaces. In: *IEEE International Conference*
683 *on Robotics and Automation, ICRA 2008*, 3058-3063.
684 (doi:10.1109/ROBOT.2008.4543675)
- 685 3. Haller CM, Buerzle W, Brubaker CE, Messersmith PB, Mazza E, Ochsenein-Koelble
686 N, Zimmermann R, Ehrbar M. 2011 Mussel-mimetic tissue adhesive for fetal membrane
687 repair: a standardized ex vivo evaluation using elastomeric membranes. *Prenat Diagn.*
688 **31**, 654-660. (doi:10.1002/pd.2712)
- 689 4. Santos R, Aldred N, Gorb S, Flammang P. 2013 *Biological and Biomimetic Adhesives:*
690 *Challenges and Opportunities*. Cambridge, UK: Royal Society of Chemistry Publishing.
- 691 5. Bianco-Peled H, Davidovich-Pinhas M. 2015 *Bioadhesion and Biomimetics: From*
692 *Nature to Applications*. Boca Raton, FL: CRC Press.
- 693 6. Hennebert E, Wattiez R, Waite JH, Flammang P. 2012 Characterization of the protein
694 fraction of the temporary adhesive secreted by the tube feet of the sea star *Asterias*
695 *rubens*. *Biofouling*. **28**, 289-303. (doi:10.1080/08927014.2012.672645)
- 696 7. Kamino K. 2006 Barnacle Underwater Attachment. In: Smith AM, Callow JA, eds.
697 *Biological Adhesives*. Berlin: Springer, 145-166.
- 698 8. Shao H, Bachus KN, Stewart RJ. 2009 A water-borne adhesive modeled after the
699 sandcastle glue of *P. californica*. *Macromol. Biosci.* **9**, 464-471.
700 (doi:10.1002/mabi.200800252)
- 701 9. Santos R, Flammang P. 2012 Is the adhesive material secreted by sea urchin tube feet
702 species-specific? *J. Morphol.* **273**, 40-48. (doi:10.1002/jmor.11004)
- 703 10. Hennebert E, Viville P, Lazzaroni R, Flammang P. 2008 Micro- and nanostructure of the
704 adhesive material secreted by the tube feet of the sea star *Asterias rubens*. *J. Struct. Biol.*
705 **164**, 108-118. (doi:10.1016/j.jsb.2008.06.007)
- 706 11. Millar A, Kraft G. 1994 Catalogue of marine brown algae (Phaeophyta) of New South
707 Wales, including Lord Howe Island, south-western Pacific. *Aust. Syst. Bot.* **7**, 1-47.
708 (doi:10.1071/SB9940001)
- 709 12. Stevens CL, Hurd CL, Smith MJ. 2002 Field measurement of the dynamics of the bull
710 kelp *Durvillaea antarctica* (Chamisso) Heriot. *J. Exp. Mar. Biol. Ecol.* **269**, 147-171.
711 (doi:10.1016/S0022-0981(02)00007-2)
- 712 13. Harder DL, Hurd CL, Speck T. 2006 Comparison of mechanical properties of four large,
713 wave-exposed seaweeds. *Am. J. Bot.* **93**, 1426-1432. (doi:10.3732/ajb.93.10.1426)
- 714 14. Seymour RJ, Tegner MJ, Dayton PK, Parnell PE. 1989 Storm wave induced mortality of
715 giant kelp, *Macrocystis pyrifera*, in Southern California. *Estuar. Coast. Shelf. Sci.* **28**,
716 277-292. (doi:10.1016/0272-7714(89)90018-8)
- 717 15. Graham JE, Wilcox LW, Graham LE. 2008 *Algae*. 2nd ed. San Francisco, CA: Benjamin
718 Cummings.
- 719 16. Osborn JEM. 1948 The structure and life history of *Hormosira banksii* (Turner)
720 Decaisne. *Trans. Proc. R. Soc. NZ.* **77**, 47-71.
- 721 17. McKenzie PF, Bellgrove A. 2009 Dislodgment and attachment strength of the intertidal
722 macroalga *Hormosira banksii* (Fucales, Phaeophyceae). *Phycologia*. **48**, 335-343.
723 (doi:10.2216/08-96.1)
- 724 18. Stevens CL, Taylor DI, Delaux S, Smith MJ, Schiel DR. 2008 Characterisation of wave-
725 influenced macroalgal propagule settlement. *J. Mar. Syst.* **74**, 96-107.
726 (doi:10.1016/j.jmarsys.2007.11.006)
- 727 19. Stevens C, Hurd CL, Smith M, Harder DL. 2001 *Durvillaea antarctica*: the strongest
728 kelp in the world? *NIWA Water Atmosphere*. **9**, 8-9.

