Milestone 3: Report

An Investigation into the Control of Bryozoan (*Plumatella and Fredericella*) Infestation of Water Pipeline Systems

John D. Orbell and Robin Mitra

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

School of Engineering and Science
Institute for Sustainability and Innovation (ISI)
Victoria University, Werribee Campus
PO Box 14428
Vic. 8001

In collaboration with
Paul Atherton, Andrew F. Barton and Steven Briggs

GWMWater

GWMWater
Regional Administration Centre
11 McLachlan Street
Horsham
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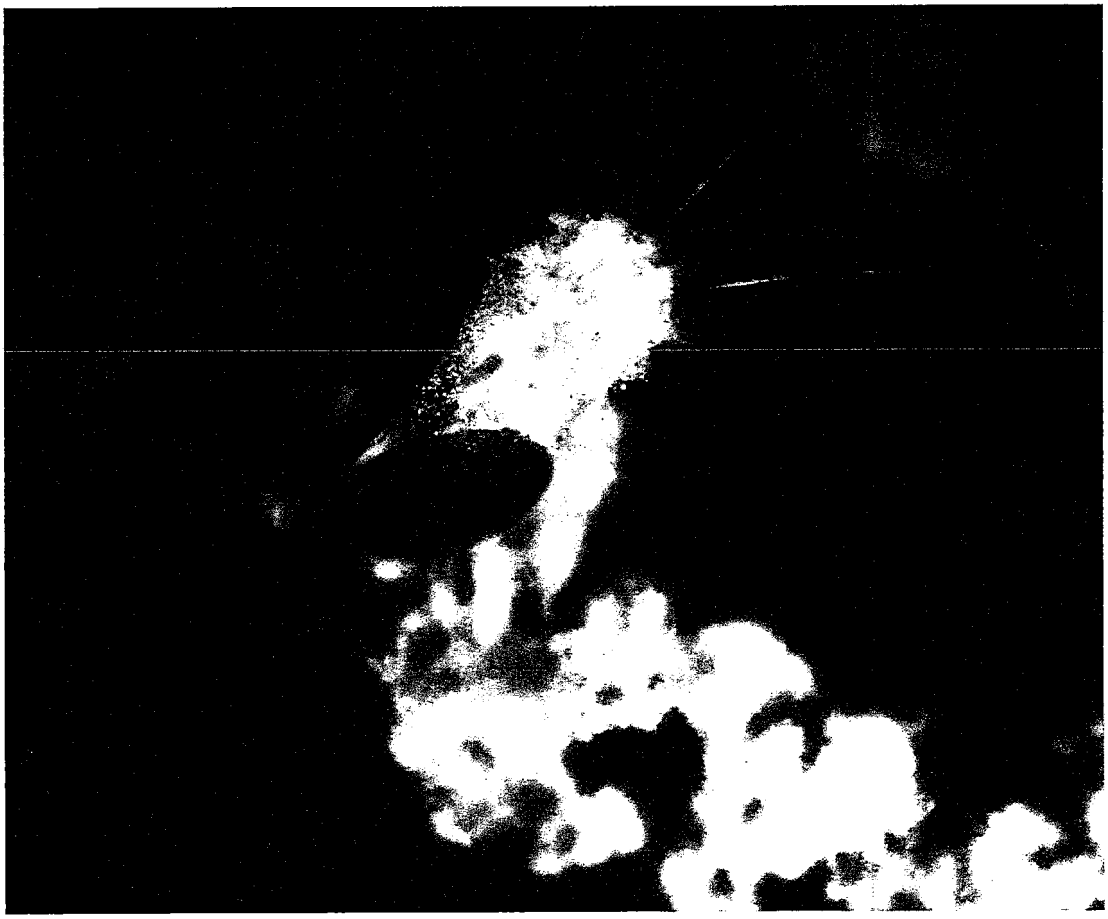


1. The Milestones

Milestone 1 (1st October 2009 to 15th December 2009)

i. Utilizing the leverage from the first year of the project, prepare and submit an ARC Linkage application for Round 2 of 2010 entitled: "Prevention of Bryozoan biofouling of water pipeline systems". This application is to include Dr. Jane Sargison of the University of Tasmania as a collaborator on the project.

ii. Attend, and present the outcomes of the first year of the existing project, at the 32nd Hydrology & Water Resources Symposium, Newcastle, NSW, 1-3 December 2009.

iii. Conduct a two day field trip in December in order to collect post-chlorination samples from the NMP and live colonies for the purpose of colony-to-colony propagation. To consolidate and deliver the Milestone 5 report from the first year of the project.

iv. Provide a copy of the ARC Linkage application - submitted on the 18th October 2009.

v. Provide a copy of the presentation delivered by Dr. Andrew Barton, Dr. Robin Mitra and Professor John Orbell at the 32nd Hydrology & Water Resources Symposium. Provide a copy of the refereed publication that has been included in the proceedings.

vi. Provide the outstanding progress report in relation to Milestone 5 for the first year of the project. Details of the December '09 field trip will be included in the Milestone 5 report. Invoice for the second instalment of $20,871.00 on Tuesday 15th December 2009.

Milestone 2 (16th December 2009 to 28th February 2010)

i. Organize all literature collected to date into a comprehensive review article on freshwater Bryozoans and their biofouling characteristics. Liaise with all team members in order to prepare a draft of this review for submission to a high quality international journal.

ii. Using the live colonies collected on the December '09 field trip, initiate colony-to-colony propagation experiments in the laboratory. The identity of these colonies is to be confirmed from the morphology of their statoblasts (SEM). Continue work on statoblast-to-colony propagation.

iii. Access the commissioned GHD report and reconcile this with our investigations – liaise with Mike Chapman and Barbara Bowles, particularly in relation to a risk management approach to the project.

iv. Arrange a team meeting in February in order to discuss matters relating to experimental design. Issues for consideration include the development of a more systematic sampling protocol, methods for assessing (qualitative and quantitative) the degree of biofouling, water quality data, access to maintenance records and importantly the design and implementation of laboratory experiments to investigate alternative (to chlorine) methods for controlling Bryozoan infestation. Imperative to the testing of alternative control methods is the supply of sufficient quantities of viable Bryozoan colonies that can be challenged in the laboratory with various chemical agents and conditions.

v. Whilst progressing, the current statoblast-to-colony and colony-to-colony methods are proving to be rather sluggish at this stage of the project. Therefore, a concurrent strategy will be initiated relating to the cultivation of colonies on transportable "plates" within the Ouyen "pit". Such plates and growth media can be transported to facilities at VU for control experiments. This "field laboratory" will also allow the issue of seasonality to be conveniently investigated. The experimental design for this will be established at the February meeting scheduled for Thursday 11th February.

vi. Progress report in relation to Milestone 2 to be submitted with invoice for third instalment of $20,871.00 on Monday 1st March 2010.

Milestone 3 (1st March 2010 to 16th May 2010)

i. Finalize and submit the review described in Milestone 2 no later than the end of March.

ii. Using SEM, subject the statoblasts obtained from the December '09 and February '10 sampling to particle size analysis as part of the continuing program to investigate the seasonality characteristics of these organisms.

iii. Continue the statoblast-to-colony and colony-to-colony cultivation of the identified Bryozoan organisms in the laboratory.

iv. Concurrently design, construct and install a "field cultivation laboratory" consisting of an array of growth plates to be suspended in the "pit" at Ouyen (see Milestone 5 report).

v. Transport the plate colonies and pit water to the purpose-built facilities at the St Albans Campus of VU and establish a methodology for assessing growth status and for carrying out static control experiments with various chemical agents. Initial experiments will be carried out using chlorine as a benchmark. Other control agents, such as hypochlorite and nano-particulate silver will then be systematically tested.

vi. Liaise with all team members to produce a draft manuscript detailing the SEM characteristics of the two NMP species also describing their geographical locations.

vii. Initiate formal discussions for the extension of the investigations to the WMP.

viii. Progress report in relation to Milestone 3 to be submitted with invoice for fourth instalment of $20,871.00 on Monday 17th May, 2010.

Milestone 4 (17th May 2010 to 30th July 2010)

i. Submit the article described in Milestone 3 to a high quality international journal by the end of May.

ii. Conduct a two day field trip in June in order to collect seasonal samples and to monitor and collect samples from the field laboratory. Evaluate the field laboratory and process all samples as in Milestone 3.

iii. Continue the static testing and acquire data in relation to the relative effects of various control agents.

iv. Draft a technical paper based on our field sampling experience for publication in appropriate journal.

v. Design, acquire and commission laboratory scale equipment whereby cultivated colonies may be systematically challenged with various control agents under flow conditions.

vi. Progress report in relation to Milestone 4 to be submitted with invoice for fifth instalment of $20,871.00 on Friday 30th July 2010

Milestone 5 (31st July 2010 to 30th September 2010)

i. Attend and present (JO and/or RM) at the International Bryozoan Association conference in Kiel, Germany, from 1-7 August, 2010.

ii. Conduct a two day field trip in late August in order to collect seasonal samples and to monitor and collect samples from the field laboratory.

iii. Continue static testing and the acquiring of data in relation to the relative effects of various control agents both under static and flow conditions.

iv. Evaluate the field laboratory and process all samples as in Milestone 3.

v. Submit the technical paper described in Milestone 4 to an appropriate journal.

vi. Make recommendations on the relative effectiveness of various control agents towards Bryozoans relative to chlorine.

vii. Progress report in relation to Milestone 5 to be submitted on Thursday 30th September 2009.

Table 1: Timetable depicting progress of the project in relation to the YEAR 2 milestones.

Milestones	Oct 2009	Nov 2009	Dec 2009	Jan 2010	Feb 2010	March 2010	April 2010	May 2010	June 2010	July 2010	Aug 2010	Sept 2010
MS 1												
MS2												
MS3												
MS4												
MS5												

1.1. Reappraisal of Milestone 2: salient points

i. Organize all literature collected to date into a comprehensive review article on freshwater Bryozoans and their biofouling characteristics. Liaise with all team members in order to prepare a draft of this review for submission to a high quality international journal.

An appropriate body of literature that addresses many aspects of freshwater Bryozoan biology and development such as laboratory cultivation and culture, nutrition and sampling, ecology and distribution etc has been collected and categorized under various sub-headings (For details refer to Milestone 2 Report March 2010, Section 2.1 pp 14). However, in this context, it is deemed appropriate to mention that the meeting between the team members to discuss the preparation of the review article is yet to take place. Conversely, the delay in the meeting has been considered as a blessing in disguise because in the interim period, the germination of the statoblasts of the freshwater Bryozoans were observed and it can now be emphasized that the knowledge gleaned from such observations may serve as a valuable input towards the review article.

ii. **Using the live colonies collected on the December '09 field trip, initiate colony-to-colony propagation experiments in the laboratory. The identity of these colonies is to be confirmed from the morphology of their statoblasts (SEM). Continue work on statoblast-to-colony propagation.**

The statoblasts were successfully germinated under laboratory conditions in petri dishes containing 'aged' and source water and the single, incipient zooids known as ancestrula were observed to emerge out of the separated valves. The colony is established from the ancestrula through successive budding (Mukai *et al.,* 1984). The petri dishes containing the germinated statoblasts were not transferred into the growth tanks but were immersed in shallow lunch boxes so that they could be observed under the microscope with least disturbance. The colony-to-colony propagation experiments are yet to be successfully established under laboratory conditions. The SEM analyses work was carried out over a period of approximately three weeks from the 10th of May to the 28th of May 2010 at Melbourne University during the course of which a protocol for SEM analyses of statoblasts was successfully developed **(Refer to Appendix 1).**

iii. **Access the commissioned GHD report and reconcile this with our investigations – liaise with Mike Chapman and Barbara Bowles, particularly in relation to a risk management approach to the project.**

To date, the meeting with Mike Chapman and Barbara Bowles in relation to the risk management approach to the project has not taken place.

iv. **Whilst progressing, the current statoblast-to-colony and colony-to-colony methods are proving to be rather sluggish at this stage of the project. Therefore, a concurrent strategy will be initiated relating to the cultivation of colonies on transportable "plates" within the Ouyen "pit". Such plates and growth media can be transported to facilities at VU for control experiments. This "field laboratory" will also allow the issue of seasonality to be conveniently investigated. The experimental design for this will be established at the February meeting scheduled for Thursday 11th February.**

As a result of a meeting held between Andrew Barton, Steven Briggs, John Orbell and Robin Mitra on Thursday the 11th of February 2010 the 'Pit apparatus also designated as the 'Field Rig' had been successfully suspended in the Ouyen Pit **(Refer to Milestone 2 Report 2010, pp 31 – 32. Figs 19a, 19b, 19c and 19d).** However according to current reports from Ouyen, the overall growth of colonies on the plates appears to be meagre and the cause has been attributed to the approaching winter. In the meantime, a decision has been reached between Robin Mitra and John Orbell to install two heaters on the growth tanks with a temperature range of 18°C to 32°C in order to maintain the growth

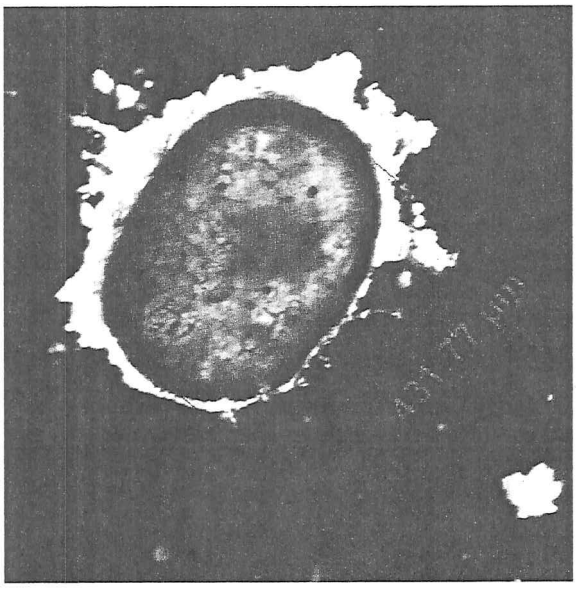
tanks at 25°C (room temperature) although the GHD Report (Anon 2009) has suggested 15°C as the appropriate temperature for initiating Bryozoan growth. However, according to Reynolds (2000), colonies of *Plumatella fungosa* were established from viable floatoblasts in his lab, in complete darkness, at a room temperature of 24.5°C. Mukai et al. (1988), germinated statoblasts of *Plumatella emarginata, Plumatella repens, Plumatella casmiana* and *Hyalinella punctata* at 25°C and cultured the germinated ancestrulae in water containing food organiams such as green algae and diatoms that were derived from a large thriving aquarium. Rao (1992), suggested that, in the laboratory, a small glass aquarium equipped with a thermostat maintained at 25°C should be used to germinate the statoblasts. Therefore, It is appropriate that the heaters are installed in the growth tanks prior to the next sampling tour so that the sampled colonies as well as the filters with entwined colonies are floated in plastic lids within growth tanks maintained at room temperature.

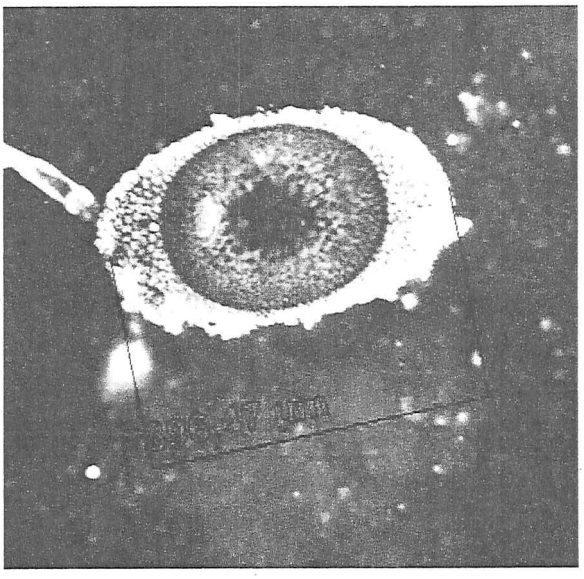
2. Addressing Milestone 3 (1st March 2010 to 16th May 2010)

2.1. Particle size analysis

Two types of control experiments are to be implemented in this project (i) control experiments conducted under static conditions and (ii) under flow conditions. The corpus of literature pertaining to control experiments related to freshwater Bryozoans is rather scanty and inchoate however control agents such as chlorine, chlorine dioxide, sodium hypochlorite, ozone and hydrogen peroxide have been deemed to be trialled in the present project. Moreover there is also a need to mimic both the ecological niche and the hydrodynamic milieu under which these biofoulers tend to thrive and hence control experiments under flow conditions are thought to be an integral aspect of the project. In this context, cultivation of identified Bryozoan colonies within annular reactors and the use of a variable slope flume suggested by Andrew Barton have been recommended to be included in the experiment. However a third proposition by Eddy Ostarcevic in the light of his query "if physical control of freshwater Bryozoans through good filtration is possible?" have been given substantial consideration in this project, for which 'particle sizing' of identified freshwater Bryozoan statoblasts seems to be an indispensible approach. The concept of physical control of the biofouling organisms in the Northern Mallee pipeline immediately warrants the designing of effective 'statoblast filters' or 'statoblast traps'. The idea for 'statoblast traps' originated in Northern Germany when aquatic seed traps were being designed to capture plant seeds during flooding (Vogt *et al.*, 2004). These seed traps were later redesigned by Hartikainen et al. (2009) to capture statoblasts of freshwater Bryozoans including those belonging to the genus *Plumatella*, by altering the pore sizes of the mesh funnel. Simon Crawford (Research Fellow / Electron Microscopist) from Melbourne University was contacted in this respect, who confirmed the availability of such software through which particle sizing of statoblasts could be conducted simultaneously with Scanning Electron Microscopy analyses. It is also noteworthy to mention here that such an option is also incorporated in the Zeiss Axioplan 2 research-grade

microscope which is available at Victoria University, St Alban's campus, **Figs 1a, 1b, 1c and 1d.** Currently, there are eight species of freshwater Bryozoans that have been identified from the Northern Mallee pipeline (NMP) system of which two *Fredericella australiensis* and *Fredericella sultana* belong to the genus *Fredericella* and the other six namely, *Plumatella emarginata*, *Plumatella reticulata*, *Plumatella minuta*, *Plumatella repens*, *Plumatella casmiana* and *Plumatella vaihiriae* all belong to the genus *Plumatella*. Of the six species of *Plumatella*, the floatoblasts of *Plumatella minuta* are very small with an overall length of 312-323 µm and an overall width of 224 – 237 µm (Wood *et al.,* 2006) and to date no sessoblast of this species has ever been discovered. The only available literature on *Plumatella minuta* in our possession at the moment are the Marato Toriumi (1955) and Wood et al. (2006) articles. Tim Wood's new book "An Introduction to the Freshwater Bryozoans of Thailand' which is known to carry some information on *Plumatella minuta* has been put on order from the university library but has not arrived as yet. To date *Plumatella minuta* has only been reported from Asia, where the organism has been detected in countries such as Japan, Formosa, Korea and Bung Borophet in Thailand (Wood *et al.,* 2006) but has never been reported from the continent of Australia. Although the recent discovery of *Plumatella minuta* in the Northern Mallee pipeline (NMP) system is a major find (unpublished), however the small size of its floatoblasts may pose a fresh challenge in the designing of the mesh pore size of the statoblast traps to be used as a means of physical control. Currently the only physical control known to exist in the potable water industry are the sand filters, the wide spread use of the sand filters have reduced the discharge of these organisms from household faucets (Wood 2005a).





