The spatial structures of hypolithic communities in the Dry Valleys of east Antarctica.

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Abstract Hypolithic communities represent important reservoirs of microbial life in hyper-arid deserts. A number of studies on the diversity and ecology of these communities from different geographic areas have been reported in the past decade, but the spatial distribution of the different components of these communities is still not understood. Moss- and cyanobacteria-dominated hypolithic communities morphotypes from Miers Valley (McMurdo Dry Valleys, East Antarctica) were analyzed by electron microscopy in order to characterize the micro-scale spatial structure. The two communities showed a high degree of internal organization, but differing according to the biological composition. In moss-dominated hypoliths, the moss plantlets are intermixed with mineral fragments of soil origin. However, in cyanobacteria-dominated hypoliths, a layered spatial organization was structured by filamentous cyanobacteria and associated extracellular polymeric components. While moss cells were lacking in cyanobacteria-dominated communities, biofilms formed by cyanobacteria and heterotrophic bacteria were observed in both community morphotypes. The water-holding capacity of both live and dead moss cells and the associated organic matrix, together with the protective properties of the extracellular polymeric substances, could facilitate the survival and activity of these communities. Similar structural strategies can favour the survival of microbial communities in different extreme environments.

Keywords Antarctica, biofilm, cyanobacteria, EPS, hypoliths, moss

Introduction

Lithic-associated niches are important reservoirs of microbial diversity in extreme environments due to their capacity to confer physical and environmental protection to the microorganisms which inhabit them (Friedmann 1982; Green et al. 1999; Cowan and Ah Tow 2004; De los Ríos et al. 2005; Cary et al. 2010; Cowan et al. 2010). Microorganisms colonizing lithic substrates, termed *lithobionts*, may adopt various ecological niches (Golubic et al. 1981), including the surfaces of rocks (epilithic), fissures and cavities within rocks (endolithic) and ventral rock surfaces (hypolithic).

Hypolithic communities are associated with a variety of translucent lithic substrates, including quartz, limestone, gypsum, carbonate and talc. These communities have been reported from many hot deserts including the Mojave (Schlesinger et al. 2003; Smith et al. 2014), Atacama (Warren-Rhodes et al. 2006; Azúa-Bustos et al. 2011; Lacap et al. 2011), Negev (Friedmann et al. 1967; Berner and Evenari 1978; Wierzchos et al. 2012) and Namib (Stomeo et al. 2013) and in other semiarid and hyper-arid regions (Tracy et al. 2010; Pointing et al. 2007; Wong et al. 2010; Weber et al. 2013).

In polar (cold) desert areas, hypolithic communities are also commonly found where suitable lithic substrates occur (Broady 1981; Cockell and Stokes 2004; Cowan et al. 2010, 2011a, 2011b; Khan et al. 2011; Chan et al. 2012). The hypolithic communities of the Miers Valley (McMurdo Dry Valleys, South Victoria Land, eastern Antarctica), which supports abundant hypolithic colonization under quartz and marble substrates (Cowan et al. 2011a), are the most extensively studied. Three different morphotypic hypolithic communities have been suggested, cyanobacteria-dominated (Type I), fungal-dominated (Type II) and moss-dominated (Type III) communities, the

most common being the Types I and III (Cowan et al. 2010). The distribution of the different morphotypes has been shown to be dependant on micro-environmental variables (e.g. soil moisture, temperature), with cyanobacteria-dominated hypolithons occurring commonly at all altitudes in the Miers Valley region, while moss-dominated hypolithons were restricted to lower altitudes (below 415 m a.s.l.) (Cowan et al. 2011a). The bacterial compositions of the different hypolithic microbial communities have been partially characterized using modern phylogenetic methods (Khan et al. 2011). These complex and diverse communities are physically dominated by cyanobacteria from the orders *Oscillatoriales* and *Nostocales* but include numerous heterotrophic bacterial phyla, together with a limited range of archaeal phylotypes and some eukaryotic components (fungi, chlorophytes and bryophytes) (Khan et al. 2011).

Given the dominance of photoautotrophic phyla, it is has been suggested that hypolithic communities are capable of both C (Cockell and Stokes 2006) and N fixation (Cowan et al 2011b). In a recent study, GeoChip analyses indicated that hypolithic communities have the genetic capacity for a very wide range of metabolic processes including interconnected autotrophic, heterotropic and diazotrophic pathways (Chan et al. 2013), and it has been suggested that hypolithic communities may represent a functionally structured consortium. The interaction with the lithic substrate and the excretion of substantial amounts of microbial extracellular polymeric substances (EPS) to create a biofilm organization (De los Ríos et al. 2003; Chan et al. 2012), may also be important in community functioning. Here we present the first electron microscopic analysis of Antarctic Dry Valley hypolithic communities, and demonstrates a high degree of internal spatial organization.

Materials and Methods

Samples

Moss-dominated and Cyanobacteria-dominated hypolith samples (MVH09-21, MVH09-122, MVH09-140, MVH09-00, MVH09-119, MVH09-127, MVH09-14, MVH09-16) were collected from the upper Miers Valley (S 78^{o} 60' E 164^{o} 00'; Fig. 1A-C) during the 2009 austral summer season. Samples (quartz pebbles and their associated hypolithic communities) were collected aseptically and placed carefully into sterile WhirlPaks®. Individual samples were placed in separate boxes in order to prevent disturbance of the overall structure, stored at <0^{o}C in the field, transported at -20^{o}C and stored at -80^{o}C in the laboratory.

Microscopy

Scanning electron microscopy with backscattered electron imaging (SEM-BSE)

Colonized rock samples were prepared according to a procedure developed for observing the rock-microorganism interface by scanning electron microscopy with backscattered electron imaging (SEM-BSE) (Wierzchos and Ascaso 1994). Rock colonized fragments were fixed in glutaraldehyde (3% v/v) and osmium tetroxide solutions (1% w/v), dehydrated in a graded ethanol series (from 30% to 100% v/v) and embedded in LR-White resin. Blocks of resin-embedded rock colonized samples were finely polished, carbon coated and observed using a Zeiss DSM-960 and FEI INSPECT SEM microscopes. Microprobe analyses were performed using an Oxford Instruments

INCA X-act Energy Dispersive Spectrometer (EDS) microanalytical system during SEM observation.