- 729 20. Taylor DI, Schiel DR. 2003 Wave-related mortality in zygotes of habitat-forming algae
730 from different exposures in southern New Zealand: the importance of 'stickability'. *J.*
731 *Exp. Mar. Biol. Ecol.* **290**, 229-245. (doi:10.1016/s0022-0981(03)00094-7)
- 732 21. Dimartino S, Mather AV, Alestra T, Nawada S, Haber M. 2015 Experimental and
733 computational analysis of a novel flow channel to assess the adhesion strength of sessile
734 marine organisms. *Interface Focus*. **5** 20140059. (doi:10.1098/rsfs.2014.0059)
- 735 22. Fagerberg WR, Towle J, Dawes CJ, Böttger A. 2012 Bioadhesion in *Caulerpa mexicana*
736 (chlorophyta): Rhizoid-Substrate Adhesion. *J. Phycol.* **48**, 264-269. (doi:10.1111/j.1529-
737 8817.2012.01113.x)
- 738 23. Ouriques LC, Schmidt ÉC, Bouzon ZL. 2012 The mechanism of adhesion and
739 germination in the carpospores of *Porphyra spiralis* var. *amplifolia* (Rhodophyta,
740 Bangiales). *Micron*. **43**, 269-277. (doi:10.1016/j.micron.2011.08.012)
- 741 24. Apple ME, Harlin MM, Norris JH. 1996 Characterization of *Champia parvula*
742 (Rhodophyta) tetraspore mucilage and rhizoids with histochemical stains and FITC-
743 labelled lectins. *Phycologia*. **35**, 245-252. (doi:10.2216/i0031-8884-35-3-245.1)
- 744 25. Moss B. 1975 Attachment of zygotes and germlings of *Ascophyllum nodosum* (L.) Le
745 Jol. (Phaeophyceae, Fucales). *Phycologia*. **14**, 75-80. (doi:10.2216/i0031-8884-14-2-
746 75.1)
- 747 26. Callow ME, Evans LV, Bolwell GP, Callow JA. 1978 Fertilization in Brown Algae. I.
748 SEM and Other Observations on *Fucus Serratus*. *J. Cell. Sci.* **32**, 45-54.
- 749 27. Callow J, Osborne M., Callow M., Baker F, Donald A. 2003 Use of environmental
750 scanning electron microscopy to image the spore adhesive of the marine alga
751 *Enteromorpha* in its natural hydrated state. *Colloids Surf. B Biointerfaces*. **27**, 315-321.
752 (doi:10.1016/S0927-7765(02)00094-2)
- 753 28. Bråten T. 1975 Observations on mechanisms of attachment in the green alga *Ulva*
754 *mutabilis* Føyn. *Protoplasma*. **84**, 161-173. (doi:10.1007/BF02075951)
- 755 29. Petrone L, Easingwood R, Barker MF, McQuillan AJ. 2011 In situ ATR-IR
756 spectroscopic and electron microscopic analyses of settlement secretions of *Undaria*
757 *pinnatifida* kelp spores. *J R Soc Interface*. **8**, 410-422. (doi:10.1098/rsif.2010.0316)
- 758 30. Chiovitti A, Heraud P, Dugdale TM, Hodson OM, Curtain RCA, Dagastine RR, Wood
759 BR, Wetherbee R. 2008 Divalent cations stabilize the aggregation of sulfated
760 glycoproteins in the adhesive nanofibers of the biofouling diatom *Toxarium undulatum*.
761 *Soft Matter*. **4**, 811-820. (doi:10.1039/B715455K)
- 762 31. Walker GC, Sun Y, Guo S, Finlay JA, Callow ME, Callow JA. 2005 Surface Mechanical
763 Properties of the Spore Adhesive of the Green Alga *Ulva*. *J. Adhes.* **81**, 1101-1118.
764 (doi:10.1080/00218460500310846)
- 765 32. Callow JA, Crawford SA, Higgins MJ, Mulvaney P, Wetherbee R. 2000 The application
766 of atomic force microscopy to topographical studies and force measurements on the
767 secreted adhesive of the green alga *Enteromorpha*. *Planta*. **211**, 641-647.
768 (doi:10.1007/s004250000337)
- 769 33. Molino PJ, Hodson OM, Quinn JF, Wetherbee R. 2006 Utilizing QCM-D to characterize
770 the adhesive mucilage secreted by two marine diatom species in-situ and in real-time.
771 *Biomacromolecules*. **7**, 3276-3282. (doi:10.1021/bm0605661)
- 772 34. Molino PJ, Hodson OM, Quinn JF, Wetherbee R. 2008 The quartz crystal microbalance:
773 a new tool for the investigation of the bioadhesion of diatoms to surfaces of differing
774 surface energies. *Langmuir*. **24**, 6730-6737. (doi:10.1021/la800672h)
- 775 35. Rosenhahn A, Finlay J, Pettit M, Ward A, Wirges W, Gerhard R, Callow M, Grunze M,
776 Callow J. 2009 Zeta potential of motile spores of the green alga *Ulva linza* and the
777 influence of electrostatic interactions on spore settlement and adhesion strength.
778 *Biointerphases*. **4**, 7-11. (doi:10.1116/1.3110182)

