**In situ** ATR-IR spectroscopic and electron microscopic analyses of settlement secretions of *Undaria pinnatifida* kelp spores

L. Petrone¹, R. Easingwood³, M. F. Barker² and A. J. McQuillan¹,*

¹Department of Chemistry, ²Department of Marine Science, and ³Otago Centre for Electron Microscopy, University of Otago, PO Box 56, Dunedin, New Zealand

Knowledge about the settlement of marine organisms on substrates is important for the development of environmentally benign new methods for control of marine biofouling. The adhesion to substrates by spores of *Undaria pinnatifida*, a kelp species that is invasive to several countries, was studied by scanning electron and transmission electron microscopies (SEM/TEM) as well as by *in situ* attenuated total reflection infrared (ATR-IR) spectroscopy. The IR spectra showed that adhesive secretion began approximately 15 min after initial settlement and that the adhesive bulk material contained protein and anionic polysaccharides. Energy dispersive X-ray microanalysis of the adhesive identified sulphur and phosphorus as well as calcium and magnesium ions, which facilitate the gelation of the anionic polysaccharides in the sea water. The adhesive may be secreted from Golgi bodies in the spore, which were imaged by TEM of spore thin sections. Additionally, an *in situ* settlement study on TiO₂ particle film by ATR-IR spectroscopy revealed the presence of phosphorylated moieties directly binding the substrate. The presence of anionic groups dominating the adhesive suggests that inhibition of spore adhesion will be favoured by negatively charged surfaces.

**Keywords:** spore adhesion; *Undaria pinnatifida*; attenuated total reflection infrared spectroscopy; electron microscopy; TiO₂; EDTA

1. **INTRODUCTION**

The biofouling of ship hulls creates a number of costly problems such as increased fuel consumption, the necessity for hull cleaning and paint removal [1]. Marine biofouling can also create problems for aquaculture by colonizing fish cages and mussel ropes. Among the range of marine biofouling species, the kelp *Undaria pinnatifida* (Harvey) Suringar (Laminariales, Phaeophyta) has spread profusely in recent years to become a problem invader for aquaculture and marine ecosystems [2,3].

*U. pinnatifida* is native to Japan, northern China and Korea, where it is cultivated for human consumption. Its international spreading probably occurs through spores transported in ballast water and their subsequent rapid growth. It has been accidentally introduced to Australia, New Zealand, Argentina, southern California (USA) and the Mediterranean Sea [4–6]. It forms dense stands resulting in competition for light and space that may lead to the exclusion or displacement of native plant and animal species [6]. The *U. pinnatifida* life cycle is characterized by microscopic haploid gametophytes and macroscopic diploid sporophytes. Mature sporophytes release microscopic flagellate zoospores from sporophylls situated on the basal part of the stipe [7]. Zoospores use surface recognition cues to detect favourable locations for settlement before developing into dioecious microscopic gametophytes [8]. Subsequently, mature gametophytes release sperm and eggs which combine to form embryonic sporophytes that grow into macroscopic individuals [9,10].

Spores are a ubiquitous natural form of algal dispersal and their fixation to a substratum is a fundamental process in the life-history phases of many macroalgae [11–13]. However, algae have widely varying settlement processes and understanding the spore settlement mechanism and the chemical functionalities involved in this process may assist in the design of effective anti-biofouling strategies. Due to its ecological and commercial importance, numerous studies have been carried out on the eco-physiology of *U. pinnatifida* [14], but no observations have been reported on the composition and physico-chemical nature of *U. pinnatifida* spore adhesive. Most previous studies have observed that algal spores prefer to colonize rough rather than smooth surfaces [15,16]. However,
Linskens [17] demonstrated that some algal species, such as *Ectocarpus fasciatus* and *Polysiphonia,* displayed a preference for settling on smooth surfaces. The settlement and attachment strength of *Ulva linza* spores are also influenced by microtopography and surface roughness [18,19]. Scardino *et al.* [20–22] investigated the response of algal cells to surface roughness, and they found that nanoscale roughness inhibits settlement and the size of surface microtexture in relation to the size of the settling cells was important in the selection of attachment sites. Other studies [23–25] have shown that the settlement of *Ulva* spores is influenced by the surface energy, wettability, modulus and lubricity. *Hinckisia irregularis* [8] and *Ectocarpus siliculosus* spores [26] have been observed to settle preferentially on uncharged hydrophobic surfaces compared with negatively or positively charged substrates. Conventional optical microscopy and digital holography [27,28] have been used to obtain detailed information on the mechanisms of surface colonization by zoospores of the green alga *U. linza* and the brown alga *Hinckisia irregularis* and adaptations to changes in surface properties.