Low Temperature Scanning Electron Microscopy (LTSEM)

Small rock fragments with hypolithic growth were sprayed with distilled water and, after eliminating excess water, were mechanically fixed onto the specimen holder of a cryotransfer system (Oxford CT1500), immediately cryofixed by plunging into subcooled liquid nitrogen, and then transferred to the microscope preparation unit via an air-lock transfer device following the protocol described in De los Ríos et al. (1999). The frozen specimens were cryofractured in the preparation unit and transferred directly via a second air lock to the microscope cold stage where they were etched for 2 min at -90ºC. After ice sublimation, the etched surfaces were gold sputter-coated in the preparation unit and the specimens placed on the cold stage of the SEM chamber. Fractured surfaces were observed using a Zeiss DSM-960 SEM microscope at -135ºC.

Results

The hypolithic communities analyzed included moss- and cyanobacteria-dominated morphotypes (Fig. 1D, 1E). Moss-dominated hypolithic communities form a spongy mat embedded in the mineral soils directly underneath the rock (Fig. 1D). However, cyanobacteria-dominated hypolithic communities were observed as green or orange pigmented biofilms adhering closely to the ventral surfaces of the translucent quartz rocks (Fig. 1E).

Moss-dominated hypolithic communities harbour numerous mineral particles of different chemical composition and of soil origin (Fig. 2A). In the hydrated state, green moss bracts could be observed, confirming the viability of at least part of the moss mat (Fig. 1D). However, microscopic analysis of the mats also showed that the mat structure included a high proportion of dead moss tissue (Fig. 2A). Dead moss tissues are distinguishable by the absence of cellular content (black arrows in Fig. 2B and Fig. 2C), whereas live tissues exhibited a high BSE signal and obvious ultrastructural detail (white arrows in Fig. 2B and Fig. 2C). LTSEM of hydrated moss-dominated hypolithic community samples clearly demonstrated that the mat was not compact, and that voids within the mat structure were ice-filled (stairs in Fig. 2B). In the hydrated state, ice occupied a higher total volume than cells. This observation suggests that the voids in the hypolithic mat structure might act as a water-retention mechanism in the naturally hydrated state.

In some areas, cyanobacterial biofilms (asterisks) were observed to be encasing moss cells (Fig. 2D). Different morphotypes of coccoid and filamentous cyanobacteria as well as putative bacterial cells appeared intermixed in these biofilms (Fig. 2E), with *Nostoc* and *Leptolyngbya* cyanobacterial morphologies being the most frequently observed. The bacterial cells were more frequently observed to be associated with living moss cells (arrowheads in Fig. 2F) than dead tissues, but always appeared to be embedded in an EPS matrix in intimate association with the moss cells (Fig. 3A). While the structure of the microbial community appears to be dominated by cyanobacterial agglutination, coccoid bacteria (arrowheads in Fig. 3B) were also immersed in the EPS matrix (Fig. 3B). In some of the moss-dominated hypolithic communities, dead mosses tissues harboured bacterial communities (arrowheads in Fig. 3C) and even showed some evidence of fungal colonization (white arrows in Fig. 3D).

Many of the moss-dominated hypolithic communities also contained a cyanobacterial cell layer adhering intimately to the rock (Fig. 4A), with an abundance of sheathed filamentous cyanobacterial cells in some areas (asterisks in Fig. 4B) and high amounts of EPS (arrows in Fig. 4A, 4C and 4D). Accumulation of fine mineral particles was observed (F in Fig. 4B).

In cyanobacteria-dominated hypolithic communities (Fig. 1E), filamentous (asterisks in Fig. 5A) and coccoid (C in Fig. 5A) cyanobacterial cells were clearly associated with larger numbers of smaller microbial cells, possibly heterotrophic microorganisms (arrowheads in Fig. 5A and Fig. 5B), and accumulations of fine mineral particles (F in Fig. 5A). As seen in the moss-dominated hypolithic communities, microbial cells in cyanobacteria-dominated communities appeared to be embedded in an extensive EPS matrix (Fig. 5B). While some communities formed narrow and relatively unstructured layers (Fig. 5C), distinctive layering was observed in more developed biofilms (Fig. 5D). These more structured communities showed densely packed thin filamentous cyanobacteria intermixed with (non-cyanobacterial) bacterial cells in close proximity to the rock-face (Fig. 5D). At a greater distance from the rock surface, close to the soil-face, structured layers were less compact and contained larger filamentous cyanobacteria (arrows in Fig. 5D). These filamentous cells showed sheath-like structures (Fig. 5E) but the frequent presence of empty sheaths was also noted (black arrows in Fig. 5F). Smaller cell morphotypes, possibly heterotrophic bacteria (although the presence of archaea cannot be disregarded), were also present in this layer (arrowheads in Fig. 5F).

Microbial biofilms were not restricted to the outer rock surfaces, but penetrated into micro-fractures and crevices in the rock: i.e., showing a ‘chasmoendolithic’ type lifestyle (Fig. 1F, 6A). The colonization observed in the fissures (Fig. 6B) was similar

to that observed in thin cyanobacterial biofilms (Fig. 5C). In thicker cyanobacterial-dominated hypolithic community structures, the penetration into the fissures was more extensive (Fig. 6C). The presence of mineral fragments of the same composition as the lithic substrate was associated with endolithic penetration (blak arrows in Fig. 6A, Fig. 6D), indicating possible biogeophysical changes to the lithic substrate. The accumulation of fine mineral deposits was also frequently associated with endolithic and hypolithic cyanobacterial cells (Fig. 6B, arrow in Fig. 6E). EDS analysis has shown that these mineral deposits are rich in Ca and P (Fig. 6F), indicating that they are not directly generated by physical fragmentation from the quartz lithic substrate. The close association to these mineral fragments with the cells, together with the chemical composition data, suggest that these mineral fragments are of biogenic origin.