- 779 36. Levi B, Friedlander M. 2004 Identification of two putative adhesive polypeptides in
780 *Caulerpa prolifera* rhizoids using an adhesion model system. *J. Appl. Phycol.* **16**, 1-9.
781 (doi:10.1023/B:JAPH.0000019034.12015.87)
- 782 37. Jordan P, Vilter H. 1991 Extraction of proteins from material rich in anionic mucilages:
783 Partition and fractionation of vanadate-dependent bromoperoxidases from the brown
784 algae *Laminaria digitata* and *L. saccharina* in aqueous polymer two-phase systems.
785 *Biochim. Biophys. Acta. BBA - Gen. Subj.* **1073**, 98-106. (doi:10.1016/0304-
786 4165(91)90188-M)
- 787 38. Pettitt ME, Henry SL, Callow ME, Callow JA, Clare AS. 2004 Activity of commercial
788 enzymes on settlement and adhesion of cypris larvae of the barnacle *Balanus amphitrite*,
789 spores of the green alga *Ulva linza*, and the diatom *Navicula perminuta*. *Biofouling.* **20**,
790 299-311. (doi:10.1080/08927010400027068)
- 791 39. Apple ME, Harlin MM. 1995 Inhibition of tetraspore adhesion in *Champia parvula*
792 (Rhodophyta). *Phycologia.* **34**, 417-423. (doi:10.2216/i0031-8884-34-5-417.1)
- 793 40. Bouzon ZL, Ouriques LC. 2007 Characterization of *Laurencia arbuscula* spore mucilage
794 and cell walls with stains and FITC-labelled lectins. *Aquat. Bot.* **86**, 301-308.
795 (doi:10.1016/j.aquabot.2006.10.010)
- 796 41. Barlow DE, Wahl KJ. 2012 Optical spectroscopy of marine bioadhesive interfaces.
797 *Annu. Rev. Anal. Chem.* **5**, 229-251. (doi:10.1146/annurev-anchem-061010-113844)
- 798 42. Petrone L. 2013 Molecular surface chemistry in marine bioadhesion. *Adv. Colloid.*
799 *Interface Sci.* **195-196**, 1-18. (doi:10.1016/j.cis.2013.03.006)
- 800 43. Burden DK, Barlow DE, Spillmann CM, Orihuela B, Rittschof D, Everett RK, Wahl KJ.
801 2012 Barnacle *Balanus amphitrite* adheres by a stepwise cementing process. *Langmuir.*
802 **28**, 13364-13372. (doi:10.1021/la301695m)
- 803 44. Parikh SJ, Chorover J. 2006 ATR-FTIR spectroscopy reveals bond formation during
804 bacterial adhesion to iron oxide. *Langmuir.* **22**, 8492-8500. (doi:10.1021/la061359p)
- 805 45. Gao Z, Bremer PJ, Barker MF, Tan EW, McQuillan AJ. 2007 Adhesive secretions of
806 live mussels observed in situ by attenuated total reflection infrared spectroscopy. *Appl.*
807 *Spectrosc.* **61**, 55-59. (doi:0003-7028/07/6101-0055\$2.00/0)
- 808 46. Bitton R, Ben-Yehuda M, Davidovich M, Balazs Y, Potin P, Delage L, Colin C, Bianco-
809 Peled H. 2006 Structure of algal-born phenolic polymeric adhesives. *Macromol. Biosci.*
810 **6**, 737-746. (doi:10.1002/mabi.200600073)
- 811 47. Vreeland V, Waite JH, Epstein L. 1998 Minireview—polyphenols and oxidases in
812 substratum adhesion by marine algae and mussels. *J. Phycol.* **34**, 1-8.
813 (doi:10.1046/j.1529-8817.1998.340001.x)
- 814 48. Schoenwaelder MEA, Clayton MN. 1998 Secretion of phenolic substances into the
815 zygote wall and cell plate in embryos of Hormosira and Acrocarpia (Fucales,
816 Phaeophyceae). *J. Phycol.* **34**, 969-980. (doi:10.1046/j.1529-8817.1998.340969.x)
- 817 49. Vreeland, Valerie, Epstein, L. 1996 Analysis of plant-substratum adhesives. In:
818 Linskens, HF, Jackson, JF, eds. *Modern Methods of Plant Analysis*. Berlin: Springer-
819 Verlag, **17**, 95-116.
- 820 50. Crayton MA, Wilson E, Quatrano RS. 1974 Sulfation of fucoidan in *Fucus* embryos: II.
821 separation from initiation of polar growth. *Dev. Biol.* **39**, 164-167. (doi:10.1016/S0012-
822 1606(74)80018-7)
- 823 51. Bitton R, Bianco-Peled H. 2008 Novel biomimetic adhesives based on algae glue.
824 *Macromol. Biosci.* **8**, 393-400. (doi:10.1002/mabi.200700239)
- 825 52. McGoverin CM, Clark ASS, Holroyd SE, Gordon KC. 2010 Raman spectroscopic
826 quantification of milk powder constituents. *Anal. Chim. Acta.* **673**, 26-32.
827 (doi:10.1016/j.aca.2010.05.014)