Few *in situ* studies have addressed the chemical nature of adhesives secreted by fouling marine organisms, in spite of the critical importance of this stage to the biofouling of surfaces. Determinations of the composition of algal spore adhesives are limited by the availability of material for chemical analysis. Previous studies have indicated that the permanent adhesive material secreted by various spores are glycoproteins, as has been shown for *Ulva* [29,30] and *Palmaria* [31]. The adhesion mechanisms suggested from the majority of studies on marine brown algae have been essentially speculative, but only Vreeland *et al.* [32] have found an adhesion mechanism which is determined by oxidase-mediated polymerization of phenolic compounds. Recent *in situ* studies of spore adhesion have employed atomic force microscopy (AFM). Callow *et al.* [33] observed by AFM that within minutes of release the *Ulva* spore adhesive undergoes a progressive curing process, becoming less adhesive and less extensible, with a 65 per cent reduction in adhesion strength after 60 min of settlement. However, AFM measurements do not give much information on the interfacial adhesion properties of the whole settled spore system, and hydrodynamic methods have been used to measure the strength of settlement of *Ulva* spores using a water jet apparatus [34]. The development of adhesive and cohesive strength was shown to be highly time-dependent, and spores that settled in groups were more resistant to detachment than single spores, suggesting that gregarious settlement behaviour may enhance the resistance to detachment forces in turbulent environments. Transmission electron microscopy (TEM) has been used to look at the ultrastructure of algal spores during both swimming and benthic stages [35]. There have been a number of EM studies of a variety of algal spores at the benthic phase, including phototactic, thigmotactic and chemotactic stimuli [13].

In the present work the ultrastructure of swimming-stage and settled *U. pinnatifida* spores was observed by TEM and field emission scanning electron microscopy (SEM). Attenuated total reflection infrared (ATR-IR) spectroscopy was applied *in situ* to the analysis of the composition of adhesive material secreted during spore settlement on germanium. This ATR-IR approach has been used recently to study the settlement of *Perna canaliculus* mussel larvae [36]. Additionally, ATR-IR spectroscopy was used to record characteristic absorptions of model compounds containing monoester-phosphate and -sulphate groups and their adsorbed species on a TiO₂ particle film, as well as interactions with calcium ions. The IR spectra obtained from the investigated model compounds and the influences of their interactions with divalent cations were used to identify functional groups involved in the spore adhesion process.

### 2. MATERIAL AND METHODS

#### 2.1. Zoospore collection

Fertile thalli of *U. pinnatifida* were collected in the vicinity of the Portobello Marine Laboratory in Otago Harbour (45°49.455’S, 170°38.529’E), New Zealand. The portions of the sporophylls bearing the sori (1–2 cm in length) were excised from the thalli, washed in a 1 per cent (w/v) sodium hypochlorite solution followed by three washes in sterilized sea water, and stored overnight in air at 15°C covered by damp paper towels. The sporophylls were then transferred to sterilized sea water at 10°C in 500 ml flasks and agitated on an orbital shaker for 4 h. Zoospores were released both during and following agitation. The sporophyll fragments were then removed using a sterilized dissecting needle. The spore concentration used for this work was $7 \times 10^8$ ml⁻¹, determined by counts with an Elzone XY 180 particle counter.

#### 2.2. SEM and EDX microanalysis

*Undaria pinnatifida* zoospores were allowed to settle on glass coverslips for periods of 30 min, 3 h or 12 h. Subsequently, specimens were washed with deionized water (Milli-Q, Millipore) to remove sea water salts, and allowed to dry. They were then mounted on aluminium stubs with double-sided carbon tape and carbon coated in a Peltier-cooled high resolution sputter coater (Emitech K575X, EM Technologies). Specimens were viewed in a field emission scanning electron microscope (JSM-6700F, JEOL) at 3 kV accelerating voltage and at 15 nm working distance. Energy dispersive X-ray analysis (EDX) microanalysis was performed on the pad of settled spores at 10 kV and at a working distance of 15 mm (JEOL 2300 EDX detector, JEOL), corresponding to a few-micrometre spatial sampling. Elements were detected from their Kα lines with highest intensity, with an acquisition time of 30–120 s and deadtime of approximately 1 per cent. This technique is sensitive to elements present above approximately 0.1 at% and can probe depths from 0.2 to 8 µm. EDX results underwent ZAF correction after calculating intensity factors of pure elements [37].
2.3. Transmission electron microscopy