Discussion

The different hypolithic morphological types described in the Antarctic Miers Valley (Cowan et al. 2010) have shown distinctly different spatial organizations. The structure of hypolithic communities was principally determined by the biological components forming the community. In moss-dominated communities, the organization of the community was determined by the structure of the moss plantlets, including both live and dead tissues. Associated microbial communities included cyanobacteria and non-photosynthetic microorganisms. Even in communities showing a macroscopic dominance by moss plants, a microbial biofilm was observed at the interface with the lithic substrate, where the spatial structure was determined by both the biological components and mineral fragments of soil origin trapped in the matrix formed by the

moss tissues. In cyanobacteria-dominated hypolithic communities, an obvious layering structure was observed, reminiscent of a microbial mat-like organization. Filamentous cyanobacterial cells and the surrounding EPS appeared to be the principal elements contributing to the observed spatial structure.

Intimate physical associations were observed between the biological components (dead and living forms) and between biological and mineral components, conferring a defined structure to the community. Various small cell morphotypes (possibly heterotrophic bacteria) were observed to be closely associated with moss and cyanobacterial cells, suggesting that recycling processes and nutrient fluxes (e.g., from the lysis products of dead moss cells) might be characteristics of these structures (De los Ríos et al. 2004; Billi 2009). Epilithic and endolithic microorganisms interact intimately with the lithic substrate, inducing physical and chemical alterations to the colonized substrate (De los Ríos et al. 2014). Although the hypolithic communities were closely associated with the quartz substrate, these effects were not detected. However, the penetration of these communities inside the lithic substrates appeared to induce biogeophysical alterations. Other mineral-microorganism interactions were clearly evident within the biofilm, observed as trapping of soil mineral fragments by the mosses in moss dominated communities and as embedding of fine mineral particles in cyanobacterial biofilms, where mineral precipitation may be induced by the organic matrix (Kornhauser and Riding, 2012).

A high bacterial phylotypic diversity has been detected in cyanobacteria-dominated and moss-dominated hypolithic communities from Antarctic Dry Valley sites (Khan et al. 2011; Makhalanyane et al. 2013), consistent with electron microscopic analyses which show a high morphological diversity of microorganisms in similar samples. The high abundance of cyanobacteria observed in the present study, and a

231 predominance of members of the orders *Nostocales* and *Oscillatoriales,* was also in
232 agreement with phylotypic analyses (Khan et al. 2011). These authors also detected
233 moss phylotypic signals (Nuclear Ribosomal Internal Transcribed Spacer region) in
234 cyanobacteria-dominated hypolithic communities, although we observed moss cells
235 only in moss-dominated communities. We speculate that these sequences may represent
236 dormant moss tissues/propagules awaiting favourable conditions for germination (Wong
237 et al. 2010; Khan et al. 2011). The presence of a cyanobacteria-rich biofilm at the
238 interface with the lithic substrate in some moss-dominated hypolithic communities,
239 together with the known tolerance to extreme conditions exhibited by this group of
240 microorganisms (Billi 2009; De los Ríos et al. 2014), suggests that cyanobacteria are the
241 first colonizers of these rocks and supports the successional sequence of hypolithic
242 morphotypes suggested by Makhalanyane et al. (2013). We suggest that structured
243 macroscopic cyanobacteria-dominated communities may evolve from thin biofilms,
244 providing microenvironmental conditions favourable for moss growth, and leading to
245 the evolution of moss-dominated communities.

246 The observation, using LTSEM, of hypolithic communities in the hydrated state
247 provides a view of the spatial structure under conditions of water sufficiency. The voids
248 existing in the spatial structure of hydrated hypolithic communities clearly have the
249 capacity to act as water reservoirs. Similar observations have been recorded in
250 hypothallus samples of the lichen species *Placopisis pycnotheca,* a primary colonizer of
251 bare soils (De los Ríos et al. 2011). Water relations in hypolithic communities are not
252 well understood. It is not clear whether hypolithic communities can take advantage of
253 the intermittent Dry Valley snow-falls, although it is known that snow-melt is minimal
254 and much of the precipitation is lost to the atmosphere by sublimation (Barrett et al.,
255 2006). Microhabitat conditions are therefore an important determining factor for the

functioning of these communities. Microenvironmental analyses through full seasonal cycles suggest that the atmospheric humidity in the hypolith zone is generally much higher than the bulk atmosphere (Chan et al. 2012), an effect that is greatest in the warmer summer months. The extent to which hypolithic communities capture water-vapour from ground-water or melted permafrost is not known, nor is the extent to which the overlying lithic substrate can act as a condensation surface (Nienow and Friedmann 1993). However, humidity values in the active zone above the melted permafrost interface, including in the hypolith community zone, are very high (90-95%), driven by the strong %RH gradient between the near-saturated soil atmosphere and dry bulk atmosphere (Barrett et al. 2006; Cary et al. 2010). The temperature in the hypolithic zone is, on seasonal averages, lower than the bulk atmosphere (Cowan, unpublished results), which might be a contributing factor to the retention of stored water in the hypolithic community zone.

The spatial organization of the hypolithic communities in a hydrated state may provide critical clues to their functional performance. The LTSEM analysis of the hydrated structures has clearly shown the presence of two components which are likely to be highly hygroscopic. Moss tissues, particularly the dead tissues, show the capacity to retain a large amount of water in the spaces between the moss plantlets and mineral fragments. Secondly, the porous structure of the EPS, in which both cyanobacterial and other bacterial cells are embedded, is also known to involved in the retention of the water in biofilms (Chenu 1993). Copious production of EPS is considered to be an important adaptive strategy in lithobiontic communities (De los Ríos et al. 2003). The implication is that both these structures may have a role in two critical water-retention elements: the active adsorption of water from either the liquid or vapour phase, and the retention of adsorbed water in sponge-like networks. The consequence of these

properties is likely to provide an extension of the metabolically active periods, particularly for active photosynthesis, a process which is very susceptible to desiccation inactivation (Schlesinger et al. 2003).

The EPS in hypolithic communities may play other roles that could be critical for the survival of these communities in Antarctic environments. For example, it has been proposed that EPS matrices contribute to the survival of microorganisms under stress conditions by acting as a cell cryoprotectant (Krembs et al. 2002; De los Ríos et al. 2004; Pointing and Belnap 2012) and by facilitating photon trapping and photosynthesis through dense cell packing (Decho et al. 2003). The physical and biochemical relationships between different microorganisms and between microbial and mineral components, facilitated by the spatial organization conferred by the EPS matrix, may be fundamentally important for the development and stability of the community (Siebert and Hirsch 1988; Dorioz et al. 1993; Billi 2009; De los Ríos et al. 2004, 2007; Pointing et al. 2009).