- 828 53. Killeen DP, Sansom CE, Lill RE, Eason JR, Gordon KC, Perry NB. 2013 Quantitative
829 Raman spectroscopy for the analysis of carrot bioactives. *J. Agric. Food Chem.* **61**,
830 2701-2708. (doi:10.1021/jf3053669)
- 831 54. Fraser SJ, Natarajan AK, Clark ASS, Drummond BK, Gordon KC. 2015 A Raman
832 spectroscopic study of teeth affected with molar–incisor hypomineralisation. *J. Raman*
833 *Spectrosc.* **46**, 202-210. (doi:10.1002/jrs.4635)
- 834 55. Smith GPS, McGoverin CM, Fraser SJ, Gordon KC. 2015 Raman imaging of drug
835 delivery systems. *Adv. Drug Deliv. Rev.* **89**, 21-41. (doi:10.1016/j.addr.2015.01.005)
- 836 56. Dimartino S, Savory DM, McQuillan AJ. 2014 Preparation of biological samples for the
837 study of wet-resistant adhesives inspired by kelps. In: *Proceedings of the 2014 Chemeca*
838 *Conference*. Perth, WA.
- 839 57. Petrone L, Ragg NLC, McQuillan AJ. 2008 In situ infrared spectroscopic investigation
840 of *Perna canaliculus* mussel larvae primary settlement. *Biofouling*. **24**, 405-413.
841 (doi:10.1080/08927010802339970)
- 842 58. Kevekordes K, Clayton MN. 2000 Development of *Hormosira banksii* (Phaeophyceae)
843 embryos in selected components of secondarily-treated sewage effluent. *J. Phycol.* **36**,
844 25-32. (doi:10.1046/j.1529-8817.2000.99059.x)
- 845 59. Kevekordes K, Clayton M. 1999 Shedding of the zygote wall by *Durvillaea potatorum*
846 (*Durvillaeales*, Phaeophyta) embryos. *Eur. J. Phycol.* **34**, 65-70.
847 (doi:10.1080/09670269910001736092)
- 848 60. Boulbene B, Morchain J, Bonin MM, Janel S, Lafont F, Schmitz P. 2012 A combined
849 computational fluid dynamics (CFD) and experimental approach to quantify the
850 adhesion force of bacterial cells attached to a plane surface. *AIChE J.* **58**, 3614-3624.
851 (doi:10.1002/aic.13747)
- 852 61. Forbes MA, Hallam ND. 1979 Embryogenesis and substratum adhesion in the brown
853 alga *Hormosira banksii* (Turner) Decaisne. *Br. Phycol. J.* **14**, 69-81.
854 (doi:10.1080/00071617900650101)
- 855 62. Forbes MA, Hallam ND. 1978 Gamete structure and fertilization in the brown alga
856 *Hormosira banksii* (Turner) Decaisne. *Br. Phycol. J.* **13**, 299-310.
857 (doi:10.1080/00071617800650361)
- 858 63. Evans LV, Callow JA, Callow ME. 1982 The biology and biochemistry of reproduction
859 and early development in *Fucus*. *Prog. Phycol. Res.* **1**, 67-110.
- 860 64. Schoenwaelder MEA. 2002 The occurrence and cellular significance of physodes in
861 brown algae. *Phycologia*. **41**, 125-139. (doi:10.2016/i0031-8884-41-2-125.1)
- 862 65. Taylor D, Delaux S, Stevens C, Nokes R, Schiel D. 2010 Settlement rates of macroalgal
863 algal propagules: Cross-species comparisons in a turbulent environment. *Limnol.*
864 *Oceanogr.* **55**, 66-76. (doi:10.4319/lo.2010.55.1.0066)
- 865 66. Scardino AJ, Guenther J, de Nys R. 2008 Attachment point theory revisited: the fouling
866 response to a microtextured matrix. *Biofouling*. **24**, 45-53.
867 (doi:10.1080/08927010701784391)
- 868 67. O. Wolff J, Grawe I, Wirth M, Karstedt A, N. Gorb S. 2015 Spider's super-glue: thread
869 anchors are composite adhesives with synergistic hierarchical organization. *Soft Matter*.
870 **11**, 2394-2403. (doi:10.1039/C4SM02130D)
- 871 68. Pereira L, Gheda SF, Ribeiro-Claro PJA. 2013 Analysis by vibrational spectroscopy of
872 seaweed polysaccharides with potential use in food, pharmaceutical, and cosmetic
873 industries. *Int. J. Carbohydr. Chem.* e537202. (doi:10.1155/2013/537202)
- 874 69. Petrone L, Ragg NLC, Girvan L, McQuillan AJ. 2009 Scanning electron microscopy and
875 energy dispersive X-Ray microanalysis of *Perna canaliculus* mussel larvae adhesive
876 secretion. *J. Adhesion*. **85**, 78-96. (doi:10.1080/00218460902782055)