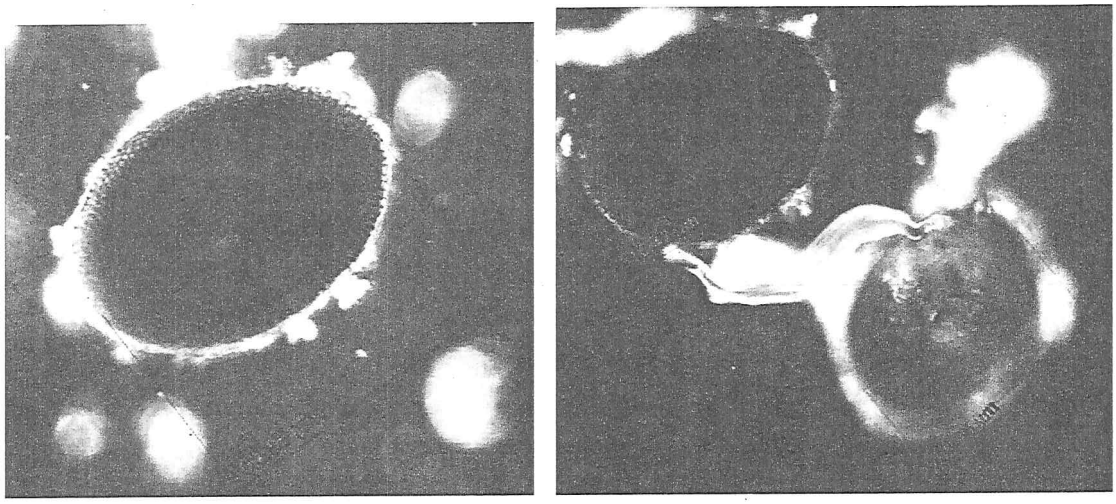


Fig 1. (a) The size of a Fredericellid statoblast (431.77 µm) possibly belonging to the species *Fredericella australiensis,* construed from the presence of the thickened rim around the edges (b) The size of a Plumatellid floatoblast (396.47 µm) frayed at the annulus area belonging to the genus *Plumatella emarginata* (c) The size of a Plumatellid floatoblast (304.56 µm) which seem to bear resemblance to the floatoblasts of either *Plumatella repens* or *Plumatella casmiana.* (d) Comparison of a Plumatellid type floatoblast (316.42 µm) to a floatoblast suspected of belonging to that of *Plumatella minuta* because of its small size (255.95 µm). Note the Plumatellid type floatoblast in Fig (c) and Fig (d) are the same, the slight variation in the sizes 304.56 µm and 316.42 µm has been attributed to human error. The sizes of the statoblast and the floatoblasts were determined using the Zeiss Axioplan 2 research-grade microscope at St Albans.

2.2. Statoblast-to-colony propagation

The germination of the statoblasts (genus *Fredericella*) and the floatoblasts (genus *Plumatella*) were carried out successfully this time. One of the ways for successfully germinating the statoblasts under laboratory conditions followed by the establishment of colonies through successive budding would be to approximate conditions that are experienced by Bryozoans in their natural environment (Wood and Okamura, 2005). Since the statoblasts are known to be resistant to adverse environmental conditions (Black and Proud 1976), the alternative way would be to subject the statoblasts to extreme conditions either by (i) implementing drought and desiccative (summer) conditions during the course of which the statoblasts are sandwiched between sheets of filter paper and dried slowly at room temperature (suggested by Timothy Wood; personal communication) for a period of one to three weeks or longer or (b) by implementing cold and dark (winter) conditions during the course of which the Bryozoan colonies are left to die in a dark, cold room at 5^{0}C for a period of 1 to 3 months. The dying colonies produce statoblasts which when transferred to a more favourable condition (25^{0}C) are convinced into believing that the onset of spring is at hand and the memory of the organisms tricked into germinating. In some strains of *Arabidopsis thaliana*, flowering is promoted by initially exposing the plants to long

periods of cold temperature, a phenomenon known as vernalization before transferring the plants to optimal conditions thereby providing the plants with a cue that the long and bleak winter is over (Sung and Amasino 2004; Wolpert *et al.,* 2007) and the time to flower is at hand. Black and Proud (1976) achieved a 100 % germination rate between 48 and 72 hours in statoblasts belonging to the freshwater Bryozoan, *Pectinatella magnifica* by storing the statoblasts at 4^{0}C for several months. However it is also appropriate to mention, that the germinating ability of the statoblasts are not only under the influence of various external factors like temperature, water or light but are also influenced by the endogenous physiological conditions (Rao 1992). In this study, a single Fredericellid type statoblast was found to germinate after three days of drying **(Refer to Milestone 4 Report July 2009 Figs 41b and 42)** however later attempts to germinate statoblasts through prolonged periods of drying in 2010 was somehow unsuccessful. Oda (1961), is of the opinion that long periods of dehydration leading to massive loss of water may affect the germinating capability to a great extent. On the contrary when freshly isolated statoblasts from dead colonies that were stored in the cold room after the summer sampling trip (8th and 9th December 2009) were added in April 2010 to the petri dishes containing previously isolated and dried statoblasts, a sudden explosion of germination was observed **Fig 2.**

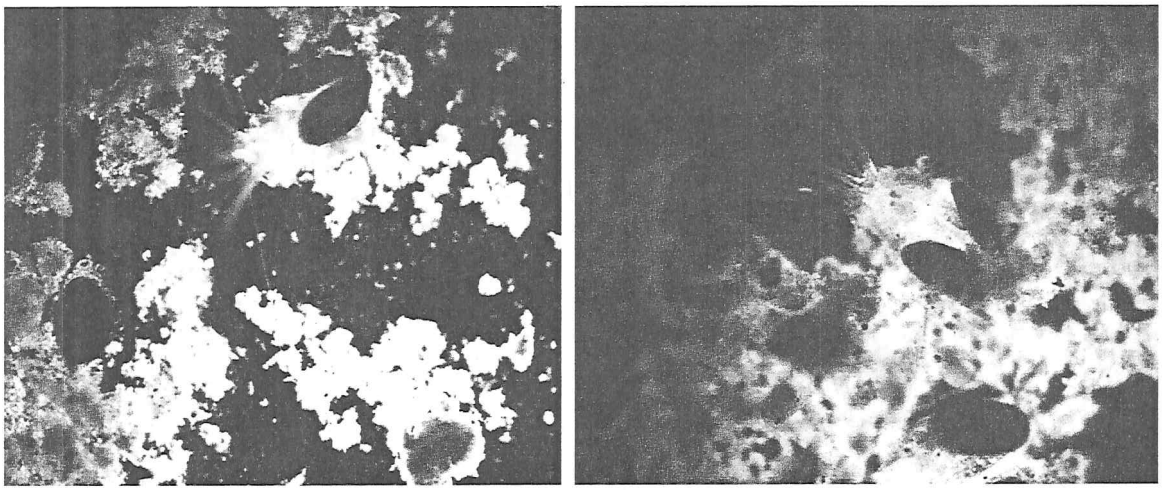


Fig 2. A sudden explosion of germination was observed after the addition of statoblasts produced by dead colonies stored in the cold room (4^{0}C) at the St Albans campus from Dec 2009 to April 2010.

Oda (1959) mentions that from a series of studies made by Rogick (1938; 1940; 1941), she concluded that statoblasts that were stored in a low temperature of about 10^{0}C provided better results with regards to germination and viability. From the current study, it can be hypothetically stated that the sudden transfer of statoblasts from the cold and dark cold room where they were stored at 4^{0}C to a petri dish contained with a mixture of 'aged' water from a thriving aquarium and source water placed at an optimal room temperature with considerable illumination could have tricked the memory of the organisms into supposing that the onset of spring was at hand. However this approach has to be

reiterated again before a more affirmative conclusion could be derived. Wood (2005b) is also of the opinion that there is a high possibility of germinating many statoblasts within a predictable period of time by first storing them under unfavourable conditions to tide over the dormancy period known as overwintering which may normally last for 3 to 5 weeks in the asexual phase. However there is also the question of seasonality to be taken into consideration. Another method that is yet to be tried out for the culture of Bryozoans is to incubate the Bryozoan colonies and statoblasts in aged and source water supplemented with Chalkley's medium (1.7 mM NaCl, 50µM KCl and 50µM $CaCl_2$) (McGurk *et al.,* 2006). Morris et al. (2002), on the other hand, has suggested the culture of Bryozoan colonies (and statoblasts?) in petridishes containing artificial freshwater of intermediate hardness (0.35 mM $CaSO_4$ ($2H_2O$), 0.5 mM KCl, 0.5 mM $MgSO_4$ ($7H_2O$), 0.1 mM $NaHCO_3$). Documented studies indicate that the growth of Bryozoan colonies are positively related to the fertility of the water, the abundance of Bryozoan colonies in unfertilized ponds are meagre compared to ponds fertilized with N-P-K (Dendy 1963). High concentrations of statoblasts belonging to the genus *Plumatella* has been reported from rivers high in nutrient concentrations and growth rates of *Fredericella sultana* in laboratory microcosms have been observed to increase with increasing nutrient content, thereby fulfilling Hartikainen et al. (2009) hypothesis that nutrient enrichment increases Bryozoan abundance.

2.3. Stages of statoblast germination (after Mukai 1982 and Mukai *et al.,* 1984)

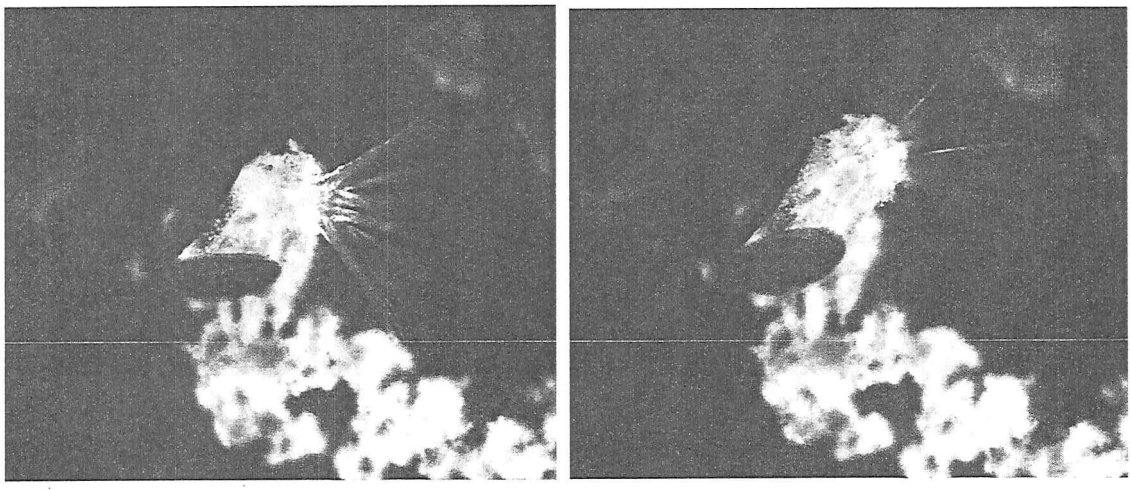
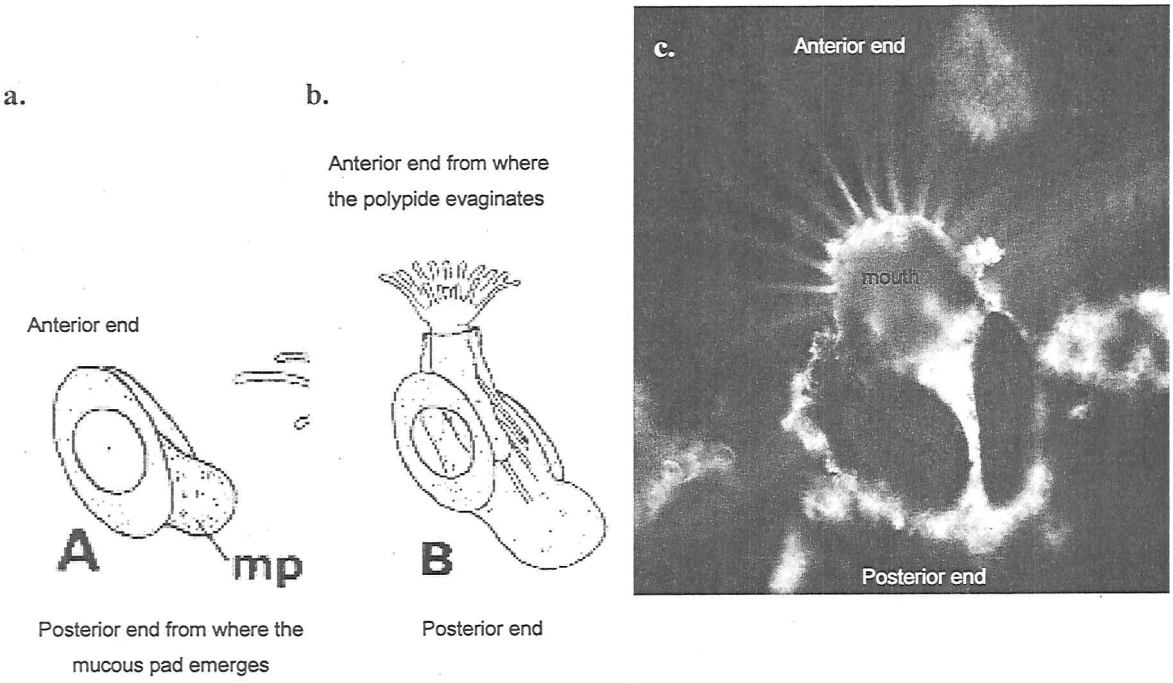


Fig 3. Reorganization of the undifferentiated tissue within the chitinous bivalves of a floatoblast and the emergence of a mature zooid of the Plumatellid type from separated valves of a floatoblast that tends to resemble that of *Plumatella emarginata* .

(i) A single zooid is known to arise from a statoblast and this incipient zooid is designated as the ancestrula. The ancestrula usually undergoes budding to give rise to one to five new zooids which in turn gives rise to multiple zooids. Proliferation of colonies or zoarium is usually observed with the rise in water temperature (Wood 2001)

(ii) Upon germination the ventral and the dorsal valve of the statoblast separates giving rise to the mucous pad (mp), Fig 4a which forms the posterior part of the ancestrula. The surface of the mucous pad is extremely sticky and the function is to enable the ancestrula to anchor to the substrate which in our case was the bottom of the petri dish. The emergence of the mucous pad was not observed, only fully matured zooids of the Plumatellid type has been photographed.



. Fig 4. (a) Emergence of a mucous pad from in between the two separated valves of a statoblast (b) Evagination of a tiny polypide (c) Mature zooid displaying rows of ciliated tentacles around a central mouth which lies at the anterior end. (Figs 4a and 4b Mukai *et al*.,1984)

(iii) Once the organism cements itself to the substrate with the aid of a mucous pad, a polypide evaginates from the anterior end. The polypide bears a prominent lophophore composed of ciliated tentacles around a central mouth, Fig 4c.

(iv) A complete unit of a colony is a zooid which is composed of two parts a polypide and a cystid. The evaginable entity of the zooid is the polypide which is composed of a tentaculated lophophore and viscera. The cystid part of the zooid represents the body wall that accommodates the polypide.