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Figure Legends:

Fig. 1 A-C, Maps of our study sites. A, B indicate the general location of the Miers Valley. C, GIS satellite map of the upper Miers Valley showing the distribution of the different analyzed samples. D, Moss-dominated hypolithic community, E, Cyanobacteria-dominated hypolithic community. F, Endolithic colonization (arrow) of a quartz fragment showing cyanobacteria-dominated hypolithic colonization.

Fig. 2 Moss-dominated hypolithic communities. A, SEM-BSE image of a moss plantlet (M) associated with soil mineral fragments (MF). B, LTSEM image of a hydrated community showing the presence of voids filled of ice (stairs) within the structure, which demonstrates the water accumulation capacity. White arrows indicate living cells and black ones, dead cells. C, SEM-BSE image of a moss plantlet showing living cells with high BSE signal (white arrows) and dead tissues (black arrows). D, SEM-BSE image of moss cells (M) immersed in a cyanobacteria-rich biofilm (asterisks). E, SEM-BSE image of a cyanobacteria-rich biofilm showing *Nostoc*-like (N) and *Leptolynbya*-like (L) cells. F, LTSEM image showing association of a bacterial biofilm (arrowheads) to living moss cells (M).

Fig. 3 Moss-dominated hypolithc communities. A, LTSEM image of a microbial colony associated to moss cells (M) harbouring cyanobacteria (asterisks) and heterotrophic bacteria (arrowhead). B, Enlargement of Fig. A showing the EPS matrix where heterotrophic bacteria (arrowheads), mineral fragments (MF) and cyanobacteria (asterisks) are inmersed. C, SEM-BSE image showing microbial colonies (arrowheads)

associated to a dead moss plantlet. D, SEM-BSE image of moss dead cells showing fungal infection (white arrows).

Fig. 4 Moss-dominated hypolithic communities. A, LTSEM image of a cyanobacteria-rich biofilm adhered to the rock. Cyanobacteria cells (C) and heterotrophic bacteria (arrowheads) immersed in an EPS matrix (white arrows). B, SEM-BSE cyanobacteria biofilm with predominance of filamentous cyanobacteria cells (asterisks). C, LTSEM image of the cyanobacteria layer showing the presence of filamentous (black asterisks) and coccoid cyanobacteria cells (C) and heterotrophic bacteria (white arrowheads) immersed in EPS matrix (white arrows). D, LTSEM image of the cyanobacteria layer at the soil-face showing the presence of a coccoid cyanobacteria cells (C) immersed in a EPS matrix (white arrows).

Fig. 5 Cyanobacteria dominated hypolithic communities. A, SEM-BSE image showing filamentous (asterisks) and coccoid (C) cyanobacteria cells associated to heterotrophic microorganisms (arrowhead) and fine mineral fragments (F). B, LTSEM image of the EPS matrix containing cyanobacteria (asterisks) and heterotrophic bacteria (arrowheads). C, LTSEM image of a thin and non-clearly structured cyanobacterial layer composed majoritary of filamentous cyanobacteria cells (asterisks); RF (Rock-face) and SF (Soil-face). D, LTSEM image of more developed hypolithic community showing a layered structure from the community rock-face (RF) to the soil-face (SF). Arrows note the presence of large filamentous cells at community soil-face. E, SEM-BSE image of filamentous cyanobacteria at the soil-face of the cyanobacterial community. F, SEM-BSE image of filaments of cyanobacteria (asterisks) together with empty cyanobacteria

490 sheaths (black arrows) and heterotrophic-like bacteria (arrowheads) at the soil-face of
491 the cyanobacterial community.
492
493 **Fig. 6** Endolithic penetration of hypolithic communities. A, SEM-BSE image showing
494 the extension of cyanobacterium hypolithic growth (H) into a quartz fissure (E) and the
495 presence of mineral fragments (MF) separated from the lithic substrate. B, SEM-BSE
496 image of a quartz fissure harbouring an endolithic biofilm (arrows) containing
497 filamentous cyanobacteria (asterisks) and fine mineral particles (F). C, SEM-BSE image
498 of extensive endolithic penetration (E) of a more developed hypolithic cyanobacterial
499 community (H). D, Fissure colonized by hypolithic growth showing associated physical
500 fragmentation of the lithic substrate (MF). E, Fissure colonized by cyanobacteria
501 showing accumulation of fine mineral deposits (F). F, EDS spectrum of the mineral
502 accumulation, indicated by an arrow in E.
503
504
505
506
507