For TEM ultrastructural visualization, specimens of the *U. pinnatifida* spores were fixed and stained as follows. Approximately 90 ml of *U. pinnatifida* spore suspension was centrifuged at 3500g for 15 min and the supernatant was discarded. The pellet was then transferred to a sample tube and 5.6 ml of primary fixative, made of 2.0 ml 70 percent glutaraldehyde, 2.8 ml 0.2 M sodium cacodylate, 800 μl distilled water and 5.6 mg ruthenium red, was added [38]. The specimen was heated at 37°C for 4 h, then centrifuged at 4000g for 5 min to remove the excess fixative and then washed for 5 min three times with 0.1 M aqueous sodium cacodylate. Afterwards, 5.6 ml of secondary fixative, made of 1.4 ml 4 per cent OsO₄, 2.8 ml 0.2 M sodium cacodylate, 1.4 ml distilled water and 5.6 mg ruthenium red, was added to the specimen and left for 24 h at room temperature. The specimen was then washed three times as before and the clean pellet was mixed with a similar volume of 3 per cent agarose in 0.1 M sodium cacodylate buffer. The pellet was chilled at 4°C for 20 min and then sliced into 1 mm cubes and loaded into a tissue processor basket. The specimen was dehydrated through an ethanol series (50%, 75%, 95% and twice 100%) and infiltrated with resin (Quetol, ProSci-Tech) using a tissue processor (Lynx el, Australian Biomedical Corporation; [39]). The resin was cured at 60°C for 24 h and then 40 nm ultrathin sections were prepared from the embedded spore specimen using a diamond knife and an ultramicrotome (Ultracut E, C Reichert AG). The sections were contrasted with uranyl acetate and lead citrate using an automatic grid stainer (LKB Ultrostain, LKB-Produker AB). The ultrathin sections were viewed in a Philips CM100 TEM (Philips/FEI Corporation) and micrographs captured using a digital camera (Olympus Soft Imaging Solutions GmbH, MegaView III).

2.4. Inductively coupled plasma-mass spectrometry

Inductively coupled plasma-mass spectrometry (ICP-MS) analysis was performed with an Agilent 7500ce instrument (Agilent Technologies). A 4.672 mg sample was digested in 2.5 ml of 6 M HNO₃. After digestion approximately 1 ml of acid remained and this was made up to 20 ml with deionized water (Milli-Q, Millipore). A 0.1 ml volume of internal standard mixture containing Sc, Rh, In, Tb and Bi was added to 5 ml of sample. Helium was used as a reaction gas.

2.5. ATR-IR spectroscopy

A specially modified ATR-IR accessory (Horizon, Harrick Scientific) containing a 13-reflection horizontal 50 × 10 × 2 mm 45° germanium prism mounted on a stainless steel trough plate was used to collect IR spectra. The accessory was equipped with water cooling channels within a stainless steel block beneath the prism, to maintain a constant temperature in the flow cell of 14°C. A schematic of the ATR-IR apparatus can be found elsewhere [36]. IR spectra were obtained using a Digilab FTS 4000 IR spectrometer equipped with a KBr beamsplitter and a Peltier-cooled DTGS detector. IR spectra from 128 co-added scans at 4 cm⁻¹ resolution were analysed with Win-IR Pro v.3.4 software. In all the presented ATR-IR spectra, absorbances were directly from measured spectra and not from deconvoluted peak data. The penetration (1/e) depth of the evanescent wave in water is 0.7 μm at 1650 cm⁻¹ [40]. The germanium prism was cleaned by polishing with 0.015 mm Al₂O₃ powder (BDH, polishing grade) on a wet polishing microcloth (Buehler) and then rinsed with deionized water (Milli-Q, Millipore). *U. pinnatifida* spore specimens were prepared for dry ATR-IR analysis with a diaphragm vacuum pump (Laboprot N810FT18, KNF) at a pressure of approximately 10 mbar for 30 min. When solutions were flowed through the trough a peristaltic pump was used to obtain a flow rate of 2 ml min⁻¹. The spectra of adsorbed species on TiO₂ particle films were recorded after coating the prism with a thin layer of colloidal anatase (Dyesol) particles, and then filling the ATR cell with the solution. The coating process was carried out by spreading approximately 1 ml of 10⁻³ M aqueous anatase TiO₂ colloidal suspension on top of the Ge, and then drying the suspension under vacuum at room temperature. The 15 nm anatase particles had a specific surface area of 67 m² g⁻¹ and the deposited particle film was approximately 100 nm thick. Chemicals in this work, O-phospho-L-serine, κ-carrageenan, were used as-received from Sigma-Aldrich and NaOH was analytical reagent grade from Ajax.

3. RESULTS AND DISCUSSION

3.1. SEM imaging and EDX microanalysis of algal spore adhesive

SEM images of *U. pinnatifida* spores after 30 min settlement on a glass microscope are shown in figure 1. The images reveal the spherical shape of approximately 4 μm diameter settled spore and the fibrillar appearance of the adhesive material surrounding the spore body. It has to be noted that in the SEM images the spores appear to be shrunken, so that the hydrated spore diameter is greater. Environmental SEM observations of other marine fouling species in their natural hydrated state [41,42] demonstrated the gel-like nature of the adhesives. Therefore, the shrinkages observed in figure 1 are artefacts of the specimen dehydration and high vacuum needed for conventional SEM. SEM images were taken at different time intervals following settlement on the glass substratum. Figure 1b shows a spore after 3 h of settlement with the beginning of the germination process producing a root-like protrusion extending across the substratum. After 12 h of settlement the spores elongated into slender tubular filaments measuring up to 400 μm in length, as commonly observed for other algal species [13,43,44].