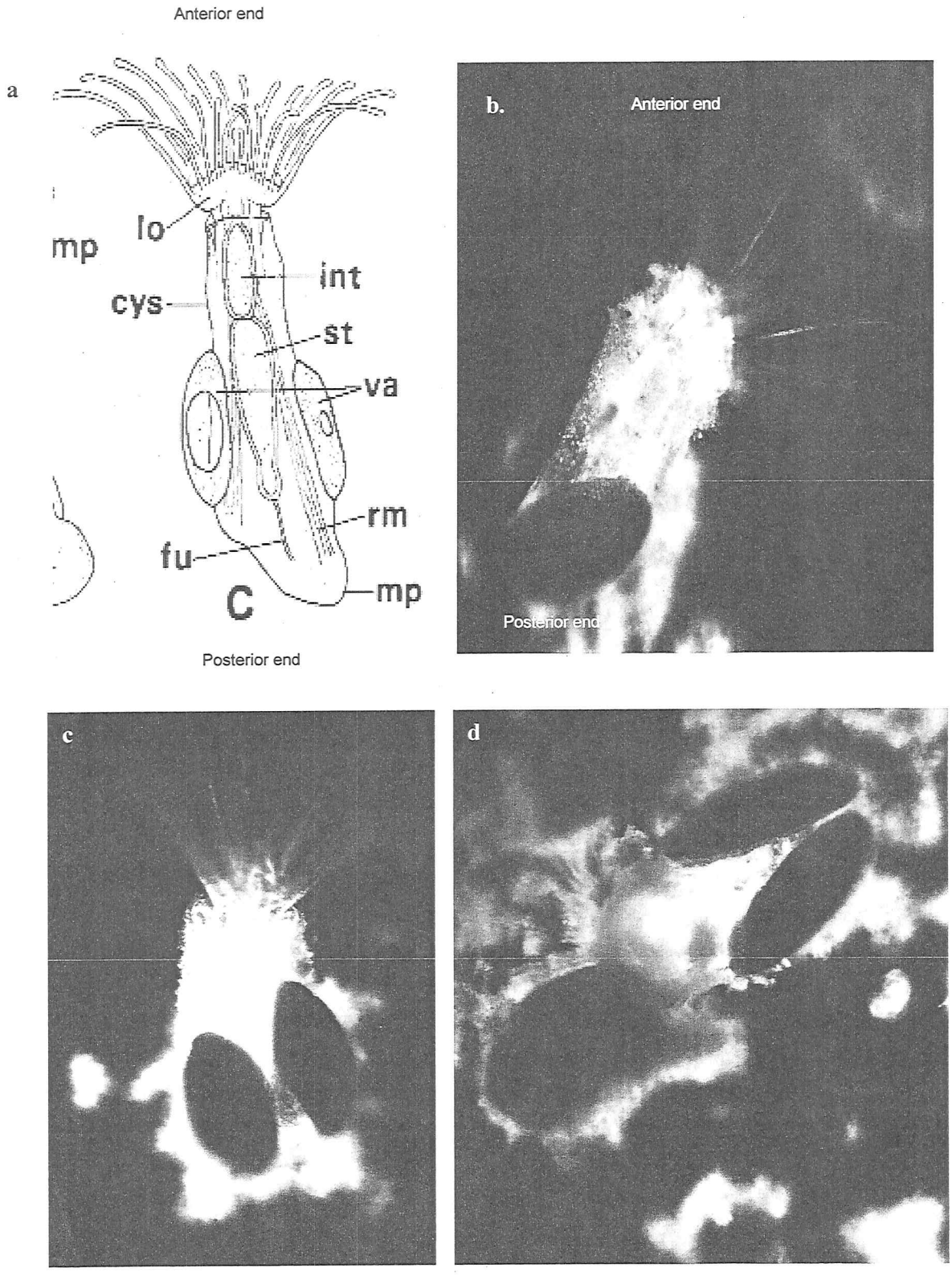


Fig 5. (a) Anatomical features of a mature zooid lo – lophophore; cys – cystid; fu – funiculus; int – intestine; st – stomach; va – valves; rm – retractor muscle; mp – mucous pad (Mukai *et al.*, 1984). (b) Mature zooid observed in a petri dish in the lab at St Albans.

2.4. Brief notes on the lophophore

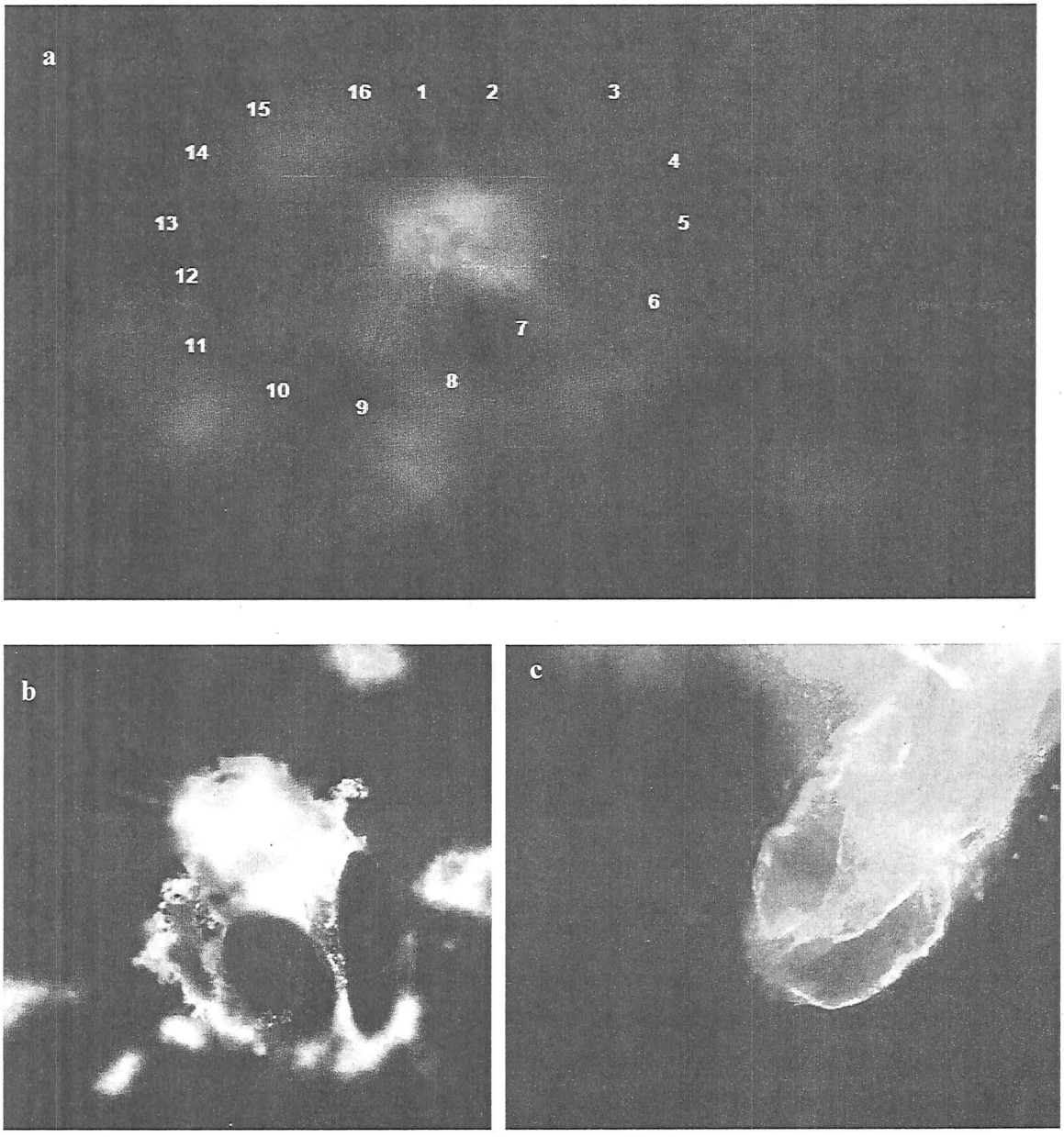


Fig 6. (a) In the members of the genus *Plumatella*, the tentacles are usually arranged in a double row. **Fig 6a** shows an extended lophophore with the number of tentacles on the fringes of the outer row as it was not possible to count the number of tentacles in the inner row. (b) the members of the genus *Plumatella* (as apparent from the micro-architecture of the separated valves) usually possess a U-shaped lophophore which however is not clearly depicted in **Fig 6b.** (c) Retracted tentacles of a zooid that had germinated from a Fredericellid type statoblast (**Refer to Milestone 4 Report July 2009 Fig 43b**).

The polypide bears a prominent lophophore, **Figs 6a** and **6b**; and **Fig 7** which is usually composed of a number of ciliated tentacles encircling a central mouth (Wood 2001). The anterior end of the polypide is usually horse-shoe or U-shaped except in the members of the *Fredericella* where it is almost circular (Mukai 1982), similar to that of the marine Bryozoans (Okamura and Doolan 1993). In the members of the genus *Plumatella*, the U-shaped lophophore bears a double row of tentacles, an outer row of long tentacles, **Figs 6a** and **6b**; and **Fig 7** and an inner row of short ones (Wood 2001), which in our case was a bit difficult to photograph. The cilia beat in coordination to create metachronal waves (Wood 2001). The number of tentacles may vary within the species for e.g. the mean number of tentacles in *Plumatella emarginata* is 38 while that in *Plumatella reticulata* is 34 (Wood 1988) .

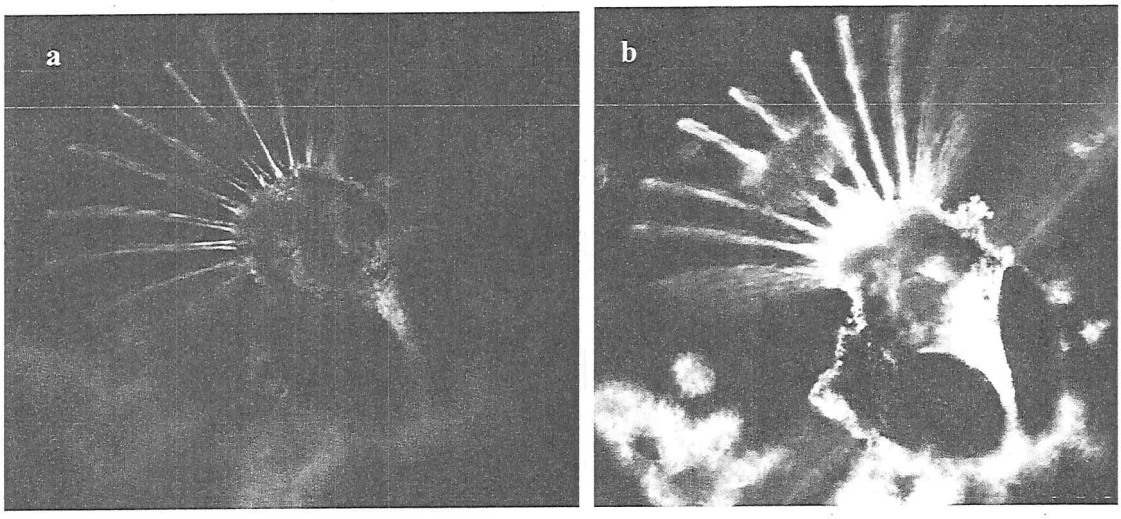


Fig 7. Crown of ciliated tentacles in a Plumatellid zooid captured in different phases of the Zeiss Axioplan 2 research-grade microscope at the St. Albans campus.

In general, the Bryozoans are known to generate their own active feeding currents with the aid of the crown of ciliated tentacles. However according to Pratt (2008), not all Bryozoan zooids are capable of feeding, only those that possess the movable part known as polypide that contains the digestive system (differentiated into pharynx, oesophagus, stomach. Intestine and an anus) and bears the lophophore retains the capacity to feed.

2.5. Colony-to-colony propagation

The 'colony to colony' now seems to necessitate a future sampling trip however from previous experience it is also now becoming apparent that the Bryozoan colonies cannot survive more than two days in sealed sampling containers. Hence it is now preferable that all the four prime sampling sites viz. Ouyen, Kiamal, Piangil and Nyah be covered in a single day with the colonies being taken to the laboratory at St Albans immediately and stored at room temperature overnight. The details of the

new approach for 'colony-to-colony propagation has been delineated in **Milestone 2 Report March 2010. Section 2.1.1. pp 15 – 16.**

2.6. Scanning Electron studies: total number of species detected from Northern Mallee pipeline (NMP) system

As mentioned earlier in Milestone 5 December 2009 (in which the first results from our early Scanning Electron Microscopy (SEM) work was reported), that apart from the hard part of the cuticle, the only consistently hard parts of the freshwater Bryozoans are the dormant buds like the sessoblasts and the floatoblasts in the genus *Plumatella* and the statoblasts in the genus *Fredericella* with their chitinous outer valves (Wood and Wood 2000). The floatoblasts and sessoblasts of the Plumatellidae are sclerotized structures that often bear minute micro-ornamentations in the form of rounded prominences called tubercles or raised reticulations thereby offering the possibility of distinguishing individual species (Wood 1979) of a particular genus. Hence, phylactolaemate systematists have long considered statoblast morphology (and the patterns of micro-ornamentations) as the primary diagnostic feature at the species level (Bushnell and Rao 1979). Apart from the tubercles and the raised reticulations, the floatoblasts of the Plumatellidae may contain tiny raised bumps known as the nodules which may be seen on the annulus region (Wood 2001). SEM analyses of isolated stato/sesso/floatoblasts of the genus *Fredericella* and *Plumatella* from the Northern Mallee pipeline system is being carried out for several months now during the course of which some more new species have been discovered and the old ones reconfirmed. The work is being carried out in the Botany department at Melbourne University, **Figs 8a and 8b.**

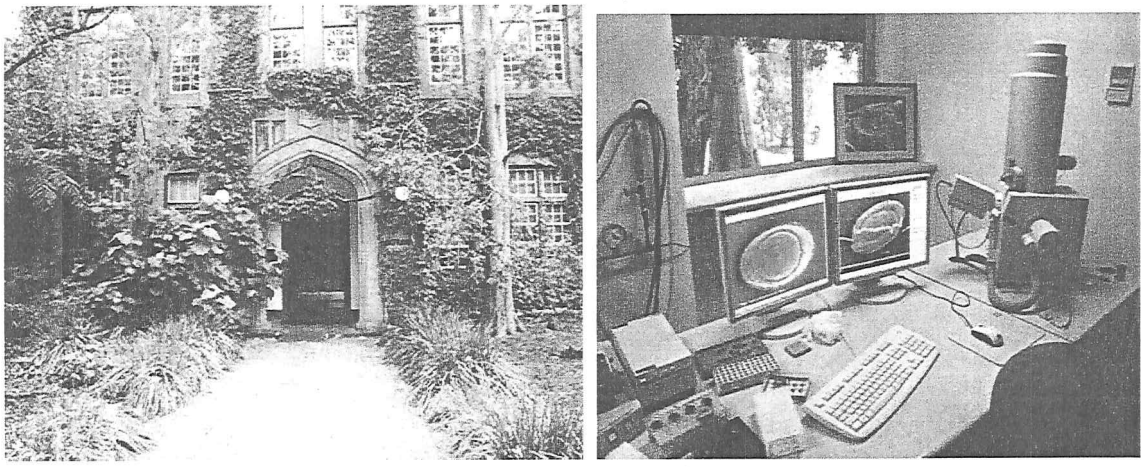


Fig 8. (a) The botany building at Melbourne University (b) SEM work in the identification of *Plumatella* floatoblasts in progress at Melbourne University

Compiling together all the reports from Bryo Technologies to date, the correspondences with Dr Tim Wood and all the micrographs from SEM analyses work from Melbourne University, all the species

from the genus *Fredericella* and *Plumatella* that has been confirmed thus far has been compiled in **Table 2.** During the course of the SEM work, a working protocol relating to the identification of statoblasts at the species level has been successfully developed with the help of Dr Simon Crawford a Research Fellow and Electron Microscopist **Fig 9** at Melbourne University.

Table 2: Taxonomy of the identified species of freshwater Bryozoans of the class Phylactolaemata and the types of asexual buds produced.