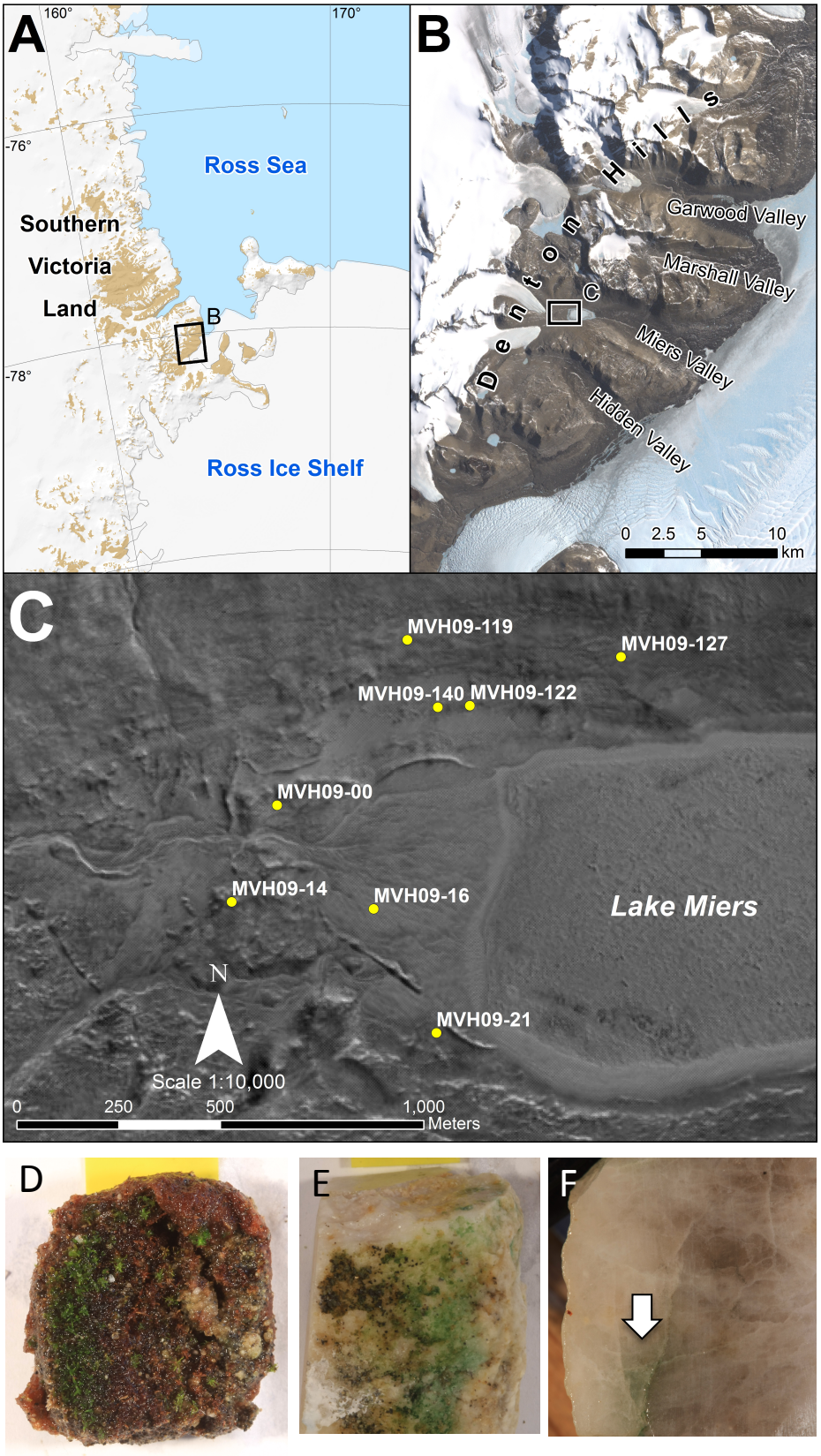

A
160°
170°
-76°
-78°
Ross Sea
Southern
Victoria
Land
B
Ross Ice Shelf
B
Dent Hills
Garwood Valley
Marshall Valley
C
Miers Valley
Hidden Valley
0 2.5 5 10
km
C
MVH09-119
MVH09-127
MVH09-140
MVH09-122
MVH09-00
MVH09-14
MVH09-16
Lake Miers
N
MVH09-21
Scale 1:10,000
0 250 500 1,000
Meters
D
E
F