- 877 70. Marshall KC, Stout R, Mitchell R. 1971 Mechanism of the initial events in the sorption
878 of marine bacteria to surfaces. *J. Gen. Microbiol.* **68**, 337-348. (doi:10.1099/00221287-
879 68-3-337)
- 880 71. Sun CJ, Fantner GE, Adams J, Hansma PK, Waite JH. 2007 The role of calcium and
881 magnesium in the concrete tubes of the sandcastle worm. *J. Exp. Biol.* **210**, 1481-1488
882 (doi:10.1242/jeb.02759)
- 883 72. Socrates G. 2004 Infrared and Raman Characteristic Group Frequencies: Tables and
884 Charts, 3rd ed. Chichester: Wiley.
- 885 73. Ragan MA. 1985 The high molecular weight polyphloroglucinols of the marine brown
886 alga *Fucus vesiculosus* L.: degradative analysis. *Can. J. Chem.* **63**, 294-303.
887 (doi:10.1139/v85-050)
- 888 74. Ragan MA, Glombitza KW. 1986 Phlorotannins, brown algal polyphenols. *Prog.*
889 *Phycol. Res.* **4**, 129-241.
- 890 75. Anno K, Seno N, Ota M. 1970 Isolation of l-fucose 4-sulfate from fucoidan. *Carbohydr.*
891 *Res.* **13**, 167-169. (doi:10.1016/S0008-6215(00)84905-8)
- 892 76. Li B, Lu F, Wei X, Zhao R. 2008 Fucoidan: structure and bioactivity. *Molecules.* **13**,
893 1671-1695. (doi:10.3390/molecules13081671)
- 894 77. Cael JJ, Isaac DH, Blackwell J, Koenig JL, Atkins EDT, Sheehan JK. 1976 Polarized
895 infrared spectra of crystalline glycosaminoglycans. *Carbohydr. Res.* **50**, 169-179.
896 (doi:10.1016/S0008-6215(00)83848-3)
- 897 78. Barth A. 2007 Infrared spectroscopy of proteins. *Biochim. Biophys. Acta BBA -*
898 *Bioenerg.* **1767**, 1073-1101. (doi:10.1016/j.bbabi.2007.06.004)
- 899 79. Vreeland V, Grotkopp E, Espinosa S, Quiroz D, Laetsch WM, West J. 1993 The pattern
900 of cell wall adhesive formation by *Fucus zygotes*. *Hydrobiologia.* **260-261**, 485-491.
901 (doi:10.1007/BF00049060)
- 902 80. Grotkopp E, Vreeland V. 1996 Aqueous algal-based phenolic type adhesives and glues.
903 US5520727.
- 904 81. Schoenwaelder MEA, Clayton MN. 1999 The presence of phenolic compounds in
905 isolated cell walls of brown algae. *Phycologia.* **38**, 161-166. (doi:10.2216/i0031-8884-
906 38-3-161.1)
- 907 82. Clayton MN, Ashburner CM. 1994 Secretion of phenolic bodies following fertilisation
908 in *Durvillaea potatorum* (Durvillaeales, Phaeophyta). *Eur. J. Phycol.* **29**, 1-9.
909 (doi:10.1080/09670269400650411)
- 910 83. Stumm W, Kummert R, Sigg L. 1980 A ligand exchange model for the adsorption of
911 inorganic and organic ligands at hydrous oxide interfaces. *Croat. Chem. Acta.* **53**, 291-
912 312.
- 913 84. Adair WS. 2012 *Organization and assembly of plant and animal extracellular matrix.*
914 San Diego, CA: Academic Press. (doi:10.1016/B978-0-12-044060-3.50002-9)
- 915 85. Kang S-Y, Bremer PJ, Kim K-W, McQuillan AJ. 2006 Monitoring metal ion binding in
916 single-layer *Pseudomonas aeruginosa* biofilms using ATR-IR spectroscopy. *Langmuir.*
917 **22**, 286-291. (doi:10.1021/la051660q)
- 918 86. Gattrell M, Kirk DW. 1992 A Fourier transform infrared spectroscopy study of the
919 passive film produced during aqueous acidic phenol electro-oxidation. *J. Electrochem.*
920 *Soc.* **139**, 2736-2744. (doi:10.1149/1.2068972)
- 921 87. Silverman HG, Roberto FF. 2007 Understanding marine mussel adhesion. *Mar.*
922 *Biotechnol.* **9**, 661-681. (doi:10.1007/s10126-007-9053-x)
- 923 88. Kamino K. 2008 Underwater adhesive of marine organisms as the vital link between
924 biological science and material science. *Mar. Biotechnol.* **10**, 111-121.
925 (doi:10.1007/s10126-007-9076-3)

