Culture of malacosporeans (Myxozoa) and development of control strategies for proliferative kidney disease

A thesis submitted to the University of Stirling
for the degree of Doctor of Philosophy

by

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April 2005

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Dedicated to my wife Sarah,
and our children John, Anna and Andrew.

Declaration

I declare that this thesis has been compiled by myself, and is the result of my own investigations. It has not been submitted for any other degree and all sources of information have been duly acknowledged.

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Abstract

Proliferative kidney disease (PKD) poses a high financial burden upon the freshwater salmonid aquaculture industry of Europe and North America. The alternate hosts of the causative agent, *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea), have been identified as freshwater bryozoans (Bryozoa: Phylactolaemata) within which spores capable of infecting salmonid fish develop. Currently, control of PKD relies upon complex management practices, with no licensed prophylaxis or treatment available.

Assessment of the nutritional preferences of phylactolaemate bryozoans allowed development of an optimised laboratory culture system. Following laboratory maintenance, bryozoans collected from PKD-endemic sites were found to be infected with the malacosporean parasites *T. bryosalmonae* and *Buddenbrockia plumatellae*. Subsequent parasitic development was observed using light-, electron- and confocal-microscopy techniques. Methods of challenging rainbow trout with *T. bryosalmonae* spores were developed, with the minimum infective dose established. The presence of Thomsen-Friedenreich and Tn epitopes within the parasite was investigated, and experimental vaccine preparations based on either these specificities or *T. bryosalmonae*-infected bryozoans were efficacy tested in rainbow trout. In addition, salinomycin and amprolium were tested as prospective chemotherapeutants for PKD.

Further insights into the development and subsequent release of malacosporean spores within their invertebrate hosts have been revealed. Long-term maintenance of *T. bryosalmonae* allowed controlled infection of rainbow trout previously vaccinated with experimental preparations. Findings of the project could potentially be utilised in future research into the development of control methods for PKD.

Acknowledgements

This project has benefited greatly from the strong supervision provided by Dr. Dave Morris and Prof. Sandra Adams with additional input from Prof. Pat Smith. Niall Auchinachie proved invaluable with his assistance towards conducting experiments within the aquarium facilities. Project funding was generously supplied by the Natural Environment Research Council, Schering-Plough Aquaculture and the Fishmongers' Company, with additional resources for conference attendances provided by Schering-Plough and the Fisheries Society of the British Isles. Trafalgar Fisheries and Test Valley Trout were accommodating in allowing sampling trips.

Dr. James Bron and Linton Brown were of great assistance with investigations in the Institute's bioimaging suites. In addition, Iain Elliot, Dr. Andy Shinn, Fred Phillips and Rodger McEwan proved most helpful in giving technical assistance with audiovisual procedures. Samples of bacteria were kindly supplied by Geoff Foster MRCVS of SAC Inverness, with subsequent technical assistance provided by Gillian Dreczkowski. Billy Struthers generously analysed water quality parameters. Thanks also to Dr. Jimmy Turnbull, Dr. Kim Thompson, Hogne "Boss Hogg" Bleie, fellow WASA members and all the staff and students at the Institute for their encouragement and good humour.

A great debt of gratitude is due to all of my friends and family, particularly my parents, for their support and patience. Sarah and our children, John, Anna and Andrew have always been extremely encouraging and supportive, even although they have been forced to be indoctrinated in the biology of bryozoans and malacosporeans over the last three years. Some comfort can be taken from the fact that everyone in John's P3 class is now fully aware of the existence and relevance of the fascinating aquatic creatures known as the Bryozoa!

Abbreviations and acronyms

3D	three-dimensional
µg	microgramme(s)
µl	microlitre(s)
µm	micrometre(s)
µM	micromolar
Ag(s)	antigen(s)
ARF	Aquatic Research Facility, University of Stirling
BW	body weight
CD	compact disc
cf.	"*confer*": compare
CFU	colony forming units
CLSM	confocal laser scanning microscopy
CM	Chalkley's medium
DMEM	Dulbecco's modified essential medium
DNA	deoxyribonucleic acid
DVD	digital versatile disc
EDB(s)	electron-dense body (bodies)
et al.	"*et alia*": and others
FP	faecal pellet(s)
g	gramme(s)
× *g*	multiples of gravity
i.e.	"*id est*": that is
i.m.	intramuscular(ly)
i.p.	intraperitoneal(ly)
JM	Jaworski's medium
kDa	kilo Dalton(s)
kV	kilo volt(s)
L	litre(s)
M	molar
mA	milliampere(s)
MAb(s)	monoclonal antibody (antibodies)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
n, no.	number
nm	nanometre(s)
OD	optical density
PAS	periodic acid Schiff
PBS	phosphate buffered saline
PC	personal computer
PCR	polymerase chain reaction
PCV	packed cell volume
PIT	passive integrated transponder
PKD	proliferative kidney disease
PKX	proliferative kidney organism unknown: 'X'
PNA	peanut agglutinin
ppm	parts per million
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
ROM	read only memory
rRNA	ribosomal ribonucleic acid
s.d.	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

sec	second(s)
sp., spp.	species
SPF	specified pathogen free
SSU	small sub-unit
TBS	tris buffered saline
TEM	transmission electron microscopy
TF	Thomsen-Friedenreich
TSA	tryptone soya agar
TSB	tryptone soya broth
UK	United Kingdom of Great Britain and Northern Ireland
USA	United States of America
UV	ultra violet (light)
vs.	"*versus*": against
VVL	*Vicia villosa* lectin
W	Watt(s)

Table of contents

List of figures

List of tables

Chapter 1: Literature review

1.1. Overview

Proliferative kidney disease (PKD) is an economically important parasitic condition, primarily affecting first season freshwater salmonid fish in areas of Europe and North America (Clifton-Hadley, Bucke and Richards 1984a; Hedrick, MacConnell and de Kinkelin 1993). The causative agent involved was originally known as PKX, denoting its uncertain taxonomic position (Seagrave, Bucke and Alderman 1980). Subsequent studies revealed that bryozoans (Bryozoa: Phylactolaemata) acted as additional hosts, and the organism was eventually named *Tetracapsuloides bryosalmonae* (Canning, Curry, Feist, Longshaw and Okamura, 1999) Canning, Tops, Curry, Wood and Okamura, 2002 and placed within the recently established class Malacosporea Canning, Curry, Feist, Longshaw and Okamura, 2000 within the phylum Myxozoa Grassé, 1970 (Anderson, Canning and Okamura 1999a; Canning, Curry, Feist, Longshaw and Okamura 1999, 2000; Canning, Tops, Curry, Wood and Okamura 2002).

Seasonal outbreaks of PKD, typically between May and September, have been linked to increased water temperatures which permit severe development of the disease in affected fish (Ferguson and Needham 1978; Foott and Hedrick 1987). Characteristic disease signs, including renal swelling with granulomatous hyperplasia encompassing interstitial extrasporogonic *T. bryosalmonae* cells are observed (Ferguson and Needham 1978). Severe disease leads to increased production costs and levels of mortality ranging from below 20% in uncomplicated cases, to 100% in fish suffering from secondary diseases (Ferguson and Ball 1979; Clifton-Hadley, Bucke and Richards 1986a). Fish that have recovered from the clinical disease exhibit apparent resistance to future challenge (Ferguson and Ball 1979; Klontz, Rourke and Eckblad 1986).