Fig. 1

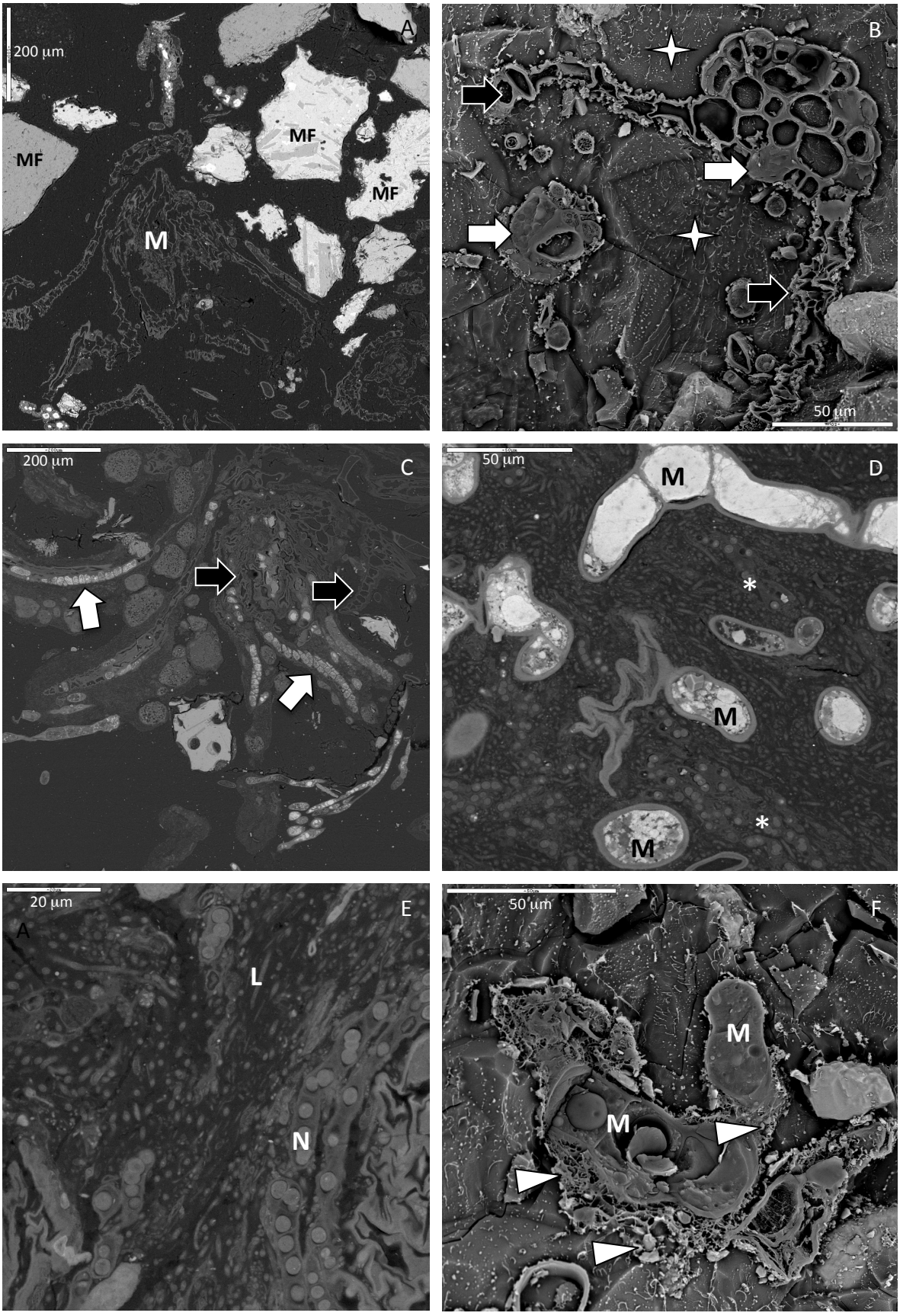


Fig. 2

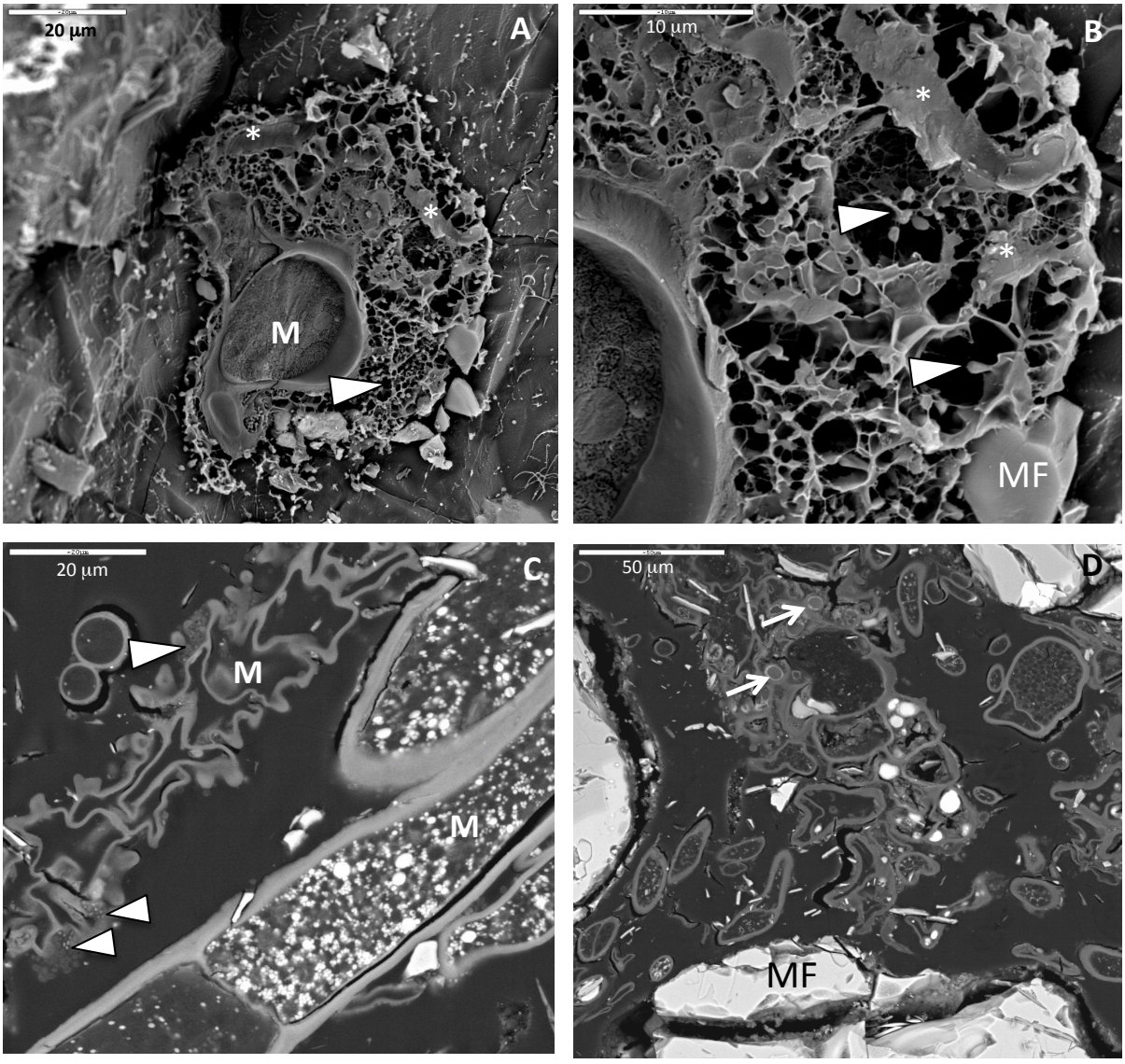


Fig. 3

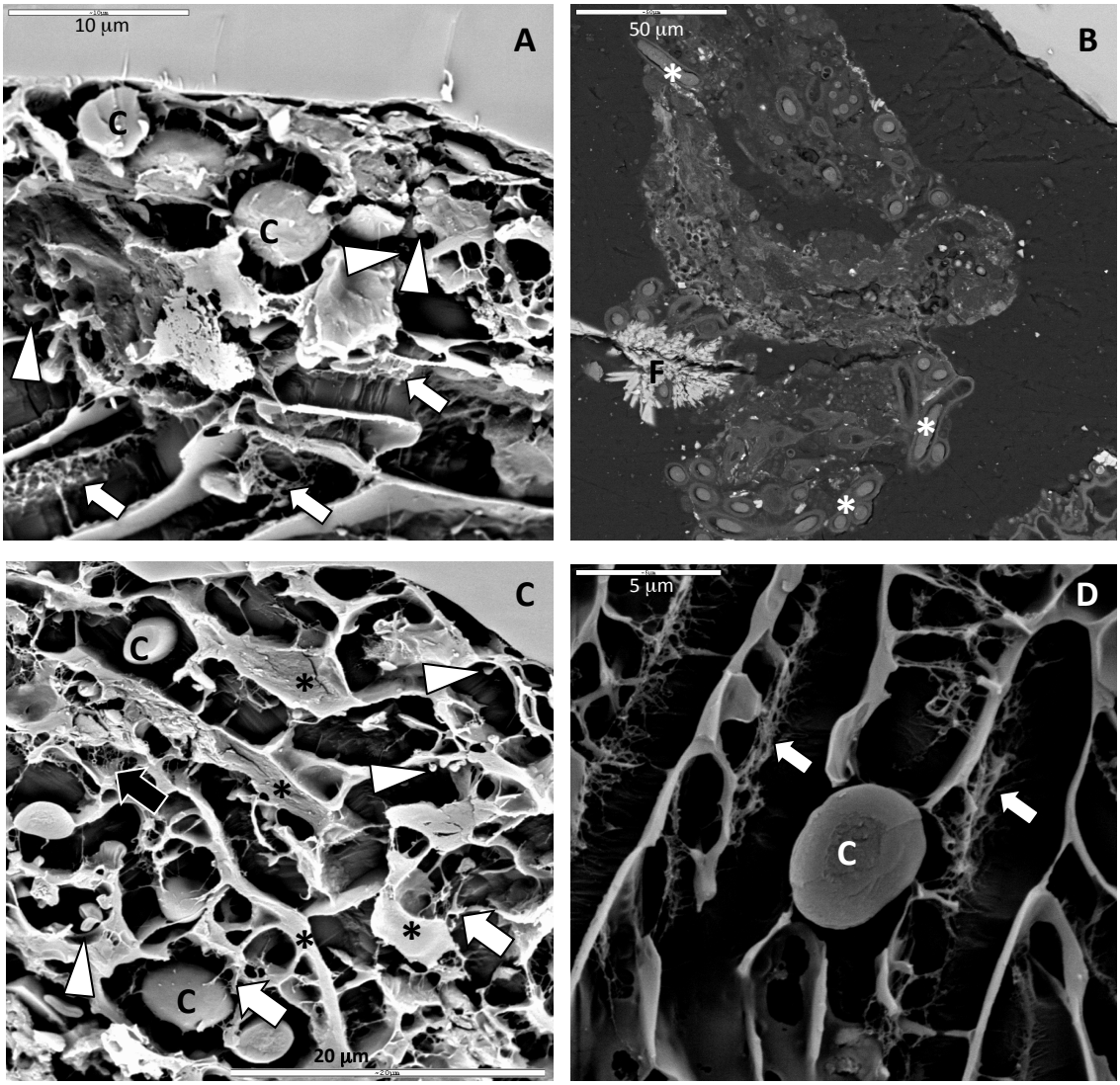


Fig. 4

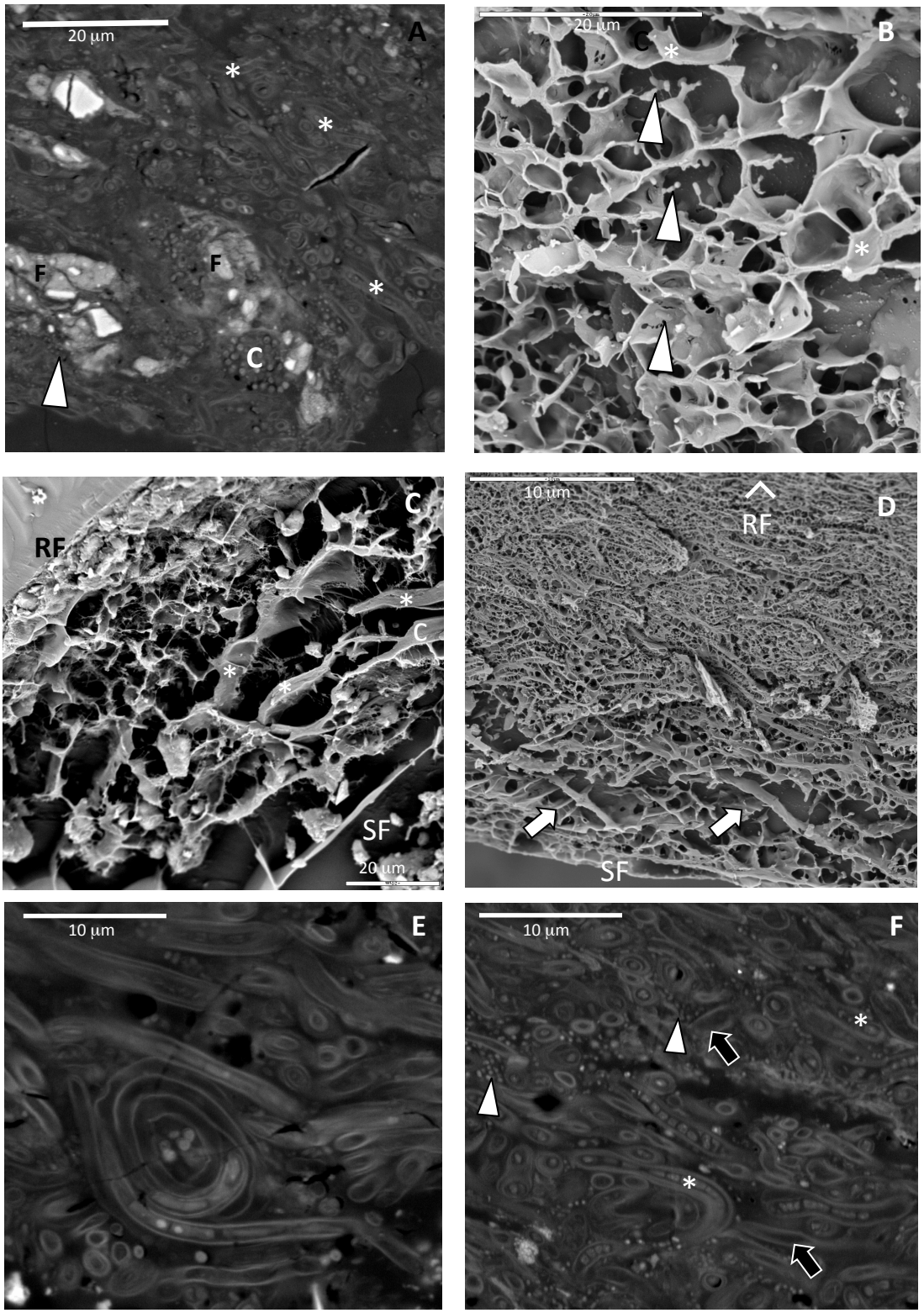