Other SEM images reveal spores mostly adhering to each other in clusters after 30 min of settlement (see the electronic supplementary material, figure S1). Glass is negatively charged at the seawater pH of 8 and the spore cluster formation suggests that the spore outer layer is also negatively charged. Rosenhahn et al. [45] have demonstrated that *Ulva* spores show a reduced
tendency to settle on negatively charged surfaces compared with neutral or positively charged surfaces. H. irregularis spore settlement has also been shown [8] to be influenced by surface hydrophobicity, and experiments revealed significantly higher settlement on hydrophobic surfaces when compared with negatively charged surfaces. Thus, the preference for adhering to each other rather than on the glass surface suggests the presence of some adhesive surface functionalities other than charged groups. This cluster behaviour may point to a survival strategy where the spores share a common pool of adhesive secretion to strengthen their adhesion, thus increasing their chances of surviving in turbulent subtidal conditions [34]. The role of anterior flagella must also be considered in initial settlement. Flagella are responsible for surface cue detection during pre-settlement exploration [46], and therefore the detection of unsuitable surface roughness, microtopography or chemistry may cause spore cluster formation.

EDX microanalysis was performed on the adhesive pad adjacent to the spore after specimens were washed three times in deionized water to remove sea water salts. The organic compound elements, carbon, nitrogen and oxygen along with phosphorus, sulphur, magnesium and calcium were detected. EDX microanalysis was also performed on the glass substrate in the proximity of the spore adhesive pad and no metal element was found. Therefore, Ca and Mg detected in the spore adhesive do not arise from traces of sea water salts, and are constituents of the spore adhesive. Jones et al. [47] suggested that calcium ions influence the cross-linkage of the algal spore adhesive since in vitro investigations demonstrated that the presence of alginate and/or calcium are essential to give strength to the spore adhesive. Chiovitti et al. [48] found that the adhesive secretion of the biofouling diatom Toxarium undulatum is composed of sulphated glycoproteins cross-linked by both calcium and magnesium ions. Thus, these findings suggest the presence of anionic functionalities, such as phosphate, carboxylate and sulphate in the adhesive of settled algal spores. The ionic radius of Mg$^{2+}$ (0.69 Å) is significantly smaller than that of Ca$^{2+}$ (0.99 Å [49]) and, therefore, the concomitant presence of these two ions would bind together negatively charged polysaccharides and proteins which possess cavities and domains of different size.

3.2. TEM of Undaria pinnatifida spores

Upon release, U. pinnatifida zoospores are generally spherical, measuring approximately 4 μm in diameter and do not possess a cell wall (see the electronic supplementary material, figure S2). A cell wall is produced soon after settlement occurs and an approximately 50 nm thick cell wall can be seen in figure 2. The only visible difference between the two TEM micrographs is the smooth outer surface of swimming spores compared with the uneven one of settled spores that indicates the presence of a cell wall. Additionally, the spore nucleus appeared roughly globular and the two varieties of chromatin, euchromatin and heterochromatin, are also visible within the nucleus [50]. Furthermore, other organelles, such as the Golgi apparatus and chloroplasts, are visible. The nucleus seems to be associated with chloroplasts wrapped around it in the centre of the spore and the Golgi apparatus in contact with the nuclear membrane. Evenly electron-dense bodies measuring up to 2 μm were observed which can be associated with lipid bodies. In fact, in algal spore lipids are the most important reserve materials and are usually found in the form of globules and droplets [51]. Small amounts of triacylglycerols have been observed in the zoospores of several algal species and are thought to fuel spore swimming [52].