Various control methods have been developed to counter PKD, with varying levels of success. Husbandry measures – including lowering summer water temperature (using bore-hole water), increasing water oxygenation, delaying transfer of naïve stocks to endemic waters, eliminating secondary pathogens and reducing feeding rates – have been implemented in attempting to limit economic losses (Bucke, McGregor, Hudson and Scott 1981). Malachite green, the antibiotic fumagillin DCH and its synthetic analogue TNP-470 have been used therapeutically with some efficacy, but concerns over toxicity to fish, residue levels and environmental issues have prevented wide adoption of these treatments (Morris, Adams, Smith and Richards 2003a). The perceived specific immunity that previously exposed fish demonstrate to *T. bryosalmonae* has led to interest in the potential development of a vaccine to combat the condition, although no such product is currently available (Petchsupa 2002).

1.2. Geographical range of diagnosed PKD outbreaks

Since the label of PKD was first attributed to the syndrome by Roberts and Shepherd (1974), the condition has been reported in many European countries, and in North America – both Canada and the USA. Descriptions of similar syndromes affecting salmonid fish date further back: kidney swelling – attributed to amoeba infection – being noted affecting rainbow trout, *Oncorhynchus mykiss* (Walbaum) and brown trout, *Salmo trutta* L. in Germany (Plehn 1924). Schäperclaus (1954) described renal disease in hatchery rainbow trout, transmissible via cell and bacteria-free filtrates from diseased kidneys and thus suspected a viral aetiology. Similar pathological manifestations were also catalogued by Besse (1956), de Kinkelin and Gérard (1977), and Ghittino, Andruetto and Vigliani (1980). These findings were thought to be consistent with the condition now known as PKD (Hedrick *et al.* 1993).

In Europe, PKD has been reported in Scotland (Roberts and Shepherd 1974), Eire (O'Brien, McArdle and Doyle 1977), France (de Kinkelin and Gérard 1977), Northern Ireland (Ferguson and Needham 1978), England (Scott 1979), Wales (Seagrave, Bucke, Hudson and McGregor 1981), Germany (Hoffmann and Dangschat 1981), Italy, Denmark, Sweden (Clifton-Hadley *et al.* 1984a), Spain (Peribáñez, Luco, García and Castillo 1997), the Czech Republic (Scholz 1999), Norway (Midtlyng, Bleie, Helgason, Jansson, Larsen, Olesen, Olesen and Vennerstrøm 2000), and Switzerland (Wahli, Knuesel, Bernet, Segner, Pugovkin, Burkhardt-Holm, Escher and Schmidt-Posthaus 2002). In North America, the original reported outbreaks were in the USA: first confirmation being in Idaho in October 1981 (Smith, Morrison, Ramsey and Ferguson 1984; Klontz *et al.* 1986). Subsequently, cases were diagnosed in California (Hedrick, Kent, Foott, Rosemark and Manzer 1985), Washington, Montana (MacConnell and Peterson 1992), Oregon, and across the border in Canadian British Columbia (Foott and Hedrick 1987) and Newfoundland (Hedrick *et al.* 1993). Retrospective analysis suggested that on the North American continent, the disease dated back to at least 1966 (probably as far back as 1958), with the condition previously known as 'lupus' being linked to PKD (Hedrick *et al.* 1985; Foott and Hedrick 1987). Henderson and Okamura (2004) suggested that PKD was originally introduced to Europe from North America, but that this event preceded fisheries activities.

1.3. Range of fish diagnosed as suffering from PKD

Although primarily a disease associated with farmed rainbow trout (Ferguson and Needham 1978; Hoffmann and Dangschat 1981; Clifton-Hadley *et al.* 1986a), PKD has also been diagnosed in various other salmonid species (Hoffmann and El-Matbouli 1994), but only detected in one species outwith the salmonids, the northern pike, *Esox lucius* L. (Seagrave *et al.* 1981).

In the European aquaculture of salmonids, the rainbow trout industry has suffered the greatest incidence of disease and economic losses (Clifton-Hadley and Alderman 1988). Major outbreaks of disease have also been reported in Scottish farmed populations of brown trout and Atlantic salmon, *Salmo salar* L. (Ellis, McVicar and Munro 1982). Infection has also been observed in Arctic charr, *Salvelinus alpinus* L. hatched from wild eggs in a hatchery also rearing Atlantic salmon fry (Bucke, Feist and Clifton-Hadley 1991). Upon exposure to river water endemic for PKD, brook trout, *Salvelinus fontinalis* Mitchill have been found to have an apparent resistance to infection, although experimental infection could be instigated via intraperitoneal injection of infected material, but without clinical signs of disease developing (Bucke *et al.* 1991; Feist and Bucke 1993). Marble trout, *Salmo trutta marmoratus* Cuvier have been reported as suffering from PKD in Italy (Berton, Beraldo, Giavenni and Galeotti 2003). Although originally anecdotically thought to be resistant to the disease, farmed golden trout, *Oncorhynchus mykiss aguabonita* (Jordan) have also been found to be susceptible (Morris, Longshaw and Adams 2003b).

PKD has been detected in wild populations of rainbow trout, brown trout and grayling, *Thymallus thymallus* L. in German rivers close to fish farms (Hoffmann and Dangschat 1981). Severe disease has been noted in grayling inhabiting rivers known to supply farms that suffered from annual outbreaks of PKD (Bucke *et al.* 1991). Clinical signs and histopathological changes were seen to be comparable with those seen in rainbow trout suffering from PKD. In grayling renal tissue, intraluminal myxozoan sporoblasts and immature spores were observed. Interpreting these findings, Bucke *et al.* (1991) suggested that PKD could have some effect on wild stocks of grayling, possibly with this species playing a role in transmission. Feist and Bucke (1993) showed that following experimental infection with PKD, grayling not only exhibited clinical and histopathological signs of severe disease, but also

demonstrated the capability of making a full recovery from infection. The severity of disease in grayling was gauged to be less than that encountered in rainbow trout, although the time course of the disease was longer with grayling, including a protracted phase of chronic infection and a lengthened recovery time.