No	Species	Genus	Family	Order	Types of asexual buds produced	Location
1	*Fredericella australiensis*	*Fredericella*	Fredericellidae	Plumatellida	Sessile statoblast	Nyah and Piangil pump stations
2	*Fredericella sultana*	*Fredericella*	Fredericellidae	Plumatellida	Sessile statoblast	Nyah and Piangil pump stations
3	*Plumatella casmiana*	*Plumatella*	Plumatellidae	Plumatellida	Sessile sessoblast, free floatoblast, free leptoblast	Nyah and Piangil pump stations
4	*Plumatella emarginata*	*Plumatella*	Plumatellidae	Plumatellida	Sessile sessoblast, free floatoblast	Nyah and Piangil pump stations
5	*Plumatella minuta*	*Plumatella*	Plumatellidae	Plumatellida	Sessile sessoblast still undetected, free floatoblast	Nyah and Piangil pump stations
6	*Plumatella repens*	*Plumatella*	Plumatellidae	Plumatellida	Sessile sessoblast, free floatoblast	Nyah and Piangil pump stations
7	*Plumatella reticulata*	*Plumatella*	Plumatellidae	Plumatellida	Sessile sessoblast, free floatoblast	Nyah and Piangil pump stations
8	*Plumatella vaihiriae*	*Plumatella*	Plumatellidae	Plumatellida	Sessile sessoblast, free floatoblast	Nyah and Piangil pump stations

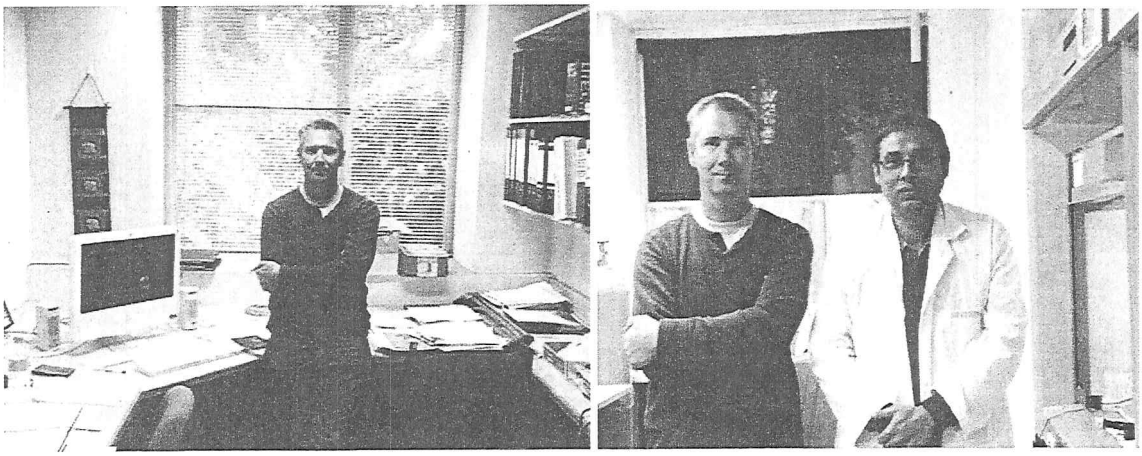


Fig 9. Dr Simon Crawford who provided the necessary training required to get acquainted with the various instruments related to sample preparation prior to SEM analysis.

2.7. Discussion on all the species detected in the Northern Mallee pipeline (NMP) system

Plumatella is the largest genus in the family with many species and therefore the most complicated from the taxonomical point of view (Rieradevall and Busquets 1990) thereby necessitating the use of Scanning Electron Microscopy (SEM) work for resolving the identification of the organisms at the species level

2.7.1. *Plumatella emarginata* Allman 1844

Floatoblasts

- The floatoblasts are broadly oval or elliptical. The float (annulus area) which is filled with a secreted gas covers the capsule more widely on the cystigenic (dorsal) side in comparison to the deutoplasmic (ventral) side (Mukai *et al.,* 1990). The deutoplasmic side is more convex than the deutogenic side by comparison.
- Small flat dorsal fenestra with smooth surface (Martinovic-Vitanovic *et al.,* 2010), the tubercles are less apparent on the dorsal side (Wood and Okamura 2005)
- Ventral valve slightly larger than dorsal, strongly convex with distinct tubercles on the fenestra (Martinovic-Vitanovic *et al.,* 2010)
- Cells of the annulus are individually convex, with distinct borders and fitted together like paving stones (Wood and Okamura 2005).

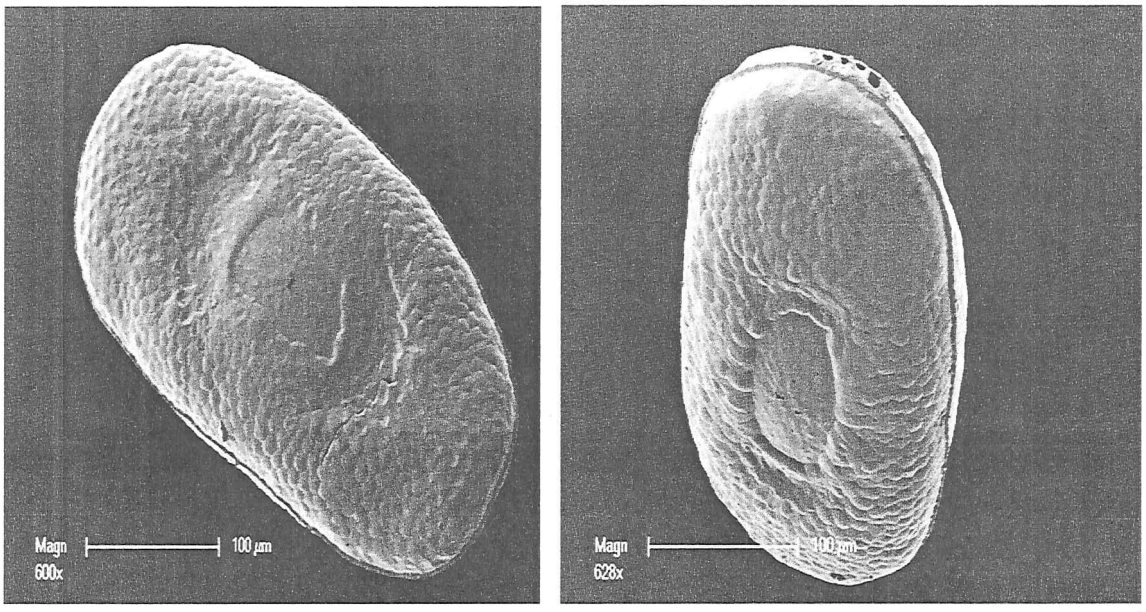


Fig 10. Dorsal (or cystigenic) valve of a floatoblast belonging to the species *Plumatella emarginata*, the fenestral area is small and oval compared to the annulus region and the tubercles are less distinct.

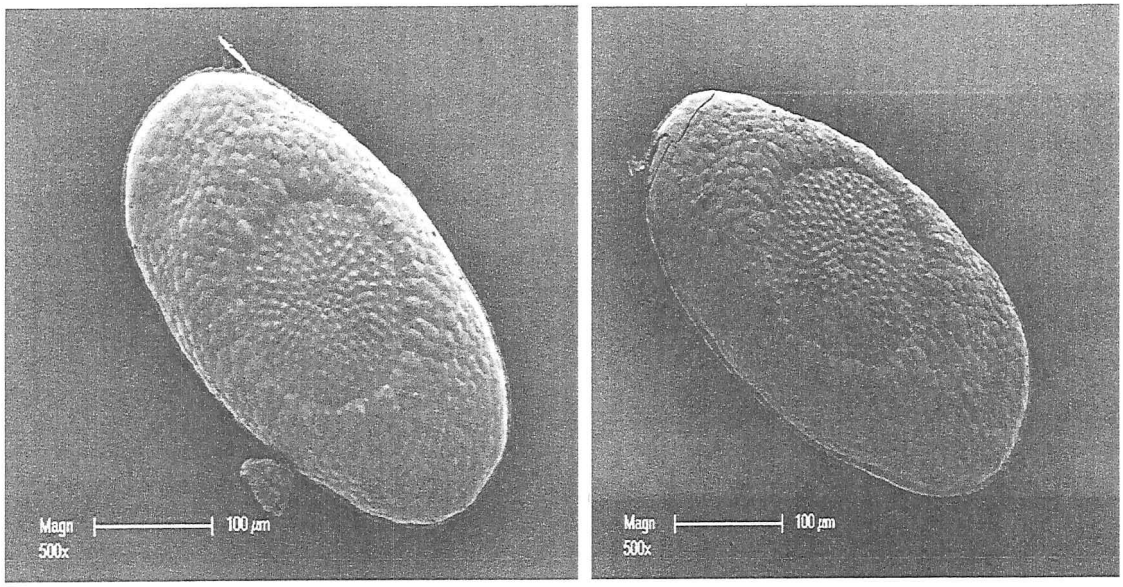


Fig 11. Ventral (or deutoplasmic) valve of a floatoblast belonging to the species *Plumatella emarginata*, the fenestral area is large and oval and bear more distinct tubercles. The ends of the floatoblasts are more tapering.

Sessoblasts

- Sessoblast exhibit raised lateral wall of the annulus (Wood and Okamura 2005)
- Tubercles present in sessoblasts are usually numerous, small and prominent (Wood and Okamura 2005)

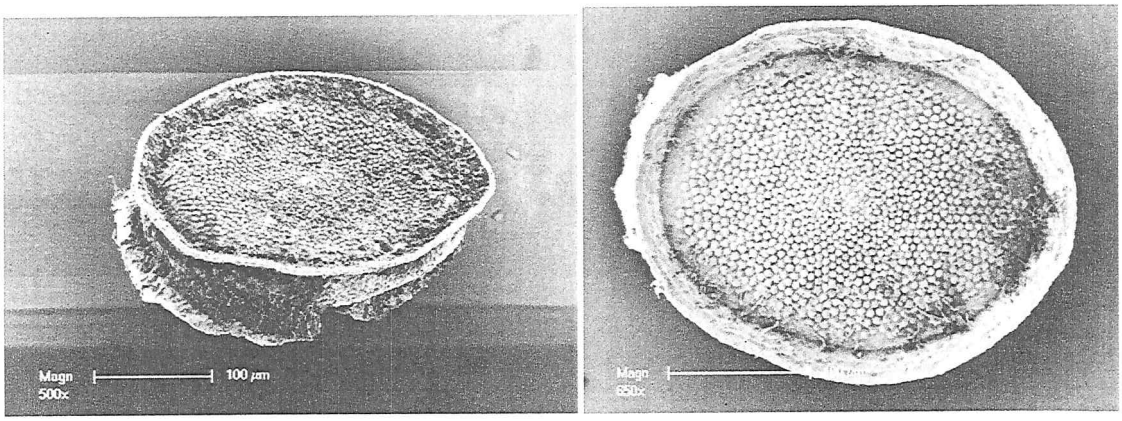


Fig 12. Judging from the raised lateral wall of the annulus and the presence of numerous small and prominent tubercles on the fenestral area, the sessoblasts depicted above could be construed to that belonging to *Plumatella emarginata*, however the absence of tubercles on the frontal valve compels one to make an alternative suggestion, the possibility of these sessoblasts belonging to *Plumatella casmiana* in which the tubercles in the frontal valve are usually reduced.

2.7.2. *Plumatella reticulata* Wood 1988

Floatoblasts

- There is a superficial resemblance between *Plumatella reticulata* and *Plumatella emarginata* for e.g. only these two species tend to share an extensive dorsal annulus on the floatoblast (Wood 1988).
- The floatoblasts of *Plumatella reticulata* are not as elongated similar to that of *Plumatella emarginata* and is more rounded while the floatoblast of *Plumatella emarginata* is more tapering by nature (Wood and Okamura 2005).
- The sides of the statoblasts are more straight in comparison to *Plumatella emarginata* and thus the opposite sides are parallel and the ends more blunt (Wood 1988; Wood and Okamura 2005).
- The valves of both *Plumatella reticulata* and *Plumatella emarginata* are almost equally convex (Wood 1988; Wood and Okamura 2005).
- The floatoblasts of *Plumatella reticulata* can easily get confused with that of *Plumatella emarginata.* These two species are frequently found to grow together (Wood 1988).

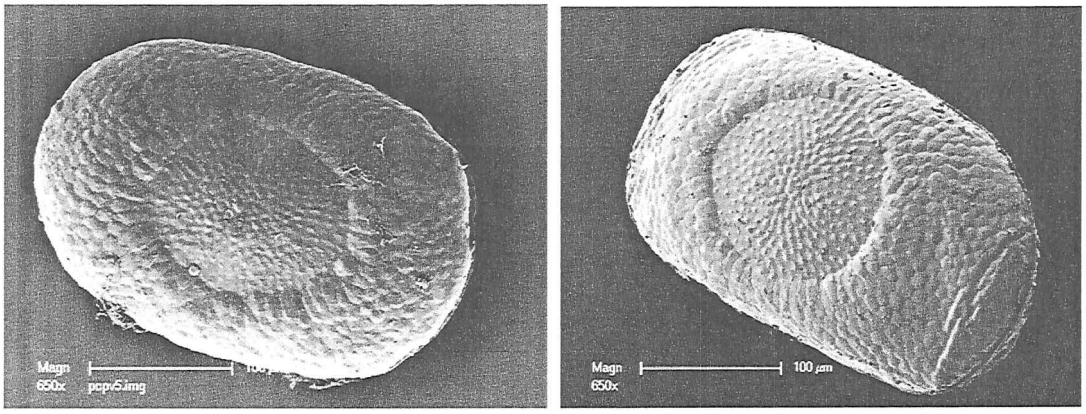


Fig 13. Ventral (or deutoplasmic) valve of a floatoblast belonging to the species *Plumatella reticulata*. Unlike the floatoblasts of *Plumatella emarginata*, these floatoblasts are endowed with parallel sides and the ends are blunt rather than tapering. The fenestral area is large and oval and bear prominent tubercles

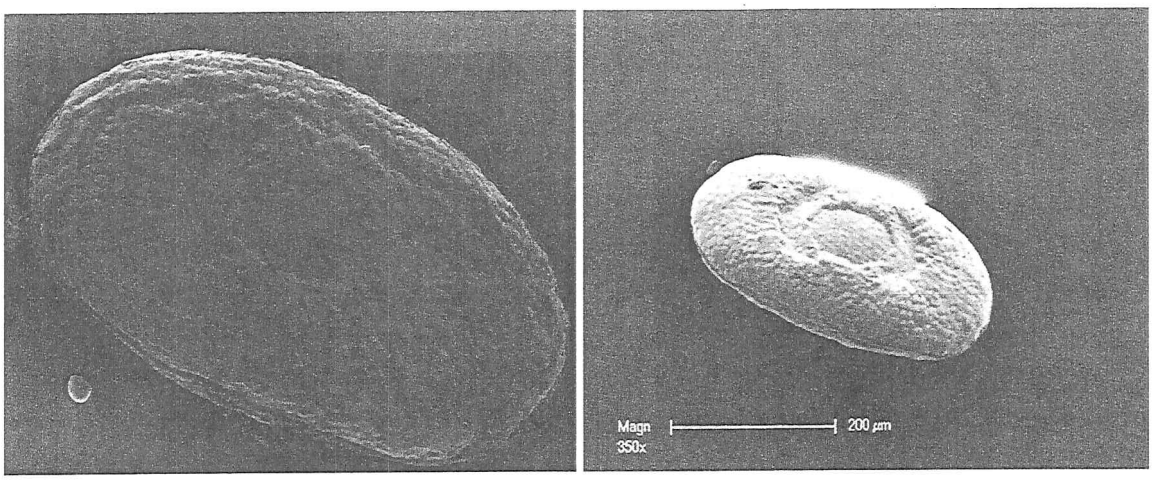


Fig 14. Dorsal (or cystigenic) valve of a floatoblast belonging to the species *Plumatella reticulata*. Judging from their roundish appearance and blunt ends as well as parallel sides, these floatoblasts have been designated as those belonging to *Plumatella reticulata*. The fenestral area is round and smooth.

Sessoblasts

- Sharply raised blade like reticulation on the frontal valve (Wood and Okamura 2005).

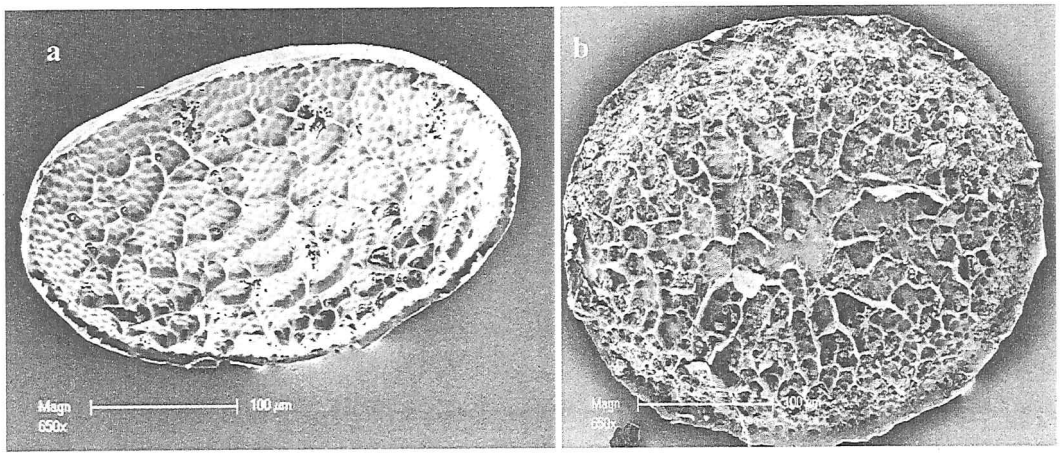
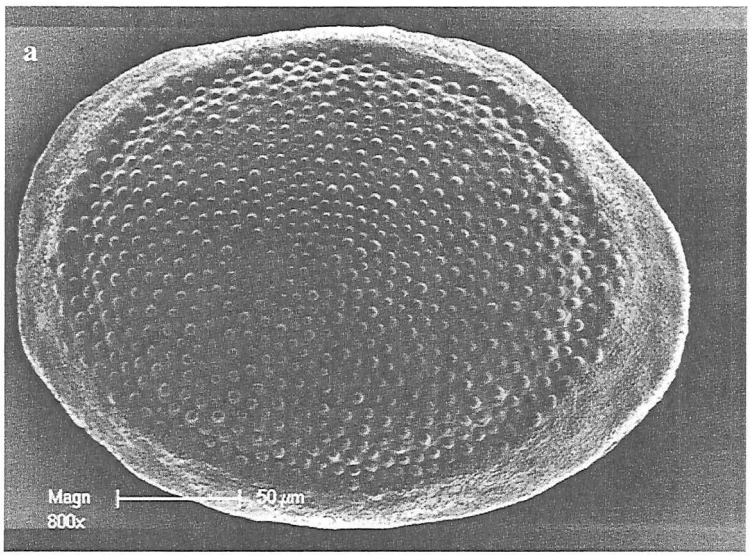


Fig 15. (a) Sessoblast of *Plumatella reticulata* (confirmed by Dr Timothy Wood) with its sharp raised reticulation. The frontal valve of the sessoblast appears to be smaller than normal (b) The sessoblast lacking in a raised frontal valve tends to resemble more to that of a reticulated *Internectella bulgarica* rather than a *Plumatella reticulata* sessoblast depicting a root like pattern on a rather low and underdeveloped frontal valve.