Electron-dense vesicles measuring 0.1–0.3 μm in diameter are evident in the TEM micrographs, and they are often found adjacent to the Golgi complex. The Golgi apparatus plays a role primarily in producing secretory vesicles for exocytosis, thus the electron-dense vesicles revealed in the TEM micrograph in figure 2 in the proximity of the Golgi complex might contain the adhesive material that is secreted during the settling process. In brown algae the Golgi apparatus is involved in the synthesis of polysaccharides [53] and in the case of Ulea spores [54] it is observed that the process of exocytosis of adhesive vesicles is rapid and typically a spore completes the adhesion process within 1 min. In another
study, Evans & Christie [55] and Stanley et al. [56] reported that the adhesive vesicles observed in the swimming zoospores of the green alga *Ulva* were absent in the settled spores, suggesting that these bodies contain the adhesive secretion. Additionally, our results revealed that the Golgi vesicles are present both in the swimming and in the settled *U. pinnatifida* spores. These findings would indicate that only a few vesicles were discharged to glue the spore to the substratum and the content of the remaining vesicles may be discharged in a subsequent period to enhance the adhesion. In situ ATR-IR spectroscopy can reveal molecules and functional groups constituting the adhesive material secreted by *U. pinnatifida* spores. After washing with deionized water, the spores were placed on the Ge prism, vacuum dried and the IR spectrum was recorded. As shown in figure 3, the high absorbance of the IR spectrum from such a small quantity of sample is owing to the sample being dried on the Ge, thus algal spores are compacted and in close contact with the prism surface where the evanescent wave is most intense. The prominent absorptions are a sharp band at 1744 cm$^{-1}$ assigned to ester vibrations of lipids [58,59], strong bands at 1647 (amide I) and 1540 cm$^{-1}$ (amide II) from protein [60,61], a band at 1456 cm$^{-1}$ arising from $\delta$CH$_2$ vibrations of alkylic moieties [62], a broad band approximately 1403 cm$^{-1}$ owing to the $v_1$(COO$^-$) mode from acidic polysaccharides [63], and a strong peak at 1237 cm$^{-1}$ probably arising from both $v_{as}$(SO$_2^-$) [64] and $v_{as}$(PO$_2^-$) modes [65]. The broad band in the region between 1200 and 950 cm$^{-1}$ contains contributions from several components including the 1157 cm$^{-1}$ band owing to $\nu$C–O–C mode and the 1082 cm$^{-1}$ peak owing to C–O and C–C stretch vibrations coupled to C–O–H bending from polysaccharides [66]. In the same spectral region lie the $v_{as}$(SO$_2^-$) [64] and $v_{as}$(PO$_2^-$) modes [65], therefore the two absorptions at 1082 and 1057 cm$^{-1}$ are also expected to contain contributions from such modes. Guan et al. [67] reported $v_{as}$(PO$_2^-$) and $v_{as}$(PO$_2^-$) vibrations for the dimethyl phosphate anion [(CH$_3$O)$_2$PO$_2^-$] at 1217 and 1083 cm$^{-1}$, and also IR spectra of lipid bilayers showed $v_{as}$(PO$_2^-$) and $v_{as}$(PO$_2^-$) at, respectively, 1226 and 1084 cm$^{-1}$ [68].

The spore cell wall consists of a mixture of carboxylated and neutral polysaccharides [51], thus the observed $v_{as}$(COO$^-$) and polysaccharide absorptions are therefore associated with it. As previously mentioned, the permanent adhesives from a variety of algal spores are polysaccharide-protein complexes and, hence, lipids are not expected to be found in the spore adhesive. Thus, the ester vibration at 1744 cm$^{-1}$ must be associated with lipid material contained in the large bodies observed by TEM. This absorption can be used to monitor the spore adhesive secretion activity at the prism/solution interface. Lastly, the absorption at 1237 cm$^{-1}$ must have a contribution from the phosphorylated moieties in chromatin. In fact, the asymmetric phosphate stretching mode originating from the phosphodiester groups of nucleic acids is usually observed at approximately 1240 cm$^{-1}$ [69].

In addition to the dry spore IR analysis, ATR-IR spectra were collected over 3 h at 14°C for a sea water

![Figure 2. TEM micrographs of settled *U. pinnatifida* spore. Internal organelles are labelled: N, nucleus; Ch, chloroplast; LB, lipid body; G, Golgi apparatus; GB, Golgi bodies; CW, cell wall. Arrows indicate mucilage. Scale bar, 1 μm.](Image 68x570 to 283x782)

![Figure 3. ATR-IR spectra of dried *U. pinnatifida* spores on Ge. Background was from the bare Ge.](Image 354x354 to 260x260)
suspension of *Undaria pinnatifida* spores covering the Ge prism to a depth of 3 mm, as shown in figure 4. The spores were observed to swim about randomly and spore settlement was recorded in the IR spectra when the spores came within the IR evanescent wave penetration depth. The bands in figure 4 spectra are less intense than those in figure 3 because the hydrated spores settling on the Ge in sea water are at a greater distance from the prism surface compared with the dried spores. Many similarities are evident between the IR spectra recorded during the spore settlement process (figure 4) and the dried spores on Ge (figure 3), with some minor band shifts in the different environment. The mid-IR region, in fact, reveals proteins and polysaccharides, which are part of both the algal spores and their adhesive secretion. However, the relative intensities of the spectral bands are dissimilar in the considered IR spectra, indicating a different chemical composition. This can be discerned by comparing the intensity of the ester absorptions associated with cytoplasmic lipid at approximately 1740 cm\(^{-1}\) with that of other bands in the two different spectra.