In North America, severe disease attributed to PKD was first identified in rainbow trout kept at the Idaho Department of Fish and Game Hatchery, near Hagerman, Idaho (Smith *et al.* 1984). Infection was also confirmed in cutthroat trout, *Oncorhynchus clarki* Richardson, and steelhead trout, *Oncorhynchus mykiss* (Walbaum) from watercourses feeding the hatchery (Klontz and Chacko 1983; Klontz *et al.* 1986). In 1983, PKD was first detected in species of Pacific salmon, in fish reared at the Mad River Hatchery, California (Hedrick, Kent, Rosemark and Manzer 1984a). Infection was described in chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), coho salmon, *Oncorhynchus kisutch* (Walbaum) and steelhead trout. Significant mortality rates, attributed to PKD were described by MacConnell and Peterson (1992) in a remote isolated population of feral cutthroat trout in Middle Creek Reservoir, Montana. The disease was not noted there until 1990, the appearance at that time could not be fully explained – State records suggested that the most recent restocking had been in 1947 – raising fears that the disease was more widespread amongst wild fish populations than previously thought. This outbreak confirmed that the disease was not confined to hatcheries, but could also have noticeable effects on feral fish populations. Arctic charr reared in St. John's, Newfoundland, Canada, were found to suffer mortality from PKD, whereas a population of landlocked Atlantic salmon, *Salmo salar ouananiche* McCarthy kept under the same conditions did not succumb to PKD (Brown, Thonney, Holwell and Wilson 1991). Infection has also been demonstrated in kokanee salmon – non-anadromous sockeye salmon, *Oncorhynchus nerka* (Walbaum) from the Puntledge River, Vancouver Island, British Columbia (Kent, Higgins, Whitaker and Yokoyama

1995a). Chinook salmon smolts reared at Vancouver Island were also seen to suffer from PKD following transfer to seawater at Nanaimo, British Columbia (Kent, Whitaker, Higgins, Blackburn and Dawe 1995b). It was postulated that the disease might impact on the survival in the ocean of salmon originating from PKD endemic waters, corroborated by the very low recorded return rates of salmon from the sea to the riverine hatchery at Vancouver Island.

Evidence suggests that the majority of species within the genera of *Salmo* L., 1758 and *Oncorhynchus* Suckley, 1861 are vulnerable to PKD, whilst more variation exists in susceptibility within the genus *Salvelinus* Richardson, 1836. Extensive analysis of wild fish specimens inhabiting PKD endemic waters has failed to reveal any additional fish hosts of PKD to those previously mentioned (Seagrave *et al.* 1981; Morris, Adams, Feist, McGeorge and Richards 2000a).

1.4. Epidemiology of PKD

Reported outbreaks of PKD have demonstrated a wide diversity in the severity of disease and the class of stock involved. The majority of reported outbreaks have affected the rainbow trout industry, perhaps in part due to its predominance in the world-wide culture of freshwater salmonids. Most incidences of PKD affecting cultured trout have taken place between May and September, after the stock has been moved from its initial environment of spring or bore-hole water to on-growing facilities fed by riverine water (Clifton-Hadley *et al.* 1984a). Ferguson and Needham (1978) described outbreaks in rainbow trout farms in Scotland and Northern Ireland with mortalities of over 75% and 10-15% respectively. In the Scottish outbreaks, mortality was evident in first season fish (0+) from two weeks following salt water acclimatisation in late July until October. The Northern Irish outbreaks followed a similar pattern with deaths in fingerlings from mid July until September. All of the first

season fish examined suffered from PKD to some extent. In 1977, the N. Irish farm experienced mortality levels of 40%. On this occasion, the fish had been fed to satiation during the warmer months, whereas in previous years the ration had been restricted to approximately 1% body weight. Seagrave *et al.* (1981) also noted morbidity levels of up to 100% on English rainbow trout farms.

In Germany, PKD was recognised more frequently in fish over one year old (1+) than in fingerlings, with variation in mortality levels (0-30%) felt to be influenced by secondary infections (Hoffmann and Dangschat 1981). In France, a mortality rate of 30% was seen in 17 month old rainbow trout kept in an indoor recirculation unit (Chilmonczyk, Thomas and de Kinkelin 1989). In Montana, USA, considerable mortalities were seen yearly in 15-20 cm cutthroat trout, with no disease diagnosed in fish longer than 27 cm (MacConnell and Peterson 1992). Ellis *et al.* (1982) recorded morbidity levels of 75% of 0+ brown trout and 60-90% of 0+ Atlantic salmon in Scottish farms between late July and October. The pattern of disease seen with the brown trout fingerlings mirrored that previously described in rainbow trout. Evidence suggested a difference in PKD susceptibility between stocks of Atlantic salmon of separate origin, with disease affecting the progeny of Norwegian stock but not Scottish stock, despite similar environmental factors. Hedrick *et al.* (1985) described PKD at a Californian rainbow trout hatchery occurring amongst groups of the largest (average weight of 45 g) and smallest fish (average weight of 7.5 g). Mortality levels of up to 20% were felt to be increased by the presence of simultaneous infections of *Ichthyophthirius multifiliis* Fouquet, 1876, *Flexibacter columnaris* Bernardet and Grimont, 1989 and unspecified gill bacteria. Mortality levels of up to 80% have been seen in the presence of various secondary infections, including furunculosis (Feist and Bucke 1993; Hoffmann and El-Matbouli 1994). Within wild salmonid stocks, the full impact of PKD has not been assessed. However, the prevalence has been found to reach 43% in brown trout sampled from

endemic waters in England (Feist, Peeler, Gardiner, Smith and Longshaw 2002), and PKD has been examined as a possible contributing factor to reduced salmonid fish catches in Switzerland (Burkhardt-Holm 2002).

1.4.1. Water conditions

Since the earliest reports of PKD, seasonality has been noted as a feature in disease outbreaks (Roberts 1978). Ferguson and Ball (1979) described a correlation between rising temperature in spring to summer and onset of disease. Ferguson (1981) further investigated these findings, examining groups of exposed fish, subsequently kept at various water temperatures. It was concluded that, in seasonal PKD development, water temperature played a more critical role than the magnitude of the infecting dose. Results also implied that a declining temperature was not essential for recovery to occur, although it accelerated recuperation. Subsequent studies revealed that under laboratory conditions, naturally infected fish developed disease at 12-18°C, but not at 9°C, with the rates of parasite multiplication and consequent disease development enhanced at higher temperatures (Clifton-Hadley, Richards and Bucke 1986b).

Foott and Hedrick (1987) concurred that the progress of PKD was temperature dependent, but suggested that infection may also depend upon the seasonal presence of the aetiological agent, as had been demonstrated with other myxozoans. The subsequent identification of *Tetracapsuloides bryosalmonae* as the causative agent, and the finding that bryozoans were additional hosts, allowed further investigation of the factors affecting the seasonality of PKD (Canning *et al.* 1999; Anderson *et al.* 1999a). Although the presence of *T. bryosalmonae* stages has been shown in bryozoans during winter months, it was discovered that water temperatures below 9°C led to cessation of multiplication of *T. bryosalmonae* in both teleost and bryozoan hosts (Gay, Okamura and de Kinkelin 2001).