2.7.3. *Plumatella minuta* Toriumi 1941

Floatoblasts

- Broadly oval in shape and laterally symmetrical (Wood *et al.*, 2006).
- Large fenestral area on both dorsal and ventral valves (Wood *et al.*, 2006).
- Tuberculation in the fenestral area could be weak and may tend to fade towards the centre (Wood *et al.*, 2006).
- Floatoblast dimensions are provided in **Fig 16b** (Wood *et al.*, 2006).
- The sessoblasts of *Plumatella minuta* has never been found (Wood *et al.*, 2006).



	Range	Mean	N
Overall length	312-323	320 ± 22	12
Overall width	224-237	230 ± 13	12
Overall length/width	1.34-1.43	1.39 ± 0.10	12
Dorsal fenestra length	178-191	185 ±13	12
Dorsal fenestra width	153-167	160 ±14	12
Dorsal fenestra length/width	1.11-1.20	1.16 ± 0.08	12
Ventral fenestra length	187-213	200 ± 26	12
Ventral fenestra width	167-180	175 ± 13	12
Ventral fenestra length/width	1.05-1.19	1.14 ± 0.14	12

Fig 16. (a) The rare and single floatoblast of *Plumatella minuta* (confirmed by Dr Tim Wood) detected from the Northern Mallee pipeline (NMP) system during SEM analyses in March 2010, however no floatoblasts of *Plumatella minuta* were uncovered during the SEM analyses in May 2010. Note the large fenestral area and somewhat narrow annulus area (b) Floatoblasts measurements for *Plumatella minuta* in micrometres (Wood *et al.*, 2006).

2.7.4. *Plumatella repens* Linnaeus 1758

Floatoblasts

- The length of the dorsal fenestra is more than half the total length of the floatoblast (Wood and Okamura 2005)
- The dorsal valve has large tubercles along the periphery of the fenestral area which tend to grow smaller and interstitial towards the fenestra's centre (Wood and Okamura 2005)
- The dorsal part of the capsule is less covered by the annular float cells than the ventral part (Rieradevall and Busquets 1990)
- Tubercles are absent on the annulus but nodules in the form of very small, rash-like bumps are present in the annulus (Wood and Okamura 2005)
- In lateral view the valves are almost equally convex that means bulging on both sides (Wood and Okamura 2005)

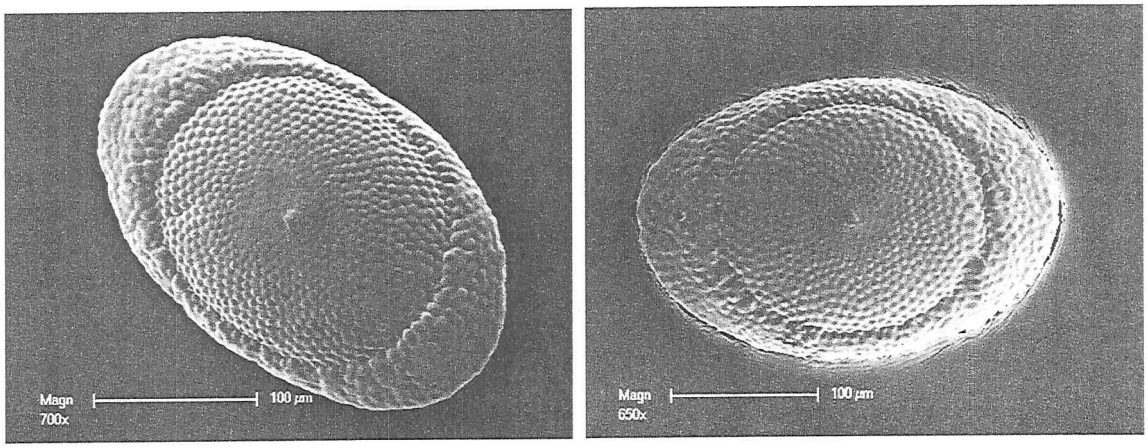


Fig 17. Based on the visual judgment from photoghraphs and sketches presented in Rieradevall and Busquets (1990) and Wood and Okamura (2005), the two floatoblasts presented on their ventral (or deutoplasmic) valves have been determined as those belonging to *Plumatella repens*. However a second opinion will be sought from Dr Timothy Wood before drawing the final decision.

Sessoblasts

- Sessoblasts have a well developed reticulated annulus and both sides of the outer annulus are reticulated (Wood and Okamura 2005)

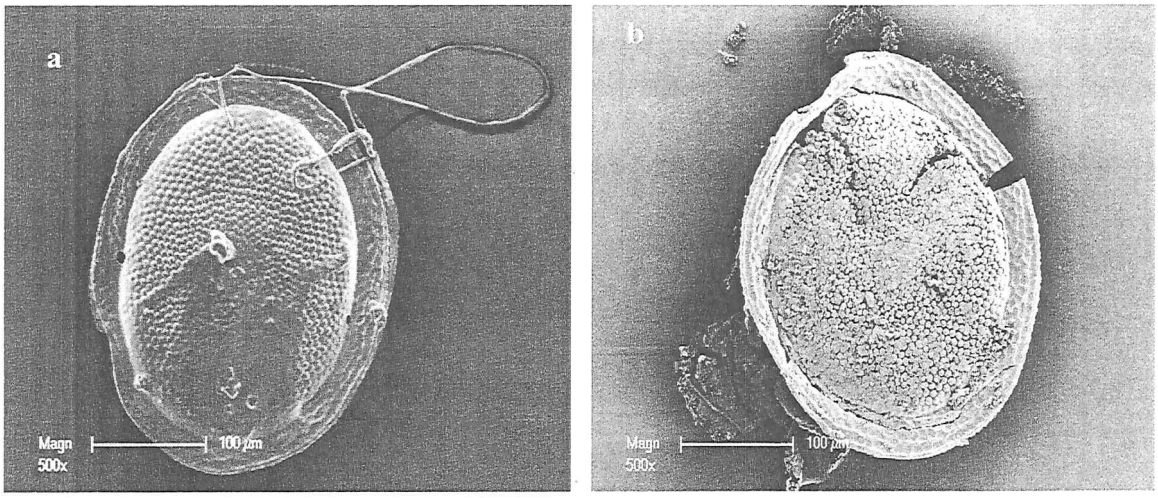


Fig 18. (a) Sressoblast of *Plumatella repens* (February lot) confirmed by Dr Tim Wood.
(b) Sessoblast of *Plumatella repens* (from the May lot). Note the well developed reticulation on the annulus.

2.7.5. *Plumatella casmiana* Oka 1907 and *Plumatella vaihiriae* Hastings 1929

The presence of *Plumatella casmiana* and *Plumatella vaihiriae* in the Northern Mallee Pipeline (NMP) system has been confirmed from the report submitted by Bryo Technologies dated the 16th of February 2010, **Figs 19a** and **19b.** However currently there seems to be some confusion in the identification and confirmation of these two species from the micrographs obtained throughout the course of the SEM analyses.

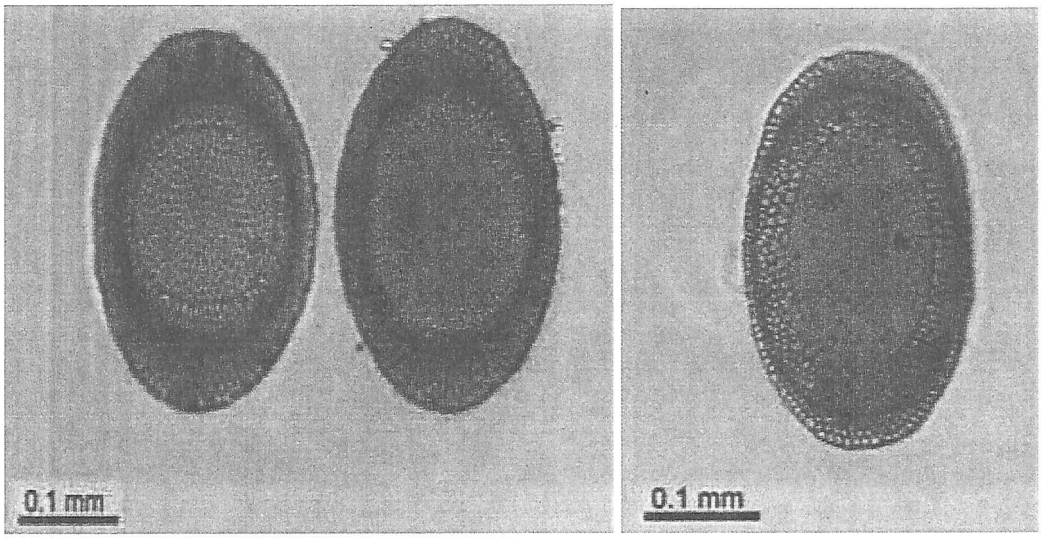


Fig 19. (a) Floatoblast valves of *Plumatella vaihiriae* from Ouyen backwash (b) Floatoblast of *Plumatella casmiana* detected from Piangil membrane filter (Bryo Technologies Report dated the 16th of February 2010).

To resolve the matter some unique floatoblasts and sessoblasts the identities of which are still ambiguous, Fig 20 have been chosen for second opinion from Dr Tim Wood.

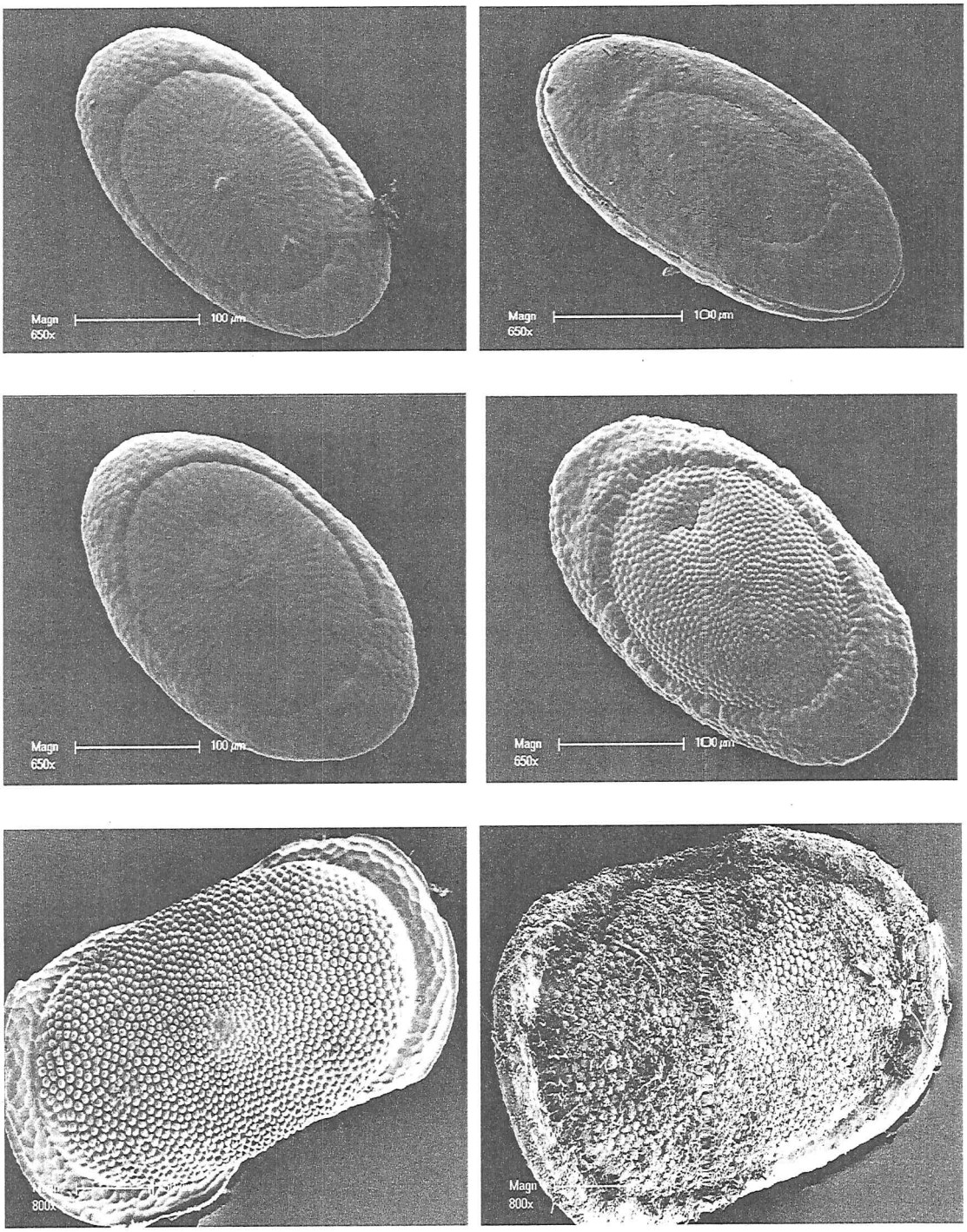


Fig 20. Some of the floatoblasts and sessoblasts whose identities are still ambiguous thereby necessitating a second opinion from Dr Tim Wood.

2.7.6. *Fredericella australiensis* Goddard 1909

There are five recognized species in the family Fredericellidae (i) *Fredericella sultana* (ii) *Fredericella australiensis* (iii) *Fredericella indica* (iv) *Fredericella brownii* (Rogick 1945 in North and Central America) and *Internectella bulgarica*. Of the five recognized species that are currently known to exist, the presence of two, namely *Fredericella sultana* and *Fredericella australiensis* have been identified and confirmed and currently the presence of a third species *Internectella bulgarica* is being suspected after the completion of the SEM analyses in May 2010.

Statoblasts of ***Fredericella australiensis***

- Statoblasts are either rounded or very broadly elliptical, not reinform and are shorter or broader compared to *Fredericella sultana* (Rogick 1945)
- Statoblast dimensions: length 375 ± 6µm, breadth 340 ± 10µm and length/breadth = 1.37± 0.07. (Wood 1998)

Measurement	Goddard (1909)	Marcus (1953)	Rogick (1945)	Bushnell (1971)	Lab-reared *F. australiensis*	Lab-reared *F. sultana*
Statoblast dimensions (µm)						
Length, range	——	320–420	331–461	——417	275–400	325–425
Length, mean	——	360–385	382	376	370 ± 14	386 ± 9
Width, range	——	250–370	266–367	——320	200–275	200–250
Width, mean	——	298–325	316	292	243 ± 9	224 ± 6
Length:width ratio	1.3*	1.19	1.21	1.29	1.52	1.72
Number of tentacles						
Range	28–30	24–27	24–28	——	22–24	21–23
Mean	——	26	26–27	——	23 ± 0.4	22 ± 0.5
Zooecium diameter (µm)						
Range	——	315–500	259–576	235–510	275–375	175–275
Mean	——	450	391	400	334 ± 10	230 ± 8

* Taken from mean length and width measurements of five figures appearing in Goddard (1909).