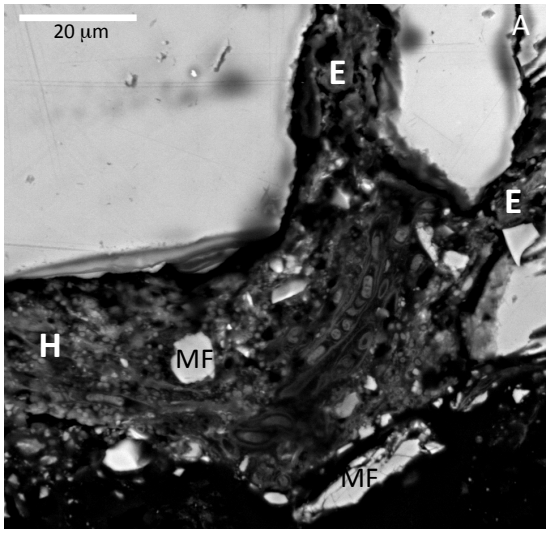
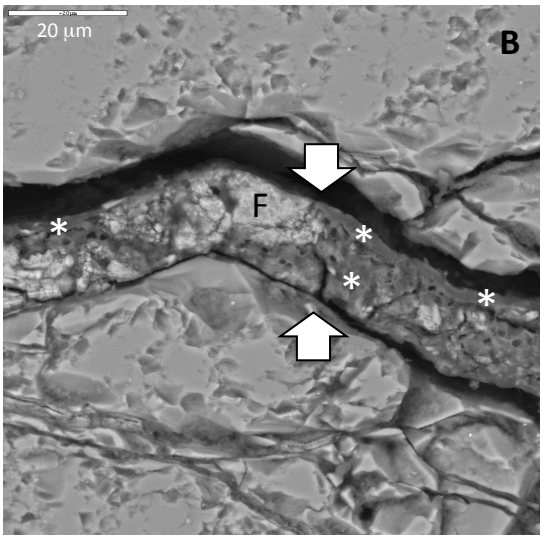
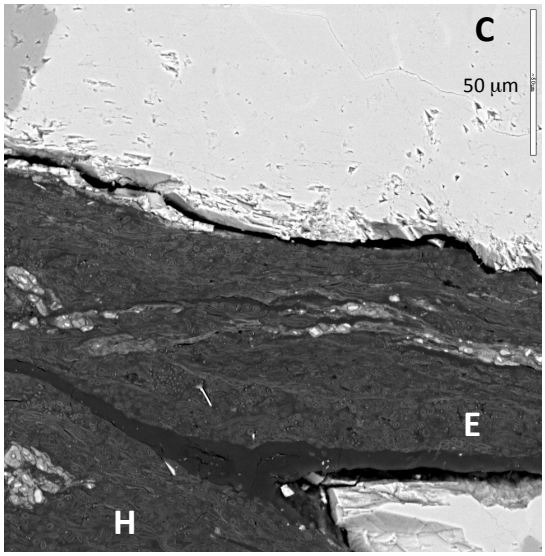
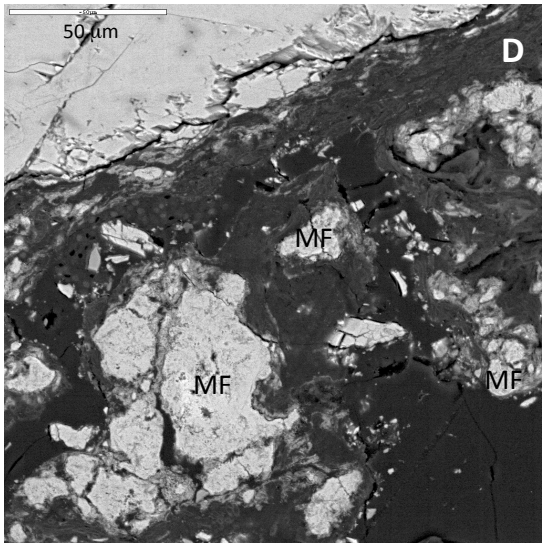