Water quality has been thought to be a significant factor in the development of PKD. In outbreaks in Scotland and N. Ireland, the water was found to be very soft (4.5 and 18 mg $CaCO_3$ L^{-1} respectively), acidic (pH 5.6 and 6.5 respectively) and oligotrophic (14.2 and 40 µg NO_3 L^{-1} respectively) (Ferguson and Needham 1978). It was suggested that poor water conditions could lead to physiological stress in stock and / or could allow significant multiplication of pathogenic organisms. It was felt that a combination of these factors might play a part in disease development. Roberts (1978) also stated that low pH could be a pertinent factor in development of PKD, as could soft water conditions during summer months (Roberts and Shepherd 1974). Smith *et al.* (1984), however, described the first North American outbreak of PKD, which occurred in good quality, slightly alkaline water. This supported the findings of Scott (1979) who described an outbreak with water sourced from a highly eutrophic alkaline (pH 7.9-8.3) chalk stream with hardness of 230 mg $CaCO_3$ L^{-1}. These findings, although seemingly paradoxical, could be consistent with the assertion that extremes of pH – acidic or alkaline – could predispose stock to the condition (Scott 1979). In a study of a waterway in southern Germany, El-Matbouli and Hoffman (2002) demonstrated a link between the organic pollution of water and the prevalence of PKD. It was suggested that changes in eutrophication level could affect the aquatic microfauna without necessarily registering on standard water quality analyses. This supported the hypothesis that water temperature was not the only extrinsic factor capable of influencing the epidemiology of PKD.

1.5. Clinical and pathological signs of PKD

Klontz *et al.* (1986) estimated the incubation period of PKD infection in rainbow trout to be approximately 40 days, with the clinical course of 60-90 days being apparently self-limiting.

1.5.1. External signs

As with many diseases of fish, a wide spectrum of clinical signs may be seen with PKD. Variations in the severity and time-course of disease manifestation may occur between different geographical areas and classes of stock, and be affected by a number of variables including water temperature (Clifton-Hadley, Richards and Bucke 1985). In the case of low-grade infections, there may be no observable abnormalities of affected fish. In severe outbreaks, with morbidity levels of up to 100%, several general presenting signs have been described.

Initially, body darkening (melanosis), gross abdominal swelling and mortalities were described (Roberts and Shepherd 1974). Melanosis has been described as an inconsistent finding (Ferguson and Needham 1978), Plehn (1924) having conversely described lightening in colour of affected stock. Typically, abdominal distension with longitudinal swellings of the body walls at the level of the lateral lines has been described (Ferguson and Needham 1978; Hedrick, Kent, Rosemark and Manzer 1984b; Ellis, McVicar and Munro 1985). Fernández-de-Luco, Peribáñez, García, and Castillo (1997) described PKD-related granulomatous myositis in rainbow trout, resulting in protuberances of macroscopic nodules and roughening of the skin surface along the lateral lines of almost 80% of surviving sub-yearling fish examined. Exophthalmos has been frequently noted – both monolateral and bilateral, with corneal cloudiness being an occasional additional sign (Ferguson and Needham 1978; Clifton-Hadley, Bucke and Richards 1987a).

Severe gill pallor, due to anaemia, has been reported as sometimes being apparent even on gross examination (Ferguson and Needham 1978; Ellis *et al.* 1985; Fernández-de-Luco *et al.* 1997). Behavioural changes have been observed in affected stock with marked nervous agitation resulting in eventual loss of equilibrium and respiratory distress (Ferguson and Needham 1978). Late in the course of the

disease, inappetence has been noted, as well as separation of affected individuals from the rest of the shoal (Hoffmann and El-Matbouli 1994). These fish were seen to become increasingly apathetic in their behaviour, resulting in diminished reflexes before death. Ferguson and Needham (1978), however, suggested that normal feeding continued up until the point of death.

As with most diseases of teleost fish, the clinical signs seen in outbreaks of PKD could not be described as being pathognomonic, with no particular changes being specific to the disease. Therefore, although the combination of clinical signs, coupled with knowledge of husbandry considerations may be suggestive of PKD, a conclusive diagnosis could not realistically be made. The frequent presence of concurrent infections during the course of PKD could also lead to changes in the clinical observations (Hedrick *et al.* 1993).

1.5.2. Internal signs

Gross and microscopic signs of PKD have rarely been reported less than four weeks post-infection. Gross renal swelling, especially of the posterior part, although not diagnostic in itself, has been found to be highly characteristic of the disease (Clifton-Hadley *et al.* 1987a). The kidney has been described as increasing in volume up to ten-fold, often causing lateral displacement of the adjacent swim bladder (Ferguson and Needham 1978; Hoffmann and El-Matbouli 1994). Ferguson and Needham (1978) described enlargement resulting in formation of swollen greyish bulbous ridges, the posterior kidney invariably affected, and the anterior portion also involved in severe cases (Ellis *et al.* 1985).

Clifton-Hadley *et al.* (1987a) divided the changes seen in affected kidneys into six categories, depending on the level of severity. Mild grades encompassed slight posterior enlargement while still remaining a normal dark red colour, through to

obvious diffuse renal enlargement with wrinkling of the overlying capsule and an overall mottled red and grey appearance. Moderate grades led to a six-fold increase in kidney volume, with marked corrugation of the surface and a blue sheen to the capsule. Severe classification entailed the kidney taking on a mottled pink and grey colour, further oedematous renal distension with adhesion of the capsule by gelatinous fluid, and release of clear fluid following incision into the parenchyma. Ascites, sometimes comprising blood-tinged peritoneal fluid, has been described as a finding in more advanced cases (Clifton-Hadley *et al.* 1987a). A grade was also included to cover the healing process when discrete cream-coloured patches or spherical nodules up to 10 mm in diameter were seen amongst dark red renal tissue. Similar lesions have also been described in hepatic, muscular and peritoneal tissues (Clifton-Hadley *et al.* 1984a; Fernández-de-Luco *et al.* 1997).

During PKD outbreaks, changes in other organs have also been noted. Reported splenic changes vary from reduction in size to gross organ enlargement with rounding of the edges (Ferguson and Needham 1978; Clifton-Hadley *et al.* 1984a; Hoffmann and El-Matbouli 1994). Such splenomegaly has resulted in a grey mottling being noted in patches under the capsule and throughout the stroma, and a roughened appearance of the surface (Ferguson and Needham 1978). Reported hepatic changes include discrete areas of greyish mottling, without hepatomegaly (Ferguson and Needham 1978), and variable anaemic colour changes to either pale or yellow tones (Clifton-Hadley *et al.* 1987a). Marked gill pallor has been reported (Ferguson and Needham 1978), with gross thickening of individual primary gill filaments noted (Clifton-Hadley *et al.* 1987a). Due to the clinical anaemia, the whole body surface and the internal organs have been described as assuming a pale colour, blood being described as noticeably thin with agglutinations present (Clifton-Hadley *et al.* 1984a; Hoffmann and El-Matbouli 1994). The finding of normal food

contents in the stomach and intestines has been cited as showing that feeding continued until death (Ferguson and Needham 1978).