Fig 21. Measurements from natural populations of *Fredericella australiensis* compared with those from laboratory-reared colonies of *Fredericella australiensis* and *Fredericella sultana* (Backus and Wood 1981)

- Possess a characteristic thickened rim or the suture rim (Wood *et al.*,1998)

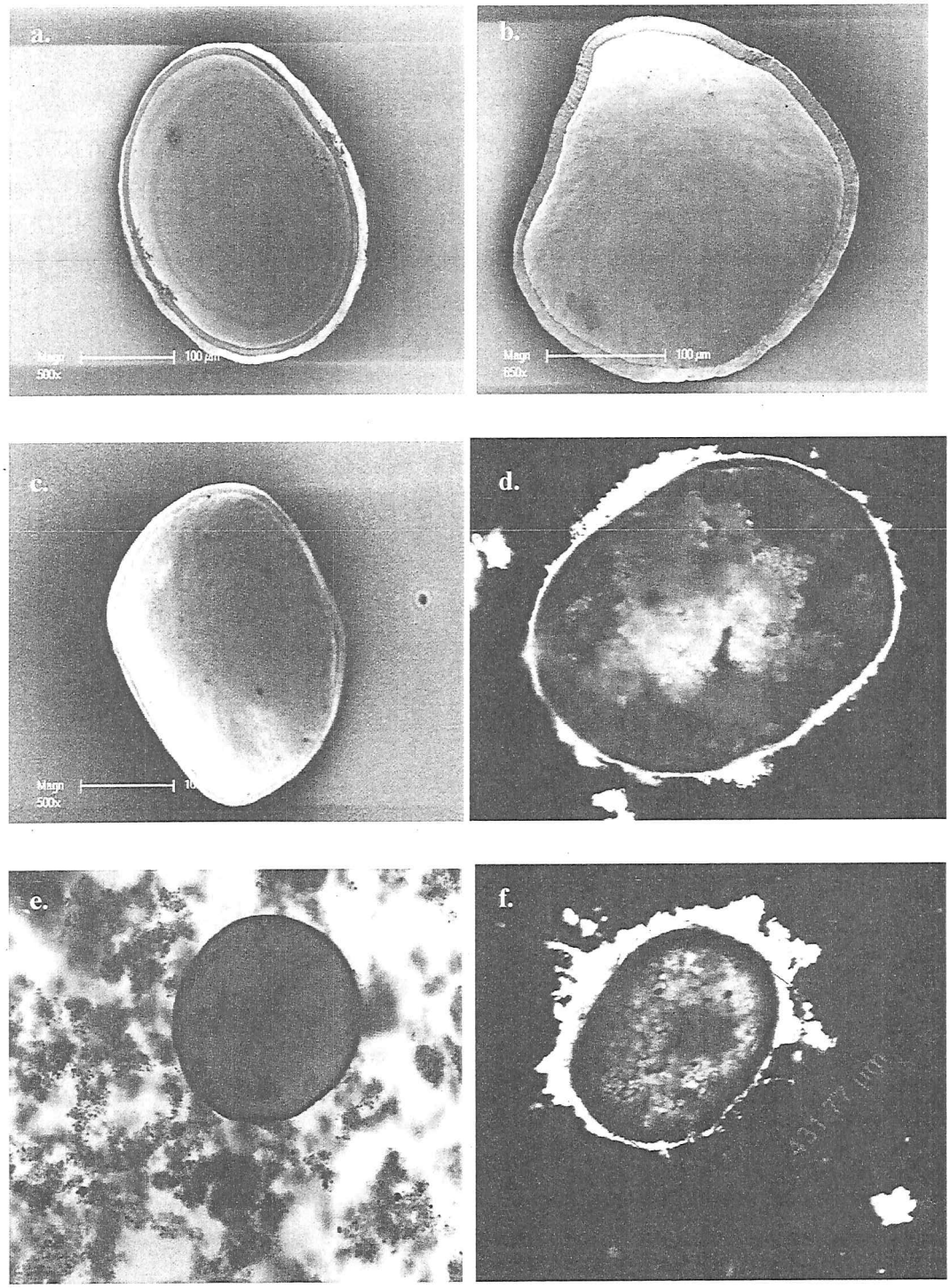


Fig 22. (a) to (c) SEM micrographs of *Fredericella australiensis* statoblasts with the characteristic thickened rim. SEM micrograph (c) confirmed by Dr Wood although the shape is a bit weird (a) somewhat resembles a sessoblast and hence requires a second opinion (d) and (e) Round statoblast of *Fredericella australiensis* with the thickened rim (f) Measure of a *Fredericella australiensis* statoblast in micrometres using the Zeiss Axioplan 2 research-grade microscope.

2.7.7. *Fredericella sultana* Blumenbach 1779

Statoblasts of ***Fredericella sultana***

- The shape of statoblasts of *Fredericella sultana* are generally oblong, oval or kidney shaped (Wood and Okamura 2005)
- The statoblasts are devoid of micro-architecture and the frontal surface appears dark, smooth and shiny. Absence of the annulus area (Wood and Okamura 2005).
- The European species of *Fredericella sultana* produce smooth, glossy statoblasts whilst the North American species are distinctly pitted on the valves (Økland and Økland 2001).

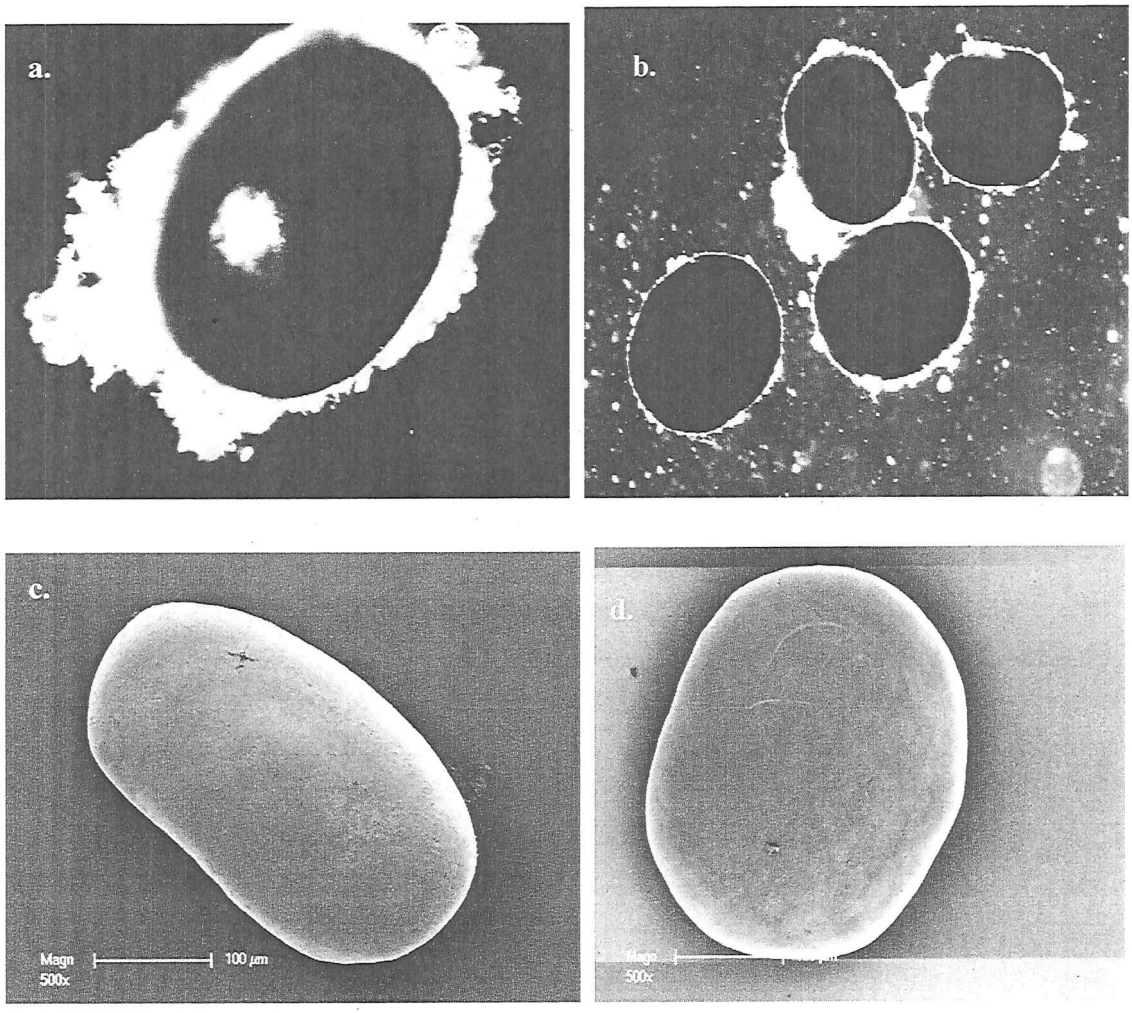


Fig 23. (a) A single dark and shiny statoblast of *Fredericella sultana* (b) A cluster of dark and shiny statoblasts of *Fredericella sultana*. Both (a) and (b) were viewed under the Zeiss Axioplan 2 research- grade microscope and photographed. (c) and (d) SEM micrographs of smooth and shiny statoblasts of *Fredericella sultana*. Unlike *Fredericella australiensis* the thickened rim is absent in *Fredericella sultana*.

2.7.8. *Internectella bulgarica* Gruncharova 1971.

Internectella bulgarica was originally detected from the lower Danube River basin (Gruncharova 1971) and heve been discovered in Thailand and much of Southeast Asia in gently flowing water or in pools left by receding flood waters (Wood *et al.,* 2006). To date, this species of freshwater Bryozoa has never been reported from Australia before. *Internectella bulgarica* is unique among the Fredericellids because it is known to form a buoyant, free statoblast which is completely enveloped by gas filled chambers and the entire structure is either grey or silvery in appearance (Wood and Okamura 2005). The free statoblast has not been detected as yet but the presence of the sessile statoblasts also known as piptoblasts of *Internectella bulgarica* which are somewhat similar to the sessoblasts of *Plumatella reticulata* is currently being suspected and will require a second opinion from Dr Timothy Wood before the presence of the organism in the Northern Mallee Pipeline (NMP) system is well and truly confirmed.

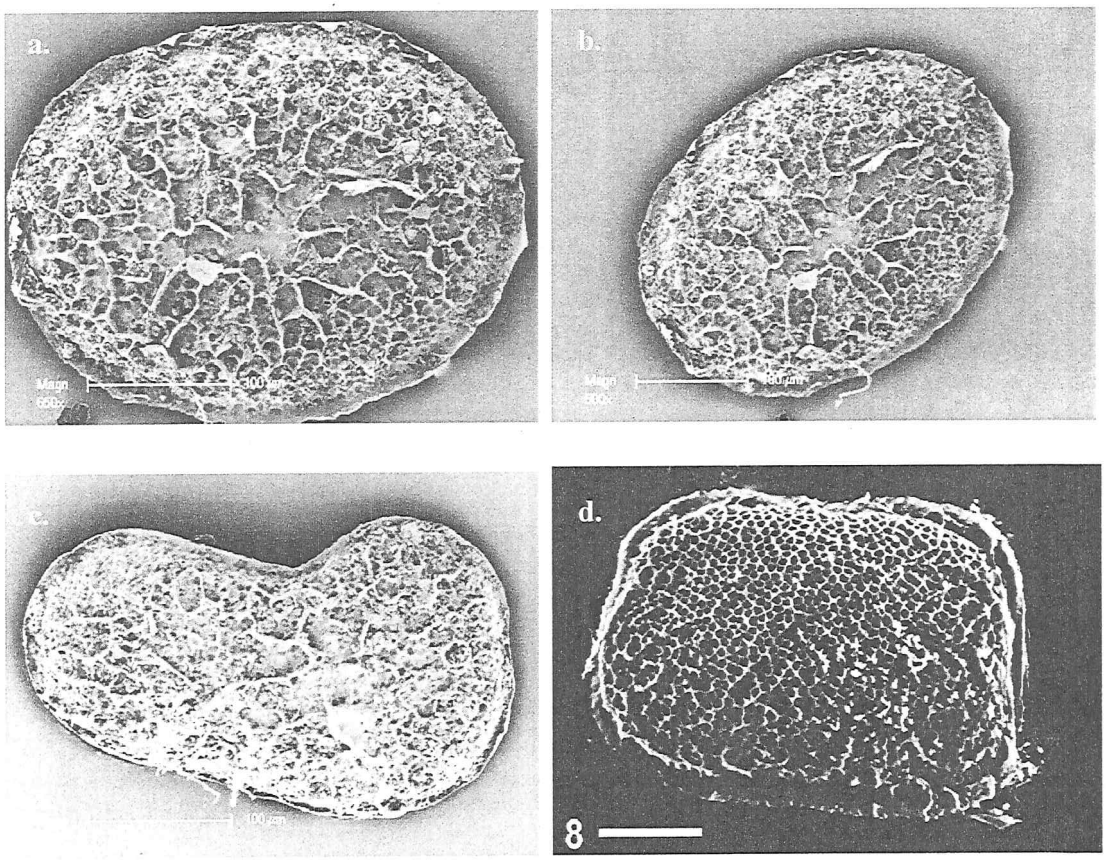


Fig 24. (a) to (c) Owing to the flat annulus, root-like patterns of raised lines on the frontal valve and unlike *Plumatella reticulata*, the absence of a raised frontal valve and a low lateral wall which is hidden beneath the annulus giving it a flattened appearance, the statoblasts depicted above has been suspected as the sessile statoblasts (piptoblasts) of *Internectella bulgarica* rather than those of the sessoblasts of *Plumatella reticulata*. (d) *Internectella bulgarica* sessoblast (piptoblast) with rough surface reticulum (Wood *et al.,* 2006).

2.7.9. Successful development of a protocol for statoblast preparation for SEM analyses .

Over the period of a few months a successful protocol for the preparation of statoblasts for SEM analyses has been developed **(Refer to Appendix One)**. The procedure for statoblast preparation involved (a) sonication, (b) dehydration with 100 % ethanol, (c) critical point drying and (d) sputter coating with gold. Provided below are the photographs of the instruments, **Figs 25, 26** and **27** that were used during the course of sample preparation for SEM.

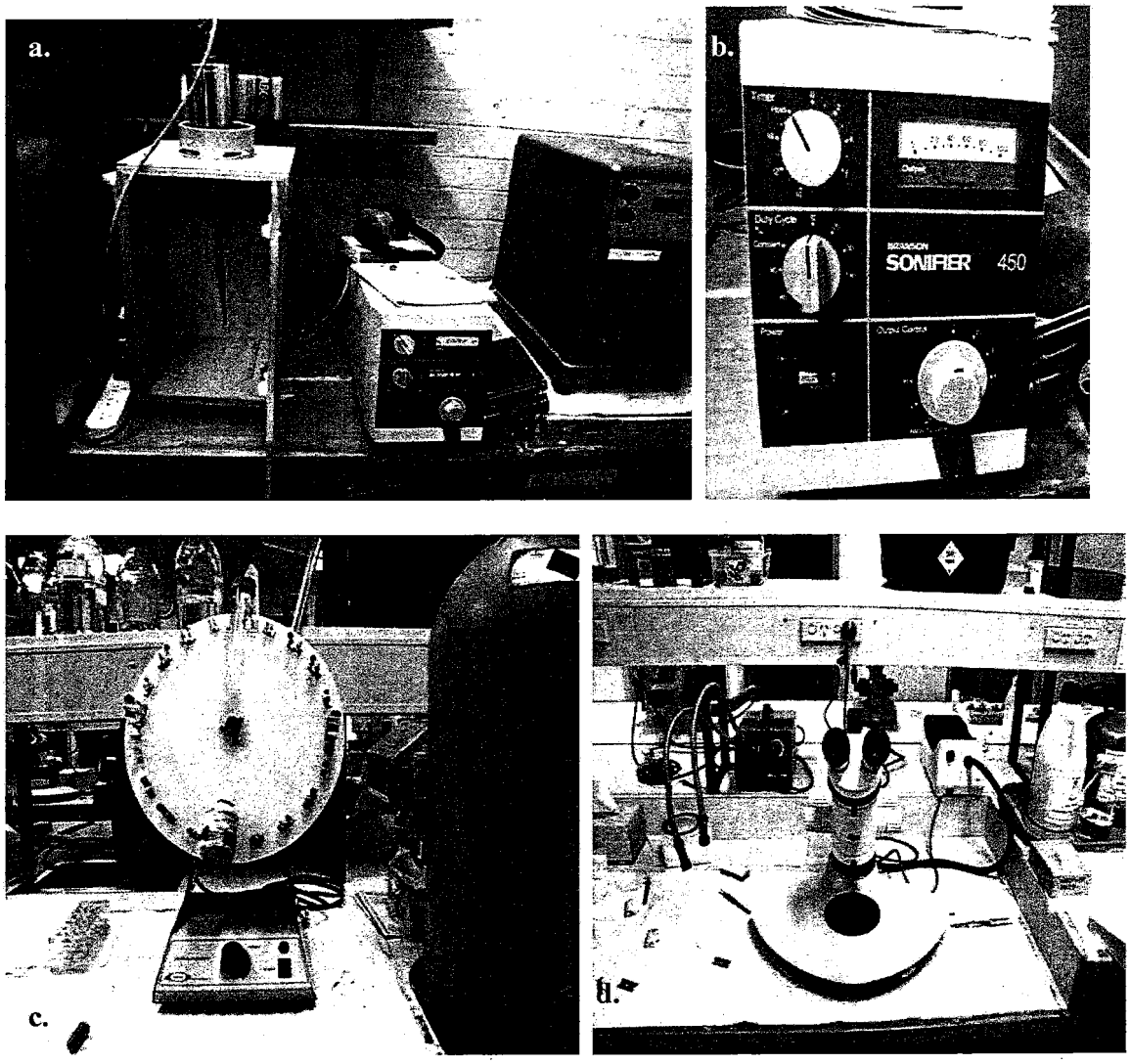


Fig 25. (a) and (b) Branson 450 sonifier that was used for removing the adhering debris from the statoblasts (c) Ratek suspension mixture that was used to remove the detergent (5% RBS-35) and also during the treatment of statoblasts with a graded series of increasing ethanol concentrations. (d) Leica binocular microscope that was used for checking the intactness of the statoblasts after sonication and critical point drying.

The Balzers CPD 030 critical point dryer, **Fig 26a** was used to dry the statoblasts slowly through the cooling (10°C) and heating steps allowing the ethanol to gradually sublimate at 40°C. Air drying of the statoblasts using filter papers under a fume hood was not carried out in order to avoid the distortion or collapse of the structure of statoblasts. To avoid distortion/collapse of the statoblasts during preparation for SEM, Wood and Wood (2000) first washed the statoblasts in distilled water and then freeze-dried them. The dried statoblasts were incubated in a desiccator overnight and loaded into SEM stubs before sputter coating them with gold using the Edwards S150B sputter coater **Figs 26b, 26c** and **26d.** The sputter coater was used to apply a thin layer (~25 nm thick) of gold, a heavy metal on the surface of the statoblasts prior to SEM analysis.