- 926 89. Waite JH, Tanzer ML. 1981 Polyphenolic substance of *Mytilus edulis*: novel adhesive
927 containing L-dopa and hydroxyproline. *Science*. **212**, 1038-1040.
928 (doi:10.1126/science.212.4498.1038)
- 929 90. Taylor SW, Waite JH, Ross MM, Shabanowitz J, Hunt DF. 1994 trans-2,3-cis-3,4-
930 dihydroxyproline, a new naturally occurring amino acid, is the sixth residue in the
931 tandemly repeated consensus decapeptides of an adhesive protein from *Mytilus edulis*. *J.*
932 *Am. Chem. Soc.* **116**, 10803-10804. (doi:10.1021/ja00102a063)
- 933 91. Brown JA, Fry SC. 1993 Novel O-D-galacturonyl esters in the pectic polysaccharides of
934 suspension-cultured plant cells. *Plant. Physiol.* **103**, 993-999.
935 (doi:10.1104/pp.103.3.993)
- 936 92. Waffenschmidt S, Woessner JP, Beer K, Goodenough IJW. 1993 Isodityrosine cross-
937 linking mediates insolubilization of cell walls in *Chlamydomonas*. *Plant Cell* **5**, 809-
938 820. (doi:10.1105/tpc.5.7.809)
- 939 93. Iiyama K, Lam TBT, Stone BA. 1994 Covalent cross-links in the cell wall. *Plant*
940 *Physiol.* **104**, 315-320. (doi:10.1104/pp.104.2.315)
- 941 94. Stewart RJ, Ransom TC, Hlady V. 2011 Natural underwater adhesives. *J. Polym. Sci.*
942 *Part B Polym. Phys.* **49**, 757-771. (doi:10.1002/polb.22256)
- 943 95. Berglin M, Delage L, Potin P, Vilter H, Elwing H. 2001 Enzymatic cross-linking of a
944 phenolic polymer extracted from the marine alga *Fucus serratus*. *Biomacromolecules*. **5**,
945 2376-2383. (doi:10.1021/bm0496864)
- 946
947