1.5.3. Microscopic findings

As many of the descriptions of fish stages of the parasite were made before it was named *T. bryosalmonae*, the term “PKX” will be used in reference to these articles. Initially, numerous typical PKX cells have been seen in blood vessels, particularly of the kidney and spleen, from approximately two weeks post-infection (Smith *et al.* 1984; Kent and Hedrick 1986; Clifton-Hadley *et al.* 1987a). Due to the initial absence of identified PKX cells in the tubules, it was suggested that primary dissemination of the parasite may be by the haematogenous rather than retrograde urinary route (Kent and Hedrick 1986; Clifton-Hadley *et al.* 1987a). As PKX cells have been noted in peritubular capillaries, but seldom within glomeruli, it has been proposed that the route of transport was via the caudal vein of the renal portal system, as opposed to the efferent glomerular arterioles (the alternative blood supply to these capillaries) (Clifton-Hadley *et al.* 1987a). In certain salmonid species, including chinook salmon, reproduction of the parasite has been reported to be very rapid during this period (Hedrick *et al.* 1993). Damage to the vascular endothelium, with thrombus formation has been reported, sometimes severe enough to lead to vasculitis and concomitant occlusion of renal and hepatic vessels (Smith *et al.* 1984; Clifton-Hadley *et al.* 1987a; Feist and Bucke 1993). Increased numbers of mononuclear cells have been noted in the interstitium, preceding the appearance of extrasporogonic cells (typical PKX cells) in the renal tissue.

The predominant histopathological change noted in outbreaks of PKD has been described as initial renal haematopoietic hyperplasia, followed by marked granulomatous interstitial nephritis (Ferguson and Needham 1978; MacConnell, Smith, Hedrick and Speer 1989). The haematopoietic hyperplastic reaction has been

compared to that seen in human bone marrow during infectious diseases (MacConnell *et al.* 1989). The cellular reaction, made up of cells resembling macrophages and lymphocytes, has been seen to be targeted on the extrasporogonic stages of the parasite (Ferguson and Needham 1978). The normal stem cell population increasingly became replaced by a mixed cellular infiltrate. MacConnell *et al.* (1989) described interstitial extrasporogonic cells with one or more macrophages adhered to the surface of each. In turn, lymphocytes were frequently seen associated with the outermost plasmalemma of the macrophages, although seldom adjacent to the parasite. In areas of extensive reaction, large amounts of collagen were noted throughout the tissue, denoting diffuse fibrosis, probably as a sequel to interstitial nephritis (Ferguson and Needham 1978).

The arrangement of the cells in histological sections has been found to adopt a distinctive radiating 'whorling' pattern. At the centre of each 'whorl', one or two large regularly round parasitic cells were observed (Ferguson and Adair 1977; Ferguson and Needham 1978). At various points between 3 to 20 weeks post-infection, macrophages were observed migrating through the basal lamina into the tubular epithelium (MacConnell *et al.* 1989). Feist and Bucke (1993) described dilation of the renal capillaries and tubules, leading to thickening of the glomerular basement membrane and widespread haematopoietic hyperplasia. This was seen to target the mesonephros, in particular, with focal areas of normal myelopoiesis observed sandwiched between the granulomata (Hedrick *et al.* 1984b). In severe cases, the numbers of tubules and glomeruli were seen to decrease, with those remaining situated in groups separated by areas of cellular reaction (Ferguson and Needham 1978).

Similar cellular reactions leading to a 'whorling' pattern have been described in the splenic tissue of infected fish (Ferguson and Needham 1978). Haematopoietic

hyperplasia, followed by diffuse inflammation including a moderate to severe degree of proliferation of the serosa was occasionally observed (Feist and Bucke 1993). In severe cases, extensive collagen formation was apparent (Ferguson and Needham 1978). In the liver, discrete areas of focal mononuclear cell accumulation within sinusoids have been noted in mild cases (Ferguson and Needham 1978; Ellis *et al.* 1985). In more severe cases, changes similar to those described in the kidney and spleen have been observed (Ferguson and Needham 1978), on occasion resulting in large areas of liquefactive necrosis (Ellis *et al.* 1985).

In the pancreas, infiltration of the tissue by mononuclear cells, leading to atrophy, necrosis and destruction of extensive areas of exocrine and endocrine tissue by granulomata was noted (Ellis *et al.* 1985). In the gills, mild clubbing of the secondary lamellae and aneurysms encapsulating PKX cells have been reported (Ellis *et al.* 1985). Ferguson and Needham (1978) described limited pathology of the muscle, affecting areas dorsal to the kidney. However, more recently a description of PKD in Spain (Fernández-de-Luco *et al.* 1997), documented incidences of granulomatous myositis in affected fish. This resulted in nodules of up to 8 mm in diameter developing in the red muscle of one or both lateral lines, and the dorsal line in some cases. The inflammatory infiltrate was composed mainly of macrophages, lymphocytes and plasma cells. Although the pathological pattern mimicked that previously observed in renal and splenic tissue, the development of inflammation was asynchronous between the organs. PKX cells were not detected in the muscle until after the peak of changes in the kidney, with increasing numbers in the muscle corresponding to renal recovery from the inflammatory process. It was proposed that the parasite cells reached the muscle via the circulation. Mild inflammation of the pyloric caeca and other areas of the gut was occasionally reported (Ferguson and Needham 1978). Limited endocardial proliferation was seen in isolated areas, but no parasites were identified in the heart (Ferguson and Needham 1978).

1.5.4. Time course of clinical and pathological signs

The sequential changes seen in fingerling rainbow trout in England suffering from PKD have been comprehensively documented (Clifton-Hadley *et al.* 1985, 1987a). The fish studied were hatched and reared in spring water on sites with no history of PKD, and in spring-time were moved to concrete tanks supplied with riverine water known to be endemic for PKD. Fish were sampled at random from the population at various times during the studies. The authors stated that several considerations including geographical area and water temperature could affect the timing of events.

From the time of exposure to disease-causing waters until four weeks later, no clinical or pathological changes were noted. In a separate study, in which fingerling rainbow trout were intraperitoneally injected with infective kidney material, evidence of parasites was first found three weeks post-infection in haematopoietic tissue, sinusoids and peritubular capillaries (MacConnell *et al.* 1989). Evidence of PKX cells was detected in rainbow trout kidneys as early as three weeks after exposure to infected water in a study in California (Kent and Hedrick 1986).

Five weeks post-exposure to infected water in England, although no gross changes were appreciated, occasional PKX cells were seen in peritubular capillaries of the kidney (Clifton-Hadley *et al.* 1985). MacConnell *et al.* (1989), and Kent and Hedrick (1986) described interstitial inflammation (mainly comprising an infiltrate of lymphocytes and macrophages) as being first evident at this time. Therefore, apparent variations in the timing of developmental features of the disease could be noted from different studies implementing differing experimental conditions. Although the exact timings varied due to a number of factors, the general sequence of changes seemed to be consistent.

In the English study, PKX cells were evident in 80% of examined kidneys and interstitial hyperplasia was apparent between 6-7 weeks post-exposure (Clifton-Hadley *et al.* 1985). At eight weeks, despite no change in colour, renal enlargement leading to crenellation of the capsular margin was seen. Numbers of PKX cells were increasing during this phase, especially in the renal vascular sinuses, leading to areas of endothelial cellular reaction. In the USA, renal tubular intraluminal sporogonic stages of PKX were first observed at seven weeks post-exposure, and were evident for at least three months after clinical recovery.