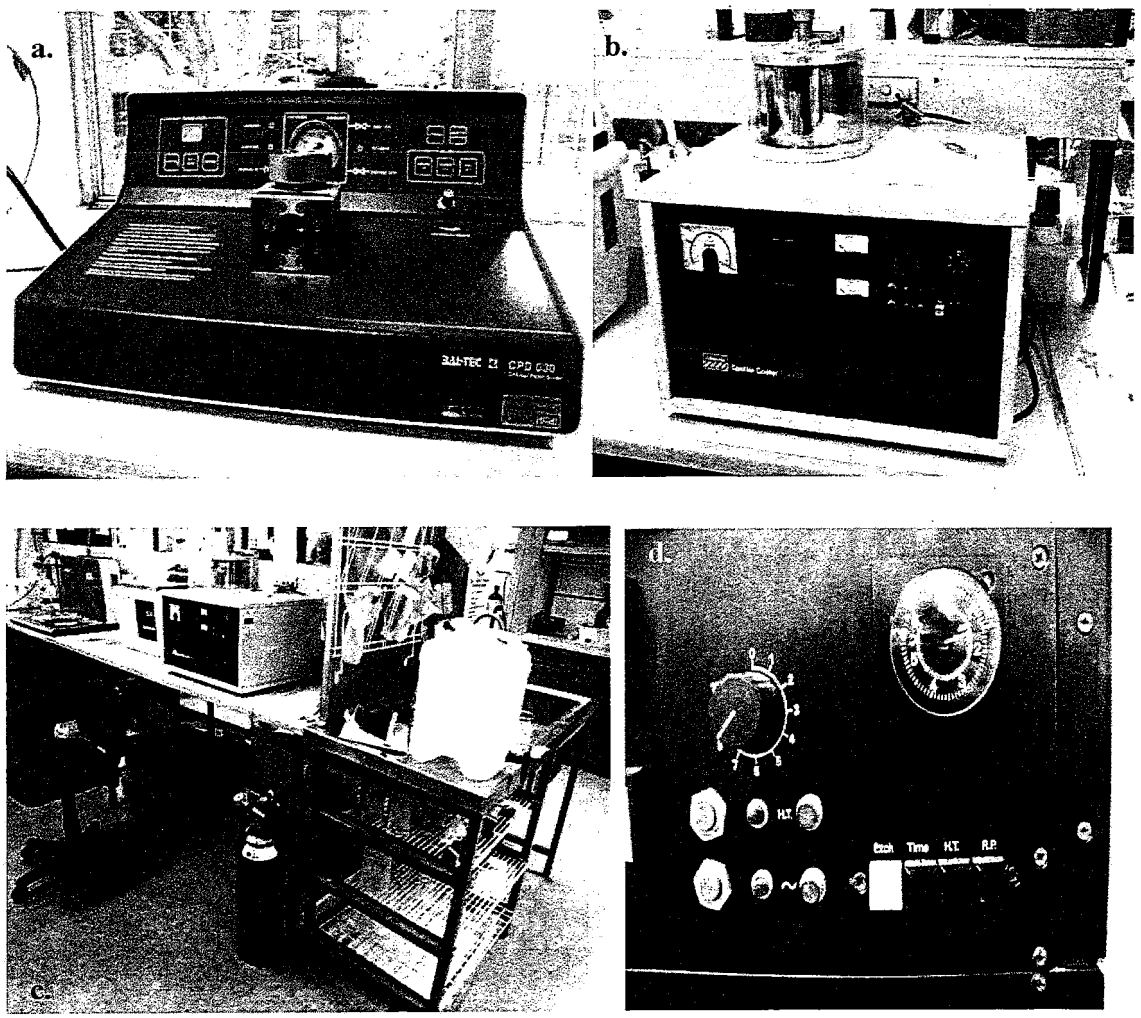


Fig 26. (a) Balzers CPD 030 critical point dryer (b) Edwards S150B sputter coater (c) Edwards S150B sputter coater and the adjacent argon cylinder. The gold coated statoblasts are bombarded with the heavy gas atoms of Argon, during sputtering. (d) Some of the functions of the Edwards S150B sputter coater like the RP (Rotary pump), the HT (High tension) buttons and the timer for the desired sputtering time used during the loading and coating of specimens.

The gold coated statoblasts on the SEM stubs were then loaded into the chamber of the scanning electron microscope by pressing the 'Vent' button on the monitor and so on.

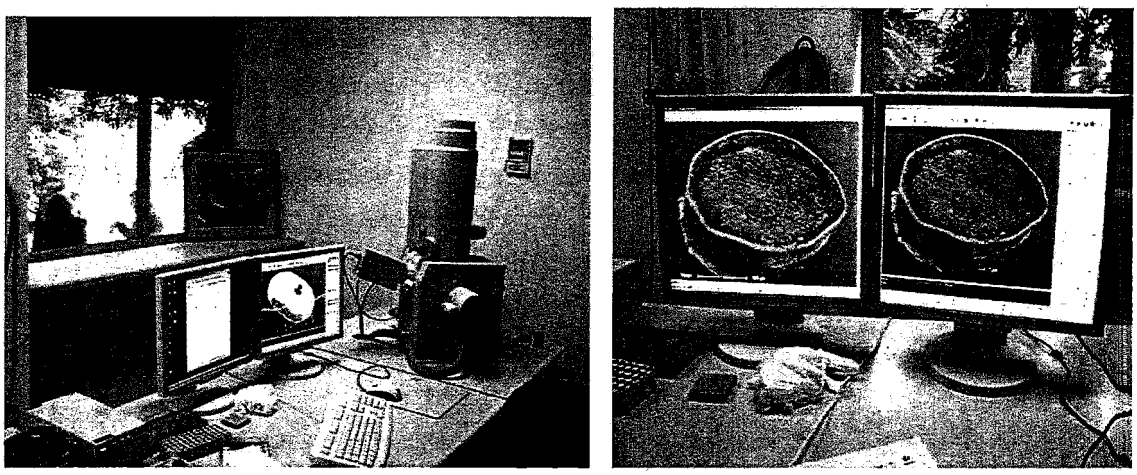


Fig 27. The XL30 FESEM Scanning Electron Microscope at Melbourne University.

3. Conclusions and recommendations

1. Finalize and submit the review described in Milestone 2 no later than the end of March.

A meeting between all the members of the research team is recommended as soon as possible to discuss the lay out of the review article.

2. Using SEM, subject the statoblasts obtained from the December '09 and February '10 sampling to particle size analysis as part of the continuing program to investigate the seasonality characteristics of these organisms.

The option for particle size analysis has been successfully trialled using the Zeiss Axioplan 2 research- grade microscope at the St Albans campus **(Refer to Section 2.1. of this report under the sub-heading 'Particle size Analysis').** Moreover, an in-depth SEM analyses relating to species identification has been carried out at Melbourne University over a period of three weeks and it is now apparent that the identification of least 5 species if not 6 can be published in a journal. On conclusion of the recent SEM studies in May 2010 there is also an indication that a new species *Internectella bulgarica* that was originally detected from the lower Danube River basin (Gruncharova 1971) could be present amongst the samples analysed from Northern Mallee pipeline (NMP), however a second opinion requires to be sought from Dr Timothy Wood Of Bryo Technologies Ohio U.S.

3. Continue the statoblast-to-colony and colony-to-colony cultivation of the identified Bryozoan organisms in the laboratory.

With the successful germination of the statoblasts, the statoblast-to-colony strategy under laboratory conditions now looks more hopeful (**Refer to Section 2.2. of this report under the sub-heading 'Statoblast to colony propagation'**). In the context of colony-to-colony cultivation in the lab, some new approaches have been outlined in Milestone 2 Report 2010 (**Section 2.1.1 pp 15-16**) which will be tried out once the next sampling tour is carried out. The filters with entwined colonies have been floated on plastic lids however the progress of their growth and development has not been checked lately due to involvement with the SEM work and milestone report preparation.

4. Concurrently design, construct and install a "field cultivation laboratory" consisting of an array of growth plates to be suspended in the "pit" at Ouyen (see Milestone 5 report). Transport the plate colonies and pit water to the purpose-built facilities at the St Albans Campus of VU and establish a methodology for assessing growth status and for carrying out static control experiments with various chemical agents. Initial experiments will be carried out using chlorine as a benchmark. Other control agents, such as hypochlorite and nano-particulate silver will then be systematically tested.

After the successful installation of the 'Pit apparatus' the growth plates at Ouyen have not been checked for Bryozoan growth as yet, however reports from Horsham mentions that the growth is meagre. A decision has been reached between Robin Mitra and John Orbell to install two heaters on the growth tanks with a temperature range of 18^{0}C to 32^{0}C in order to maintain the growth tanks at 25^{0}C (room temperature) prior to the next sampling tour. .

5. Liaise with all team members to produce a draft manuscript detailing the SEM characteristics of the two NMP species also describing their geographical locations.

After an extensive SEM analyses over a period of three weeks at Melbourne University relating to species identification it is now possible that not 2 but the identification of least 5 species if not 6 can be published in a journal

6. Initiate formal discussions for the extension of the investigations to the WMP.

7. Progress report in relation to Milestone 3 to be submitted with invoice for fourth instalment of $20,871.00 on Monday 17th May, 2010.

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APPENDICES

APPENDIX ONE

Scanning Electron Microscopy Protocol

1. Sonication

- The Plumatellid and Fredericellid type asexual dormant buds collectively designated as statoblasts were isolated from the Bryozoan colonies that were collected from both the sand and membrane filters at Nyah and Piangil pump stations respectively. The statoblasts were then sorted out tentatively according to their exterior morphology as viewed under the light microscope and put out into different eppendorfs immersed in source water collected from the Nyah pump station. The eppendorfs were duly labelled as FS (*Fredericella sultana*), FA (*Fredericella australiensis*), PE (*Plumatella emarginata*), PR (*Plumatella reticulata*), PM (*Plumatella minuata*), PV (*Plumatella* vaihiriae), PC (*Plumatella casmiana*) and MISC (Miscellaneous). The contents of the eppendorfs were analysed, one eppendorf at a time, over a period of three weeks at Melbourne University.

- The statoblasts from each eppendorf were emptied into a 10ml yellow capped tube (Techno Plas U.S). The source water was replaced with 5 ml of 5 % RBS – 35, a detergent which is used for cleaning glassware and laboratory equipment made of stainless steel, plastic, porcelain etc. The detergent treatment coupled with sonication were used to remove the adhering organic debris from the samples.

The duty cycle was fixed at 50 %

The Output Control was maintained at a minimal of 1

Fig 1. Branson 450 Sonifier

- The statoblasts were then subjected to sonication using Branson 450 sonifier. Prior to sonication, the tip of the horn of the sonicator was wiped clean with 70 % ethanol. The Duty Cycle was fixed at 50 %. In the pulsed mode, the ultrasonics are pulsed at a fixed repetition rate of one/pulse per second. If the duty cycle is fixed at 50% the ultrasonics will be on for 50 % of every second. The Output Control, which controls the amplitude, was maintained at a minimal of 1 in order to avoid the damage of the statoblasts. The timer was set at 20 cycles for some samples and 30 cycles for others.

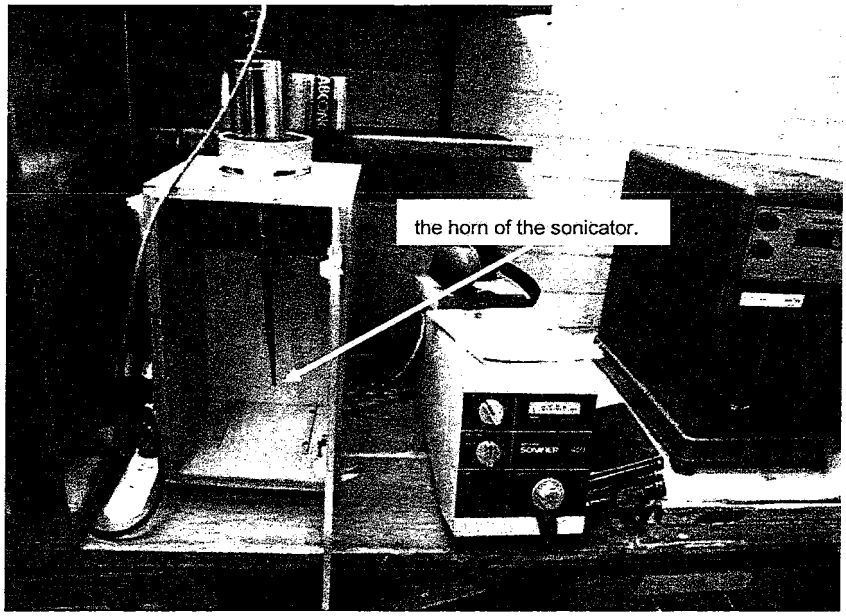


Fig 2. Branson 450 Sonifier – the horn of the sonicator.

- After sonication, the intactness of the statoblasts were observed by viewing them under the dissection microscope.

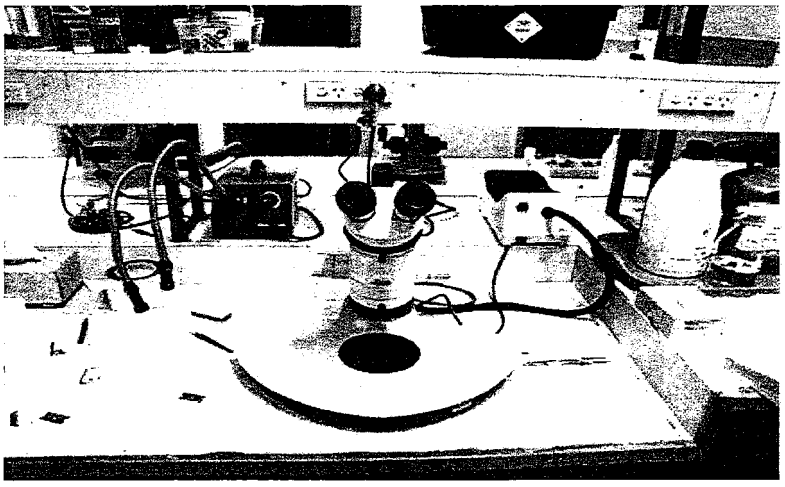


Fig 3. The dissection microscope at the Melbourne University lab.

- The statoblasts were then rinsed 3 X in ddH_2O. Initially the detergent within the yellow tipped tube in which the statoblasts were immersed was replaced by an equal amount of ddH_2O. The yellow tipped cap was then clamped to a rotating wheel for 5 minutes. The procedure was reiterated 3 X for a thorough removal of the detergent.

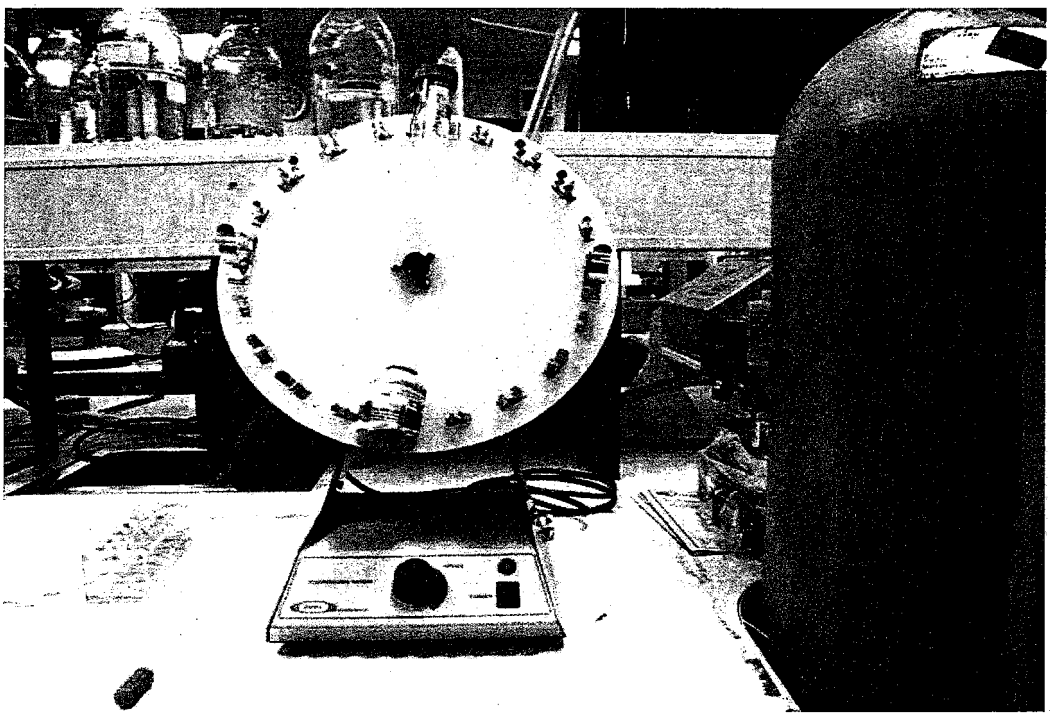


Fig 4. The rotating wheel that was used to remove the detergent from the samples. Melbourne University lab.