Despite the fairly constant increase over time in figure 4 protein-related amide signals, other spectral features did not behave similarly. In the 1200–950 cm\(^{-1}\) spectral region there is initially a broad yet featureless band indicating a mixture of polysaccharides [70]. After approximately 1 h, peaks begin to emerge in this region with a maximum at 1056 cm\(^{-1}\) along with less intense shoulders at 1084, 1037, 1157 and 1016 cm\(^{-1}\). At approximately 1400 cm\(^{-1}\) there is a detectable peak corresponding to carboxylate groups of proteinaceous residues [71,72] within the algal spores. Figure 5 shows the plot of the time-dependence of absorbance of the various figure 4 IR spectral bands during the first 50 min of the *U. pinnatifida* spore adhesion. The peak absorbances in the first 10 min rapidly increase, indicating that the algal spores are reaching the Ge surface within the range of the evanescent wave. After approximately 15 min the slopes of the curves dramatically decrease, indicating that the spore settlement rate has declined, which is partly owing to the decrease in the unoccupied surface area remaining from increased spore surface coverage. At this stage the absorbance of the peak at 1742 cm\(^{-1}\) associated with lipid vibrations suddenly decreases. This must correspond to the initial release of the adhesive secretion, with the spores being forced away from the prism surface causing the decrease of the lipid absorbance [73]. During the following 35 min there is a constant rate of increase in the protein and polysaccharide band absorbances corresponding to the continued release of adhesive material.
After 3 h the initial featureless broad band in figure 4 is now well resolved into distinct peaks including those at 1084, 1056 and 1037 cm⁻¹. The peak at 1084 cm⁻¹ becomes dominant over the amide I band at 1642 cm⁻¹ and the sharp polysaccharide peaks at 1056 and 1037 cm⁻¹. The amide I band can furnish information about the protein secondary structure, and a peak at 1642 cm⁻¹ is characteristic of random coil structures [74]. The final carboxylate absorption peak is at 1413 cm⁻¹, which corresponds to carboxylated polysaccharides in the spore adhesive secretions. After approximately 2 h, the spore germination process begins as observed by SEM (figure 1b). Therefore, the growth of the bands at 1084 and 1413 cm⁻¹ assigned to carboxylated polysaccharides in the last phase could arise from two factors: (i) the extension of germination stalks across the surface, spreading secreted polysaccharide and thereby increasing its signal; (ii) the cell wall formation, which begins shortly after settlement. Thus, the ATR-IR data of the U. pinnatifida spore settlement indicate the presence of proteins and anionic polysaccharides, probably forming a glycoprotein. The ATR-IR data do not refer exclusively to molecules and functional groups right at the adhesive/prism interface, but are the average signals of compounds within the penetration depth of the evanescent wave. Therefore, the bulk of spore adhesive material dominates the signal rather than functional groups and molecules directly involved in the binding to the substrate.

3.4. The influence of EDTA on the IR spectra of settled algal spores

The EDX microanalysis showed the presence of metal ions in the spore adhesive. The use of high affinity metal-binding chelators such as EDTA has been shown to inhibit bacterial growth by disrupting surface adhesion and preventing biofilm production [75,76]. Therefore, the influence of EDTA treatment on the spore spectra was evaluated. Following the settlement of the algal spores on Ge, an aqueous NaOH solution at pH 8.0 was introduced via the flow cell to remove any unsettled spores and sea water salts, and after 30 min an IR spectrum was recorded. The major observed spectral changes are loss of intensity of the broad band between 1200 and 950 cm⁻¹, with less-resolved peaks at 1073 and 1013 cm⁻¹. A significant component of this intensity loss is from sulphate ion, one of the major constituents of sea water at a concentration of approximately 0.001 M [77], which possesses a strong IR absorption at 1097 cm⁻¹ [37]. The absorbance of the bands at 1244 (ν_{as}(PO_{2}) and ν_{as}(SO_{4})), 1413 (ν (COO⁻)), 1549 (amide II) and 1657 cm⁻¹ (amide I) also decreases. Such spectral changes may be attributed to several factors: unattached algal spores being removed from the prism surface, algal spore adhesive components being more soluble in basic aqueous solution, and the removal of IR-active sea water salts. The amide I band at 1657 cm⁻¹ was upshifted by 15 cm⁻¹, probably owing to changes in the protein secondary structure attributable to the transition from random coil to α-helix structures [74]. The absorbance of the amide I band decreased with respect to the amide II band, possibly because of an absorbance loss at approximately 1640 cm⁻¹ associated with the bending mode having different absorption intensity in the hydration water of sea water salts compared with that in bulk water.

Next, 60 mL of 10⁻³ M EDTA solution at pH 8.0 (EDTA^{4⁻}) was flowed over the settled algal spores for 30 min to remove cations entrapped in the spore adhesive secretion. A broad band peaking at approximately 1255 cm⁻¹ appears less intense. This band contains the ν_{as}(PO_{4}) and ν_{as}(SO_{4}) absorptions and this loss of intensity may be related to absorbance losses from ν (PO_{2}) and ν (SO_{4}) groups contributing to lower band intensity in the polysaccharide characteristic region at approximately 1100 cm⁻¹. The before-mentioned ATR-IR spectra are shown in the electronic supplementary material, figure S3. Subsequently, the EDTA wash solution containing material removed from the settled spores was collected and the ICP-MS analysis revealed the presence of 0.10 at% Mg and 0.17 at% Ca. Hence, these metals were present in the settled algal spores and sequestered by the EDTA molecules. A control experiment performed under the same experimental conditions without spores did not reveal the presence of such metal ions upon EDTA washing, confirming the origin of the Ca and Mg ions.