From 9 to 12 weeks, clinical signs were observed, including abdominal distension, melanosis, exophthalmos, and pallor of the gills and internal organs. The kidney was massively enlarged, the capsule appearing ribbed with a blue sheen. Red and grey mottling of the kidney was apparent, with dark red foci also seen in the spleen, liver, abdominal fat and gills. These foci corresponded with areas where blood vessels were occluded by PKX cells and cellular debris. Large regions of endothelium were damaged, with minimal fibrin deposition evident. The grey patches of diseased kidneys corresponded to disorganised interstitial cellular reaction surrounding PKX cells. The predominant cell types consisted of macrophages, lymphocytes and fibroblasts, few erythrocytes being present. As the reaction progressed, the presence of excretory elements in the affected patches diminished, until only reactive tissue was evident. Occasional PKX cells were detected in glomerular capillary tufts and tubular epithelium. Inflammatory foci were also observed in the liver, spleen, pancreas, gills, skeletal muscle and intestinal submucosa at this time.

MacConnell *et al.* (1989) reported PKX cells within renal tubules, 10 weeks post-injection with infected material. PKX cells with attached macrophages seemed to be degenerating; however the previously noted internal daughter cells were usually intact. Haematopoietic hyperplasia became more prominent than granuloma

formation, the former being mainly composed of macrophages, lymphocytes and thrombocytes. Signs of tubular degeneration were perceived by electron microscopy.

From 12 to 20 weeks post-exposure, renal swelling became less marked, with fewer red foci, and the grey areas becoming paler in appearance (Clifton-Hadley *et al.* 1985). Microscopically, many of the PKX cells had lost their distinct margins, apparently suffering from necrosis. In previously damaged segments, resurgence in the numbers of capillaries, erythrocytes, melanin and excretory structures was observed, signifying organisation and resolution of the tissue. A small number of the renal and splenic grey reactive zones did not follow this progression, instead white nodules up to 10 mm in diameter of chronic inflammatory tissue containing intact PKX cells developed. After 21 weeks, the healing process was well established, with only minor renal distension apparent in some fish.

Despite variations in the timing of pathological changes between the studies (in part attributable to the contrasting exposure methods), and even between individuals from each outbreak, an overall impression of the stages involved in the development of disease, and subsequent recovery can be gained. A number of factors, not least water temperature and environmental stressors could conspire to alter the course of clinical development, possibly leading to mortality in some individuals, as opposed to long-term recovery.

1.5.5. Pathophysiology

Anaemia is the most profound reported pathophysiological change described in association with PKD. Severe disease has been found to lead to clinical anaemia in affected fish, resulting in packed cell volume (PCV) levels as low as 11.2% (Ferguson and Needham 1978; Clifton-Hadley, Richards and Bucke 1987b), although more typically PCV levels in the range of 28-30% have been reported

(Hoffmann and Lommel 1984; Clifton-Hadley *et al.* 1987b). Apparently, as the pathological changes progress, the level of anaemia becomes more marked with fish showing more advanced signs (such as oedema) exhibiting lower PCVs than lesser-affected individuals (Clifton-Hadley *et al.* 1987b). Alongside the diminished PCV, reduced haemoglobin levels (measured by standard photometrical methods), lowered red blood cell counts – with erythrocyte polychromasia, anisocytosis and poikilocytosis were reported (Hoffmann and Lommel 1984; Clifton-Hadley *et al.* 1987b). Reports of white blood cell counts have varied, ranging from leucopaenia to leucocytosis, although neutrophilia was consistently reported, becoming more marked as the condition progressed (Hoffmann and Lommel 1984; Clifton-Hadley *et al.* 1987b; Foott and Hedrick 1990).

Traditionally, classification of anaemia has included three broad categories: haemorrhagic, haemolytic and hypoplastic. Initially, Roberts (1978) described the anaemia of PKD to be hypoplastic – correlating with decreased red blood cell production. However, Ferguson and Needham (1978) discovered a large component of immature microcytic blood cells in diseased fish, therefore suggesting the anaemia not to be truly hypoplastic. Subsequently, the resulting anaemia was compared to that caused by protozoal diseases in humans and was categorised as a chronic haemolytic anaemia (Hoffmann and Lommel 1984). Clifton-Hadley *et al.* (1987b), however, described features of both hypoplastic and haemolytic anaemia. Haemolysis, resulting in the formation of haemoglobin crystals was reported, related to the progressive development of intravascular cellular aggregations, possibly hindering newly produced blood cells from entering the circulation. Comparison with the anaemia of human chronic renal failure was made, and it was concluded that although sharing features of the haemolytic and hypoplastic categories, the anaemia of PKD was more akin to the latter group (Clifton-Hadley *et al.* 1987b).

Changes in serum protein levels have also been documented. While Hoffman and Lommel (1984), Olesen and Vestergård Jørgensen (1986), and Clifton-Hadley *et al.* (1987b) recorded significant decreases in total protein levels (particularly in advanced cases), other studies showed changes in the relative concentration of serum proteins without an overall reduction in total protein (Scott 1984; Klontz *et al.* 1986; Foott and Hedrick 1990). In particular, the ratio of albumin : globulin was seen to be substantially lower in affected fish, with absolute concentrations of prealbumin and albumin falling, and a rise in the globulin fractions. These findings led to the suggestion that, despite the development of anaemia, renal filtration and hepatic function were not impaired until the disease became severe – resulting in the manifestation of oedema (Clifton-Hadley *et al.* 1987b).

Foott and Hedrick (1990) described increased plasma concentrations of magnesium and calcium in severely affected fish, impaired renal tubular function leading to compromised excretion of these divalent ions. Unlike human chronic renal failure, blood urea levels have not been seen to be uniformly elevated in PKD, presumably because excretion of urea is primarily via the gills rather than the kidneys in salmonid fish (Clifton-Hadley *et al.* 1987b; Hoffmann and El-Matbouli 1994).

1.6. Causative agent

1.6.1. Taxonomy

Since the earliest reports of syndromes resembling PKD, suspicion has fallen upon the possible role of infectious organisms. Plehn (1924) suspected an amoebic cause, while Ferguson and Adair (1977), and Ferguson and Needham (1978) reported that the implicated organism possessed characteristics of both amoebae (including pseudopodia) and myxosporeans, although failure to find spores prevented further classification. Seagrave *et al.* (1980) first used the term ‘PKX’ (proliferative kidney

organism unknown: ‘X’) to denote the organism, and demonstrated similarities to members of the class Haplosporea Caullery, 1953 within the phylum Haplosporidia Caullery and Mesnil, 1899. In particular, the observation of cytoplasmic electron-dense bodies was described in the primary cells of PKX, similar to haplosporosomes seen in members of the genus *Marteilia* (Grizel *et al.*, 1974) Perkins, 1976 comprising invertebrate parasites especially of oysters. Also, the mechanism of internal cleavage with formation of daughter PKX cells resembles the sporulation sequence in *Marteilia* spp., with other similarities including the presence of multivesicular bodies and an amorphous cell wall being noted.