2. Dehydration with 100 % ethanol

- After the third rinse, dehydration with 100 % ethanol was carried out by subjecting the statoblasts to a graded series of increasing ethanol concentrations at each step, until the final two steps of dehydrating with 100 % ethanol was achieved. The yellow tipped tube in which the different concentrations of ethanol were prepared was labelled as 'Ethanol in H_2O'. The same tube was used to prepare 10%, 30%, 50%, 70%, 90% and two100% ethanol concentrates for which the steps are delineated below : -

- 10 % ethanol. A fresh yellow tipped 10 ml tube was taken to which 1 ml of absolute ethanol and 9 ml of ddH_2O was added to prepare a solution of 10 % ethanol. Water from the sample was replaced with 10 % ethanol and clamped back on the rotating wheel for 10 minutes.

- 30 % ethanol. In the 10 ml tube, 3 ml of absolute ethanol and 7 ml of ddH_2O was added to prepare a solution of 30 % ethanol. 10 % ethanol from the sample was replaced with 30 % ethanol and the tube was then duly clamped back on the rotating wheel for 10 minutes.

- 50 % ethanol. In the 10 ml tube, 5 ml of absolute ethanol and 5 ml of ddH_2O was added to prepare a solution of 50 % ethanol. 30 % ethanol from the sample was replaced with 50 % ethanol and the tube was again clamped back on the rotating wheel for 10 minutes.

- 70 % ethanol. In the 10 ml tube, 7 ml of absolute ethanol and 3 ml of ddH_2O was added to prepare a solution of 70 % ethanol. 50 % ethanol from the sample was replaced with 70 % ethanol and the tube was then clamped back on the rotating wheel for 10 minutes.

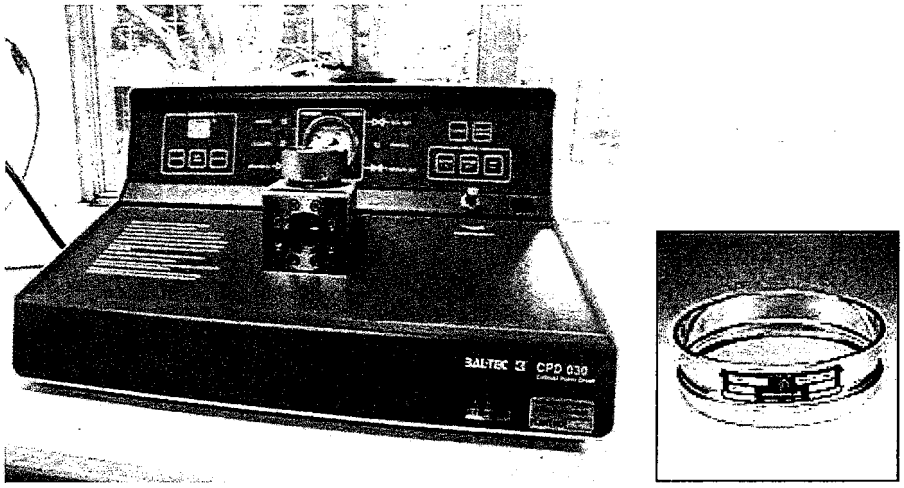
- 90 % ethanol. In the 10 ml tube, 9 ml of absolute ethanol and 1 ml of ddH_2O was added to prepare a solution of 90 % ethanol. 70 % ethanol from sample was replaced with 90 % ethanol and the tube was then clamped back on the rotating wheel for 10 minutes.

- 100 % ethanol. In the 10 ml tube, 10 ml of absolute ethanol was added and 70 % ethanol from the sample was replaced with 100 % ethanol and the tube was then duly clamped back on the rotating wheel for 10 minutes.

- 100 % ethanol. The step was reiterated. In the 10 ml tube, 10 ml of absolute ethanol was added and 100 % ethanol from the sample was replaced again with 100 % ethanol and the tube was then duly clamped back on the rotating wheel for the final time of 10 minutes.

3. Critical Point Dryer

- The ethanol dehydrated samples were then emptied into fine meshed sample holder



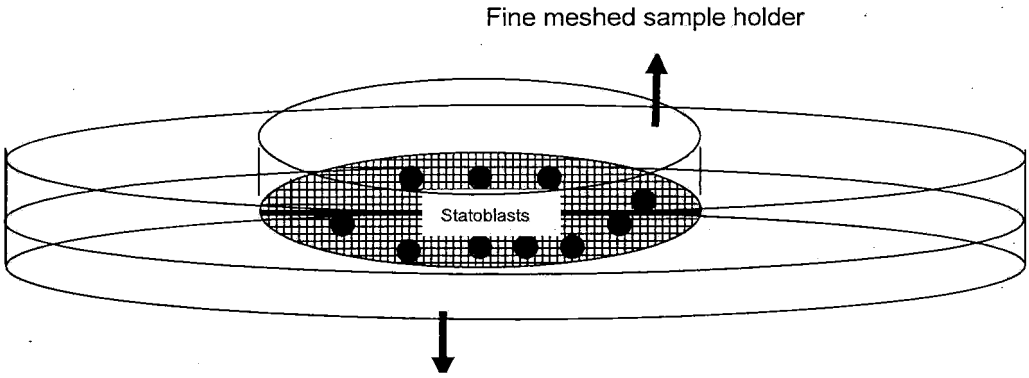


Fig 4. Balzers CPD 030 Critical Point dryer, sample holder and the schema of a fine meshed sample holder in a petri dish containing 100 % ethanol.

- The lid of the Critical Point Dryer was opened and four fine meshed sample holders, spare on top, followed by *Fredericella*, another spare and finally *Plumatella* at the bottom to prevent mixing up were placed inside and was then filled with ethanol until it covered the top. However, one sample holder and a spare are used for a single set of sample.

3.1: Critical Point Dryer – the cooling step.

Fig 5. Balzers CPD 030 Critical Point dryer.

- The COOLING button was pressed which lit up with a green light and the temperature was lowered to 10°C.

- The dry CO_2 cylinder was opened and the STIRRER button was pressed for the ethanol and dry CO2 to mix.

- Once the temperature reached 10°C, The MEDIUM IN button was pressed until the ethanol reached an appropriate level and the MEDIUM IN button was pressed again to stop the flow of ethanol into the chamber. A pause of 5 minutes was maintained and then the MEDIUM OUT button was pressed until the ethanol drained out of the chamber completely. The MEDIUM OUT button was pressed again to stop.

- The MEDIUM IN and MEDIUM OUT procedure was a repeated a total of six times at the end of which a thick upsurge of bubbles was observed during the MEDIUM OUT phase.

- The MEDIUM IN button was turned on again but the COOLING / STIRRER button was turned off.

- The HEATING button was turned on until the temperature riose to 40°C. Then the GAS OUT button was pressed to allow the gas out by controlling the METERING VALVE and making sure that the 'black ball' sits on 10 at all times and that the black ball does not hit the roof in a sudden burst of outflow. The HEATING button stays on.

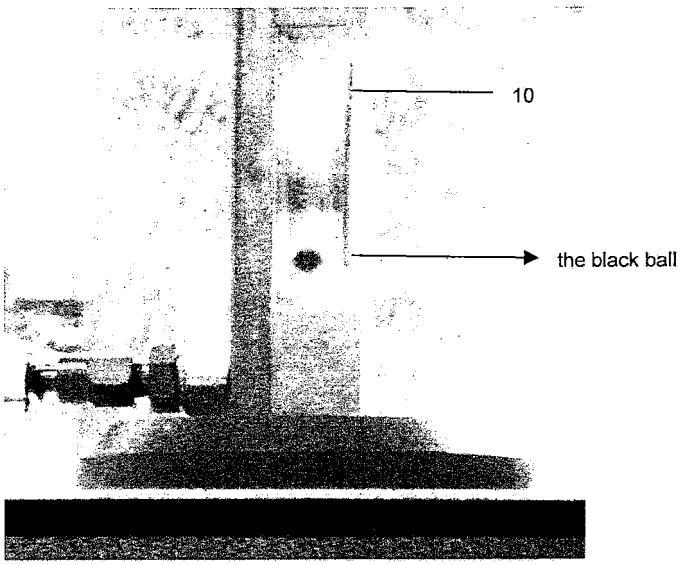


Fig 6. Balzers CPD 030 Critical Point dryer – the black ball.

- When the needle in the pressure chamber drops to 0, the METERING VALVE is turned off and the dry CO_2 cylinder is closed. Finally press MEDIUM IN to drain out the gas. CAUTION: Make sure that METERING VALVE is closed at all times during the operation of the critical point dryer or there is a possibility that a sudden burst of outflow could distort the samples.

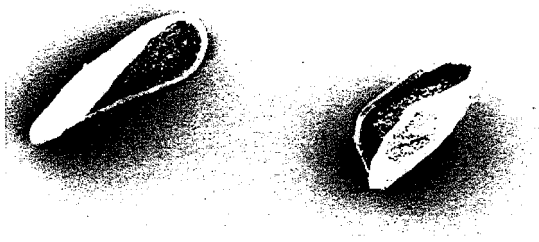
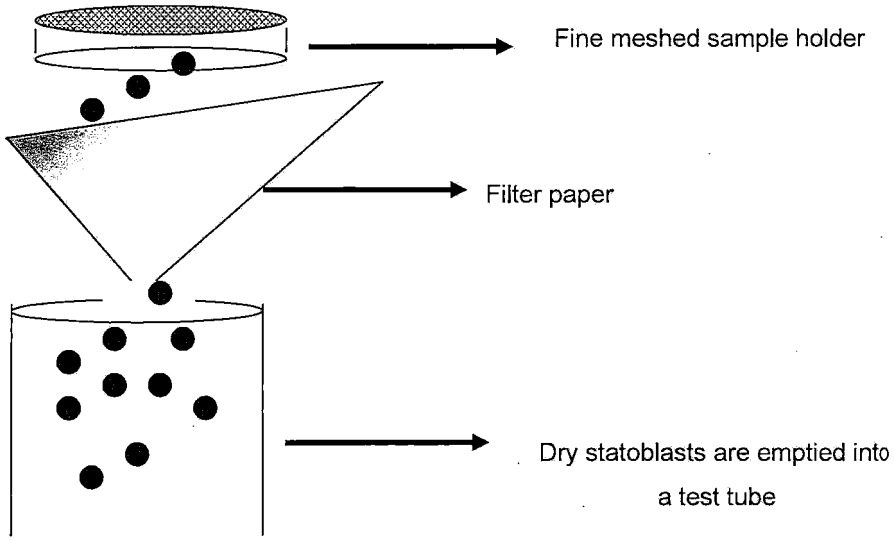


Fig 7. Distorted statoblasts



- Stored overnight in a desiccator ready to be loaded into a SEM stab.

OPERATION OF EDWARDS S150B SPUTTER COATER

(PROTOCOL PROVIDED BY SCHOOL OF BOTANY, MELBOURNE UNIVERSITY)

TO OPEN CHAMBER:

- Open Argon cylinder valve and flow meter valve.
- Open the gas/air admit valve the flow of gas can be heard.
- The chamber can be opened after about 30 seconds.
- Close the gas/air admit valve immediately in order to curtail waste of Argon.

LOADING SPECIMENS:

- Wearing gloves, remove the outer ring and specimen holder from the chamber.
- Put the specimen to be coated onto the holder; return the holder onto the base plate. inside the chamber. Return the outer ring into the groove surrounding the specimen holder making sure they are not in contact.
- Switch on the electric supply. The green mass indicator lamp lights up.
- Press the RP (rotary pump) button. The pump starts and the Pirani gauge (vacuum gauge) is switched on.
- Leave pumping for 10 minutes, or until the pressure reaches less than 6×10^{-2} mbar.

COATING:

- Open gas/air admit needle valve to fill chamber with Argon for 30 seconds, gauge needle should go to the far right.
- Close gas/air admit valve for 30 seconds. Repeat this argon flush 3X.
- Gradually open the pressure control needle valve until the pressure rises to about 3×10^{-1} mbar. (between 2 – 4 on the outside scale).
- Set the HT (high tension) voltage control to 7.5
- Set the timer to the desired sputtering time. (1.5 minutes in our case). However 1.0 minute is plenty if specimens are properly prepared for FESEM).
- Press the HT button and the TIME button simultaneously. The red HT indicator lamp lights.
- When the set time has elapsed, the timer switches off the HT and the red glow discharge is terminated.

REMOVING SPECIMENS:

- Press the HT and the timer button.
- Close the pressure control valve.
- Turn off Rotary pump by pressing the RP button, and turn off at the power-point.
- Open the gas/air admit valve until gas flow is heard.
- The chamber can soon be opened to remove specimens.
- Turn Argon cylinder valve off wait until gas flow ceases then close flow meter valve.
- Close gas/air admit valve.
- Remove specimens, replace specimen holder and ring in chamber, then close lid of chamber.

NOTE: The sputter coater is left in vacuum when not in use:

- Press RP button. The pump starts. Pump 2 minutes.
- Turn off the pump by depressing RP button.
- Turn power off.

Fig 8. Edwards S150B sputter coater.

INSTRUCTIONS FOR USING XL30 FESEM

(PROTOCOL PROVIDED BY SCHOOL OF BOTANY, MELBOURNE UNIVERSITY)

LOADING SAMPLES:

- Make sure to select a working distance (Z) of +30, then press 'Go To'.
- Press 'Vent' button – will be able to gently pull door open after about 1 minute.
- Load samples (Wear powderless gloves), lock sample holder into place.
- Press 'Pump' button (hold door closed initially) and wait for 'Vac OK' message.
- Select kV 2.0 and spot 2 under 'Beam' menu, aperture 1 on microscope column.
- Press '2.0kV' button, wait for image to appear, adjust brightness to suit, lower the magnification and locate stub.
- Go up one or two steps in magnification, do a rough focus and then press 'OK' in 'Conform focus' box.
- Select a 'Z' height or working distance of 10 and press 'Go To' stage will move to new position.
- Choose something small and roundish (e.g. pore or hole) to focus on it at high magnification (10,000 X or above).
- Focus as best as you can.
- Expand the 'Beam' page, click on the 'Less modulation' box. If there is a lot of movement in the image, stabilize it rotating the two knobs near the aperture control on LHS of column. Uncheck box.
- Check for astigmatism in image, go to 'Imaging' pages – position cursor in cross hairs of 'Stigmator' box and hold down left mouse button, move cursor around perimeter of screen until image quality improves, then release button. Repeat this until best image is obtained when the cursor is in the middle of the screen.

MOVING AROUND:

- Recommend moving to the 6 '0' clock position of the carousel and rotating until the first stub comes into view (look for black line indicating position 1 is to the left).
- To find a new stub simply use the rotating button to bring it into view.
- Move around stub by holding down left mouse button, point cursor in the direction one wants to move.
- To rotate the image use the 'scan rotation' preferably, and remember to reset to zero after capturing an image.

TAKING PICTURES:

- Select an area of interest, increase the magnification through 2, 3, 5 or 6 steps and focus, return to original magnification.
- Under In/out menu, 'Databar' allows you to choose what information, one has on the picture.
- Under 'Scan' menu, select 'Slow Scan' 2 or 3. 'Slow Scan' 2 was selected in our case.
- Turn on 'Videoscope' button, and adjust contrast and brightness until the range falls within the upper and lower limits of 'videoscope' graph. When corrected turn off 'videoscope' graph.
- Under 'Filter' menu select 'Integrate 1'.
- Wait until scan is completed and the image is frozen (Frozen or snow flake symbol lights up)
- Go to 'In/Out' menu and choose 'Image' to save image to file (click on [..] and chose appropriate folder in which to store file), start each filename with an X, name file and choose 'Save'.
- Return to live image by clicking on TV symbol, or choosing 'Live' from 'Scan' menu.

CLOSING DOWN:

- Go to 'TV' image and go to lowest magnification, beeping indicates lowest magnification available.
- Increase working distance (Z) to '+30' and click 'Go To' – wait for stage to move.
- Turn 'Beam' button off and press 'Vent' remove samples after one minute.
- After removing samples close door and press 'Pump' – leave SEM under vacuum.
- Put time and details of use in log book.

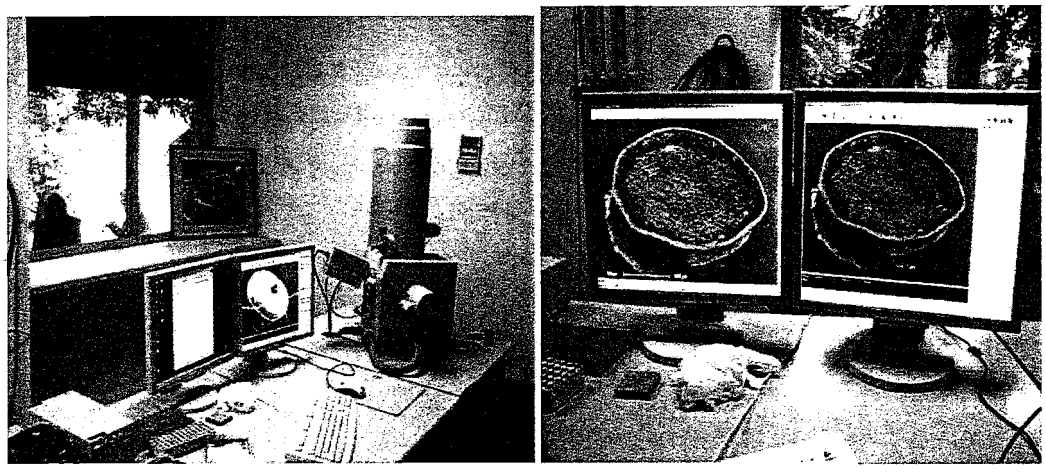


Fig 9. The XL30 FESEM Scanning Electron Microscope