3.5. Ca^{2+} interactions within settled Undaria pinnatifida spores

Following the EDTA treatment, an aqueous solution at pH 8.0 was flowed over the settled spores to remove the EDTA molecules from the flow cell and, afterwards, a 10⁻² M calcium CaCl₂ at the same pH was introduced to the metal ion-depleted settled algal spores. Figure 6 (solid line spectrum) shows the difference spectrum in the 1350–900 cm⁻¹ region resulting from exposure to CaCl₂ solution where the major spectral changes were found. The spectral changes were rapid in response to the Ca^{2+} addition.

There is a generally increased absorption across the region with distinct peaks in figure 6 at 1161, 1085, 1054, 1038 and 970 cm⁻¹. This intensity increase is thought to originate from the gelling of negatively charged polysaccharide chains caused by interactions with Ca^{2+} ions [78]. The formation of a gel results in denser aggregates at the prism/solution interface, leading to the observed increased absorances in the ATR-IR spectrum. A doublet peak was also observed at 1236 and 1224 cm⁻¹. Dobson et al. [79] reported a strong doublet absorption at 1238 and 1200 cm⁻¹ owing to ν_{as}(SO_{4}) of sodium dodecylsulphate both in solution and adsorbed on metal oxide surfaces, and a sharp band at 1061 cm⁻¹ assigned to ν (SO_{4}). Also Cabassi et al. [80] have studied the IR spectra of a number of sulphated carbohydrates and the 1254–1230 cm⁻¹ doublet has been assigned to the ν (SO_{4}) mode. Thus this observed doublet peak probably contains contributions from sulphated polysaccharides involved in the Ca^{2+}-induced gelation.
Confirmation of this suggestion was obtained in a parallel work with calcium ions on \( \kappa \)-carrageenan, a model sulphated polysaccharide. Similar spectral changes in the 1350–900 cm\(^{-1} \) spectral region were recorded for the corresponding experiment as shown in figure 6 difference spectrum (dotted line). The interaction of calcium ions with monoester-sulphate groups of the \( \kappa \)-carrageenan led to the enhancement of the \( \nu_{\text{as}}(\text{SO}_3^{2-}) \) band in the 1300–1170 cm\(^{-1} \) spectral region. A similar absorbance increase was recorded for the interaction of Ca\(^{2+} \) with settled algal spores along with a similar enhancement of the broad polysaccharide band, having major peaks in the 1100–1000 cm\(^{-1} \) spectral region. Thus, the comparison between IR spectra in figure 6 gives spectroscopic evidence for the presence of monoester-sulphated polysaccharide in the adhesive of settled algal spores. Some other spectral changes are not so readily explained, which may be owing to structural rearrangements of the polysaccharide backbone in response to the Ca\(^{2+} \) binding.

3.6. ATR-IR spectroscopy during spore settlement on TiO\(_2\)

The settlement of \( U. \) pinnatifida spores was also evaluated by ATR-IR spectroscopy on an anatase TiO\(_2\) particle film covering the germanium prism surface. Interfacial contributions to spectra are emphasized when a particle film is present because of the much larger surface area relative to the Ge prism alone [81]. Anatase TiO\(_2\) possesses an isoelectric point between pH 5.0 and 6.0 [82]. Therefore, the TiO\(_2\) surface will have a negative charge in the sea water at pH of 8.0. This surface charge reduces charge-induced adsorption but allows any coordinative adsorption to occur. Figure 7 shows the ATR-IR spectra between 1750 and 900 cm\(^{-1} \) originating from the settlement of \( U. \) pinnatifida spores onto a TiO\(_2\)-coated Ge prism over 3 h. The background was from the algal spore suspension at time zero.

Compared with figure 4 ATR-IR spectra from the spore settlement on Ge, figure 7 shows an additional weak absorption at 1723 cm\(^{-1} \), probably arising from the \( \nu_{\text{C=O}} \) of unsaturated esters [83]. The main difference between the spore settlement spectra on the two surfaces is the dramatic enhancement of the absorption in the spectral region between 1200 cm\(^{-1} \) and 900 cm\(^{-1} \), with distinct bands at 1122, 1100, 1027 and 994 cm\(^{-1} \). Such enhancement must be indicative of interactions between the TiO\(_2\) particles and specific functionalities in the algal spore adhesive. However, the carboxylate absorption region is not significantly affected.