Subsequently, the features that PKX shared with the haplosporeans were also found to be applicable to members of the phylum Myxozoa, class Myxosporea Bütschli, 1881. Electron-dense bodies resembling haplosporosomes known as sporoplasmosomes (Lom, Feist, Dyková and Kepr 1989), endogenous internal cleavage, the presence of multivesicular bodies and microtubules in secondary and tertiary cells have all been described in various myxosporean species (Kent and Hedrick 1986; Lom and Dyková 1995). Kent and Hedrick (1985a, 1986) described the development of sporogonic stages and immature spores in rainbow trout, suggesting that the causative agent of PKD belonged to the phylum Myxozoa, this proposition being supported by other workers (Feist and Bucke 1987; Clifton-Hadley and Feist 1989).

Although they were considered unique in their ability to produce multicellular spores, the members of the phylum Myxozoa were originally classified with the parasitic protozoa – Apicomplexa Levine, 1970, Microspora Sprague, 1977, and Ascetospora Sprague, 1979. Also, close similarities were noted in morphogenesis between myxozoan polar capsules and nematocysts (stinging cells laced with poison) of members of the metazoan phylum Cnidaria Hatschek, 1888 (Lom and Dyková 1995;

Zrzavý, Mihulka, Kepka, Bezdek and Tietz 1998; Canning *et al.* 2000). Molecular analysis of 18S rRNA sequences (Smothers, von Dohlen, Smith and Spall 1994) and of Hox gene sequences (Anderson, Canning and Okamura 1998) demonstrated an affinity with triploblasts, showing a relationship with the nematodes. Interpretation of further comparisons of myxozoan 18S rDNA sequence data with that of the parasitic narcomedusan *Polypodium* spp. Ussov, 1885 suggested that the myxozoans could, in fact be diploblasts, being degenerate relatives of the cnidarians (Siddall, Martin, Bridge, Desser and Cone 1995; Siddall and Whiting 1999). Studies of the nematode-like parasite of freshwater bryozoans, *Buddenbrockia plumatellae* Schröder, 1910 demonstrated the concurrent presence of myxozoan features (including polar capsules) and a worm-like bilaterian structure (Okamura, Curry, Wood and Canning 2002). It was proposed that these findings demonstrated confirmation of earlier studies suggesting triploblastic features. Moreover, it has been suggested that the myxozoans are derived from the bilateria, and that bryozoans are their ancestral hosts (Anderson *et al.* 1998; Anderson, Canning and Okamura 1999b; Okamura *et al.* 2002). The weight of evidence has led to the myxozoans now being considered as metazoans (Smothers *et al.* 1994; Saulnier, Philippe and de Kinkelin 1999).

Originally, the phylum Myxozoa contained two classes: the Myxosporea and the Actinosporea Noble and Levine, 1980 (Lom and Dyková 1992, 1995). More than 1350 species have been allocated to approximately 52 genera of Myxosporea (Kent, Andree, Bartholomew, El-Matbouli, Desser, Devlin, Feist, Hedrick, Hoffmann, Khattra, Hallett, Lester, Longshaw and Palenzeula 2001). These have principally occurred in the tissues and body cavities of teleost fish, but also have been recognised in elasmobranchs, lampreys, myxines, aquatic and chelonid reptiles, amphibians, and invertebrates (Lom and Dyková 1995). In addition, a myxozoan-like parasite has been observed in the brain of a mole, *Talpa europaea* L. (Friedrich, Ingolic, Freitag, Kastberger, Hohmann, Skofitsch, Neumeister and Kepka 2000), and

suspected infection has also been noted in birds (Lowenstine, Rideout, Gardner, Busch, Mace, Bartholomew and Gardiner 2002). Myxozoan spores have even been found in human faeces samples from immunocompromised patients, with their persistence suggesting that they were produced by true infection rather than remaining unchanged from ingested infected fish tissue (Canning and Okamura 2004). Members of the much more limited class Actinosporea, have been found to be mainly parasitic in annelid and sipunculid worms (Sommerville 1998). The discovery that the actinosporean *Triactinomyxon* sp. Štolc, 1899 in the worm *Tubifex tubifex* Müller was a stage in the life cycle of the myxosporean *Myxobolus cerebralis* Hofer, 1903 led to the realisation that actinospores represented alternate life cycle stages of organisms already established in the class Myxosporea (Markiw and Wolf 1983). Myxozoan life cycles incorporating stages in annelid worms have been documented in more than 25 species of freshwater fish parasites (Kent *et al.* 2001). It was suggested that the class Actinosporea should be suppressed in favour of a single class Myxosporea (Kent, Margolis and Corliss 1994). However, some researchers suggested that new species of actinosporeans should be named using the traditional binomial system, and cited the protocol of the Linnean system as adopted by the International Code of Zoological Nomenclature (Lester, Hallett, El-Matbouli and Canning 1998). This was contested by Kent and Lom (1999) who proposed a novel naming system. Although this proposal was not accepted, it was agreed that the International Commission on Zoological Nomenclature should resolve the matter (Lester, Hallett, El-Matbouli and Canning 1999). Two orders exist within the class Myxosporea, each containing multiple families: the order Bivalvulida Shulman, 1959 and the order Multivalvulida Shulman, 1959. Differentiation is made according to spore morphology, in particular the number of valves, the Bivalvulida having two and the Multivalvulida having up to 13 valves (Canning *et al.* 2000; Whipps, Adlard, Bryant and Kent 2003).

Traditionally, myxozoans have been primarily classified on the basis of the structure of their spores (Lom and Dyková 1992, 1995). Initially, spores are composed of multiple cells (4-16), which during sporogenesis become transformed into sporal components. Between 1-13 capsulogenic cells develop, enclosing the polar capsules that contain coiled polar filaments and are located near the spore apex. Two to 13 valvogenic cells differentiate into the outer protective shell valves composed of resistant nonkeratinous protein, sometimes coated with a mucus envelope, which may aid distribution by increasing buoyancy. One or two sporoplasmogenic cells become the sporoplasm, located in the middle or posterior section of the spore, containing electron-dense bodies known as sporoplasmosomes which although not PAS positive are similar in appearance to the haplosporosomes of the Haplosporidia.

Myxosporeans have been characterised by having complex life cycle phases within their hosts. Originally, it was assumed that following infection, released sporoplasms travelled via the circulation to the target organ to undergo sporogonic development. Subsequently, proliferative stages were also identified (typically in different tissues from the sporogonic stages), which could result in very heavy infections. These extrasporogonic developmental stages lead to the formation of doublets of cells within cells, and have been extensively studied in *Sphaerospora* spp. Thélohan, 1892 (Lom and Dyková 1992, 1995). Feist and Bucke (1987) compared the development of secondary and tertiary cells of PKX with stages described of several myxosporeans, although those mentioned normally formed large polysporoblastic plasmodia, not the small pseudoplasmodia usually associated with PKX. However, PKX secondary cell characteristics such as prominent Golgi apparatus, double cell membranes, bundles of microtubules and numerous cytoplasmic ribosomes were consistent with the recognised morphological features of myxosporeans.