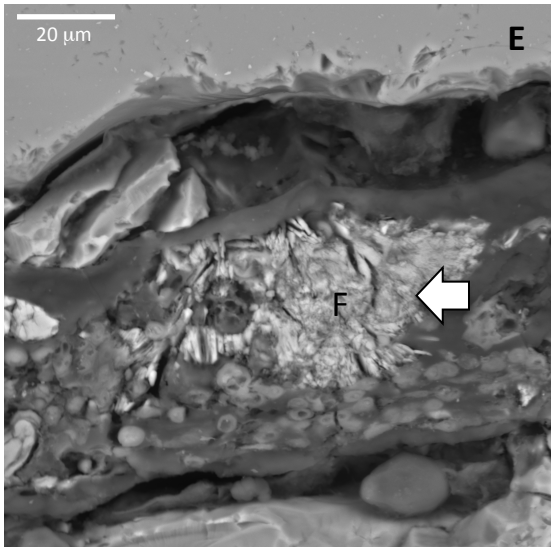
Fig. 5


20 µm
A
E
E
H
MF
MF


20 µm
B
F
*
*
*
*


C
50 µm
E
H


50 µm
D
MF
MF
MF


20 µm
E
F

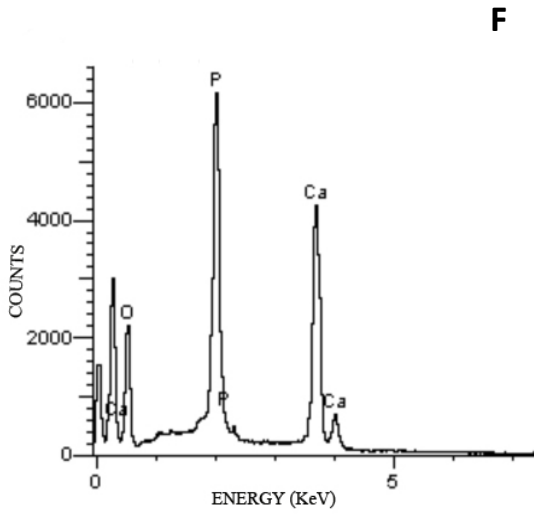

F
6000
4000
2000
0
COUNTS
P
Ca
O
Ca
P
Ca
0
5
ENERGY (KeV)

Fig. 6