Interpretations of the IR region between 1250 and 950 cm\(^{-1} \) can be ambiguous owing to the concomitant presence of polysaccharide, sulphated and phosphorylated compound absorptions. In order to test for the presence of these functional groups in the algal spore adhesive and to assist with the interpretation of the IR spectra in figure 7, ATR-IR adsorption studies were conducted with model compounds containing phosphorylated and sulphated groups. An IR study on the interactions of the model phosphorylated amino-acid O-phospho-L-serine (p-Ser) with a mineral oxide surface was undertaken. Figure 8a shows the IR spectra from \( 10^{-3} \) M p-Ser solution at pH of 8.0 and that of figure 8b p-Ser adsorbed onto TiO\(_2\) from \( 10^{-3} \) M solution at pH 8.0. p-Ser contains a monophosphate group with \( pK_1 = 2.1 \) and \( pK_2 = 6.5 \) [84], thus at pH of 8.0 the phosphate group is almost fully deprotonated with the \(-\text{PO}_3^{2-} \) part possessing approximately \( C_3v \) symmetry, having equivalent P–O bands of bond...
order 4/3. Based on the IR spectroscopic work of Tejedor-Tejedor & Anderson [85] and Gong [86], a 
PO3\(^2\) species with C\(_{3v}\) symmetry shows three P–O stretch bands owing to the degenerate 
v\(_{as}(P–O)\) and non-degenerate v\(_{s}(P–O)\) modes. Hence, the bands at 1091 and 980 cm\(^{-1}\) in figure 8a are assigned, respectively, to the v\(_{as}(P–O)\) and v\(_{s}(P–O)\) modes of 
–OPO3\(^2\) groups, with the higher wavenumber shoulder of the 1091 cm\(^{-1}\) peak being owing to the splitting of the degenerate v\(_{as}(P–O)\) mode. Figure 8b shows the IR spectrum of p-Ser adsorbed onto TiO\(_2\) from 10\(^{-3}\) M solution at pH 8.0. Upon coordination of two of the phosphate O atoms with the Ti(IV) ion, the equivalence of the P–O bonds in the phosphate group is lost and the p = O stretch absorption appears at 1144 cm\(^{-1}\). The other peaks at 1089 and 1000 cm\(^{-1}\) are assigned to, respectively, v\(_{as}(P–O)\) and v\(_{s}(P–O)\) modes of phosphate groups coordinating Ti(IV) ions. Connor & McQuillan [87] investigated the adsorption of \(n\)-butyl phosphate, a monosubstituted phosphate, onto TiO\(_2\) at pH 6 and observed similar peaks at 1150, 1098 and 1004 cm\(^{-1}\) owing to the three P–O stretching modes of phosphate–titanium complexes on the titania surface.

On the basis of the study of the adsorption p-Ser onto TiO\(_2\), the peaks at 1122 and 1100 cm\(^{-1}\) in figure 7 arising from the algal spore settlement on the same substratum are assigned, respectively, to the degenerate and the symmetric stretching modes of dianionic phosphate groups, whereas the bands at 1027 and 994 cm\(^{-1}\) are owing to, respectively, v\(_{as}(P–O–Ti)\) and v\(_{s}(P–O–Ti)\) of phosphate–titanium complexes on the surface. Furthermore, it is worth noting that the asymmetric phosphate stretching mode originating from the phosphodiester groups of nucleic acids is usually observed at approximately 1240 cm\(^{-1}\) [69]. Therefore, the bands found at this wavenumber in figures 3, 4 and 7 may have a contribution from the asymmetric stretching vibration of phosphodiester groups in nucleic acids.

Distinct differences between the ATR-IR spectra of the algal spores settled on the bare Ge crystal and onto the TiO\(_2\)-coated Ge prism were observed. Such spectral changes are attributed mainly to the affinity of phosphate groups for the titania surface, as found for the adsorption of a phosphorylated model compound (p-Ser) on the same surface. In general, sulphate ligands do not bind as strongly to mineral oxide surfaces as phosphate groups. Furthermore, among the model compounds adsorbed on TiO\(_2\) which have been studied, only the phosphate monoester compound phosphoserine gave spectral features similar to those obtained from the algal spore settlement on TiO\(_2\).

4. CONCLUSIONS

The adhesion processes and associated chemistry involved in the initial stage of settlement of U. pinnatifida algal spores on solids from sea water has been studied using EM and in situ IR spectroscopy. The SEM images of the approximately 4 \(\mu\)m spores settled on glass showed clusters of spores held together by secreted adhesive material and indicating their
IR and EM analyses of Undaria spores  L. Petrone et al.

propensity to adhere to each other in preference to the negatively charged glass substrate. The TEM images of spore thin sections clearly showed the cell ultrastructure containing different electron-dense Golgi-derived vesicles, which are probably associated with the secretion of adhesive material. The IR spectra from the spore settlement process initially showed the presence of protein and polysaccharide components. Subsequently, the growth and changes in the IR spectral absorptions indicated that the secretion of the adhesive began approximately 15 min after initial settlement and that the germination of the spore took place within 3 h. The IR spectra of the secreted adhesive showed the presence of carboxylate groups associated with the poly- saccharides. The presence of sulphorylated groups was confirmed by spectral changes from EDTA chelation and calcium ion addition. Phosphorylated anionic groups were also confirmed by comparing IR spectra from model compound adsorption experiments on titanium dioxide with those from spore settlement on the same substrate. EDX microanalysis of the spore adhesive by interactions with the anionic polysaccharides. The abundance of anionic functionalities in the U. pinnatifida spore secretions suggests that the spores are most likely to adhere to the positively charged mineral substrates, although there is evidence for other adhesive mechanisms, such as ligand coordination to mineral metal ions, which are not determined primarily by charge interactions.

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