948 **Figure captions**

949 Figure 1: Optical micrographs of cultured germlings on glass substrates. A, B and C: *D.*
950 *antarctica* at 6, 30 and 140 h after fertilization, respectively. D, E and F: *H. banksii* at 6, 42
951 and 140 h after fertilization, respectively. The arrows indicate where the thallus detaches from
952 the surface allowing the germlings to stand upright.

953 Figure 2: SEM images of *H. banksii*: A) Cluster of unfertilized eggs displaying numerous
954 peripheral vesicles, B) Magnification of the polydisperse vesicles covering the eggs surface,
955 C) Germings 1 h after fertilization, remnants of sperms and antheridia (jacket originally
956 enclosing sperm) are visible on the bottom-left germling and in the background, both
957 germling are characterized by extremely smooth surface, D) Magnification of the adhesive
958 threads produced 24 h after fertilization.

959 Figure 3: SEM images of *D. antarctica*. A) Unfertilized egg having irregular surface with
960 crater-like structures. B) Germlings 1 h after fertilization displaying smooth surface with
961 scattered surface vesicles. An adhesive footprint composed of an array of small adhesive pads
962 is also visible. C) Germlings 24 h after fertilization with significant development of the
963 adhesive pad. The rhizoidal cell is covered by radial adhesive threads. Additional connective
964 material is present underneath the apical cell. D) Magnification of the interconnected network
965 of adhesive fibrils produced by the germlings 24 h after fertilization.

966 Figure 4: ATR-FTIR spectra from settled eggs of *D. antarctica* (bottom line) and *H.*
967 *banksii* (top line). Background from seawater.

968 Figure 5: Band evolution from *H. banksii* germlings in the first 24 h after fertilization.
969 Background is settled eggs at the end of inoculation of sperm. The temporal resolution was 20
970 min, lines plotted every 2 h.

971 Figure 6: PCA analysis of the IR spectra collected during the development of *H. banksii*
972 germlings. Pale samples representing earlier timepoints and darker for later timepoints. (a)
973 The scores plot represents 98 % of spectral variance. (b) The loadings plots describing the
974 spectral features contributing to spectral variance in comparison to some representative
975 spectra.

976 Figure 7: Band kinetics of developing germlings of *H. banksii* normalized to 1.

977 Figure 8: Band evolution in the first 24 h of *D. antarctica* germlings following fertilization
978 with sperm. Background is settled eggs at the end of inoculation of sperm.

979 Figure 9: PCA analysis of the IR spectra collected during the development of *D. antarctica*
980 germlings. Pale samples representing earlier timepoints and darker for later timepoints. (a)
981 The scores plot represents 94 % of spectral variance. (b) The loadings plots describing the

982 spectral features contributing to spectral variance in comparison to some representative
983 spectra.

984 Figure 10: Band kinetics of developing germlings of *D. antarctica* normalized to 1.

985 Figure 11: PCA analysis of IR spectra collected during the development of *D. antarctica*
986 and *H. banksii* germlings. Pale samples representing earlier timepoints and darker for later
987 timepoints. (a) The scores plot represents 94 % of spectral variance. (b) The loadings plots
988 describing the spectral features contributing to spectral variance in comparison to some
989 representative spectra.