Similarities between PKX and members of the myxosporean family Sphaerosporidae Davis, 1917 became evident through various studies (Kent and Hedrick 1986; Kent, Kumi, Whitaker and Margolis 1993a; Hoffmann and El-Matbouli 1994). In California, Kent and Hedrick (1985a) described similarities between the intraluminal stages of PKX and trophozoites of the Sphaerosporidae. The secondary cell of PKX was morphologically compared with the monosporous pseudoplasmodium of the Sphaerosporidae, and was seen to be analogous in size and shape to that of *Mitraspora* spp. Fujita, 1912 (family Sphaerosporidae) from kidney sections of goldfish, *Carassius auratus* L. Although similarities were noted, the Sphaerosporidae showed obvious valvogenic cells surrounding sporoplasmic and capsulogenic cells, which were not observed upon examination of PKX. Kent and Hedrick (1986) described the sequential development of PKX in rainbow trout, culminating in the formation of renal intraluminal myxozoan spores. These authors described further similarities between these spores and immature spores of *Sphaerospora*, sharing a characteristic elongated shape, alongside small polar capsules and poorly defined valves, unlike mature *Sphaerospora* spores which, consistent with their nomenclature are spheroid.

Similarities to the spores of PKX were noted with those of the genus *Parvicapsula* Shulman, 1953 which are elongated with two polar capsules. Although members of this genus were known to sporulate in the renal tubular epithelium of salmonids, their development seemed to usually result in the formation of large plasmodia and pansporoblasts, unlike PKX. In the UK, these findings were corroborated by Clifton-Hadley and Feist (1989), although the intraluminal PKX stages demonstrated a pleomorphic nature in the development of the maturing spore. Examination of wild sticklebacks, *Gasterosteus aculeatus* L. and *Pungitius pungitius* L. revealed life cycle stages of *Sphaerospora elegans* Thélohan, 1892 in the choroidal *rete mirabile* and in renal interstitial haematopoietic tissue (Feist, Chilmonczyk and Pike 1991). These

were similar to PKX in size, location (intercellular in the kidney), and morphology (the presence of secondary and tertiary cells), although the distinctive electron-dense bodies associated with PKX were absent. Further comparisons were made between PKX and *Sphaerospora truttae* Fischer-Scherl, El-Matbouli and Hoffman, 1986 (Walter, Odening and Bockburst 1991), *Sphaerospora oncorhynchi* Kent, Whitaker and Margolis, 1993 (Kent *et al.* 1993a; Kent, Whitaker and Margolis 1993b; Kent *et al.* 1995a), and *Sphaerospora renicola* Dyková and Lom, 1982 (Voronin and Chernysheva 1993), although confirmation of the identity of PKX could not be established. It was considered that PKX could be an extrasporogonic stage of *S. oncorhynchi*, that only sporulates in sexually mature salmonids (Kent *et al.* 1993b, 1995a).

Using a rapid lectin-based staining procedure (employing the lectin GS-1 produced by *Griffonia simplicifolia* Baillon), differences in carbohydrate moieties were demonstrated between PKX and *Sphaerospora* sp. from brown trout (Hedrick, Marìn, Castagnaro, Monge and de Kinkelin 1992). These findings were confirmed and expanded upon by comparing PKX to an increased range of *Sphaerospora* spp. and also using monoclonal antibodies alongside lectin probes (Marìn de Mateo, McGeorge, Morris and Kent 1996). Characteristic differences in staining properties and morphogenesis were noted between PKX and *S. renicola*, *S. elegans*, *S. truttae*, and *Sphaerospora* sp. from Atlantic salmon. Whereas a previous study had shown a positive staining of spores and trophozoites of *S. oncorhynchi* with GS-1 (Kent *et al.* 1993a), these findings were not confirmed, although some antigenic similarities with PKX were inferred from the cross-reactivity of monoclonal antibody MAb B4 (Marìn de Mateo *et al.* 1996). It was stated by the authors that conclusive deductions on the relationship between PKX and the genus *Sphaerospora* could only be made by implementing experimental transmission trials and making genetic comparisons.

Molecular studies in France led to the cloning and sequencing of two genes of PKX (Saulnier, Brémont and de Kinkelin 1996; Saulnier and de Kinkelin 1997). While the first gene reported (GenBank™ accession number U59916) encoded an antigen targeted by serum from PKX-infected rainbow trout, the second gene (GenBank™ nucleotide sequence number U70623) encoded the small sub-unit ribosomal RNA (SSU rRNA) of PKX and strongly supported classification of the parasite in the phylum Myxozoa. A subsequent study, using PKX-specific primers developed using data from the SSU rRNA code, demonstrated a close relationship between PKX samples from four geographically separate locations, apparently confirming the presence of a single species (Kent, Khattra, Hervio and Devlin 1998). Comparison of SSU rDNA of PKX with sequences from *S. oncorhynchi*, *S. truttae*, *S. elegans* and *Parvicapsula minibicornis* Kent, Whitaker and Dawe, 1997 showed marked differences between PKX and these species, although close similarities were seen between members of the Sphaerosporidae. These findings apparently dismissed the theory of PKX being closely related to *Sphaerospora*; in fact PKX did not appear to be closely related to any known myxosporeans. Preliminary phylogenetic analysis implied that PKX had roots in the phylum Myxozoa before divergence of the orders Multivalvulida and Bivalvulida. Later, the position of PKX as a sister group of Myxozoa was confirmed by three separate phylogenetic tree reconstruction methods, the results being consistent regardless of the species used for comparison (Saulnier *et al.* 1999). Analysing 18S rDNA sequences, Anderson, Canning and Okamura (1999a) not only demonstrated that PKX parasitised several species of the class Phylactolaemata Allman, 1856 within the phylum Bryozoa Ehrenberg, 1831 from North America, but also demonstrated a relationship with the previously described bryozoan parasite *Tetracapsula bryozoides* Canning, Okamura and Curry 1996. Recent phylogenetic analysis has suggested that discrete genotypes of *T. bryosalmonae* exist in North America and Europe (Henderson and Okamura 2004).

Subsequently, PKX was also detected by molecular methods in bryozoans from the UK (Longshaw, Feist, Canning and Okamura 1999). Phylogenetic analysis of partial sequences of 18S rDNA sequences of myxozoan organisms from Bryozoa and salmonid fish suggested that PKX and *T. bryozoides*, although discrete species, formed a novel clade within the Myxozoa, apparently distinct from the existing orders Multivalvulida and Bivalvulida (Anderson *et al.* 1999b). *Tetracapsula bryozoides*, originally observed developing in coelomic cavities of the phylactolaemate bryozoan, *Cristatella mucedo* Cuvier, 1798, was originally classified in the class Myxosporea, order Multivalvulida (Canning, Okamura and Curry 1996). The family Saccosporidae and genus *Tetracapsula* were established to accommodate this newly described myxozoan whose characteristics seemed dissimilar to any described parasites at that time.

Initial taxonomic classification of PKX placed the organism within the genus *Tetracapsula*, due to morphological, behavioural and molecular similarities to *T. bryozoides* (Canning *et al.* 1999). The former species was named after the two hosts that it was known to parasitise, and thus the label PKX was disregarded in favour of *Tetracapsula bryosalmonae* (Canning *et al.* 1999). Experimental transmission trials demonstrated that naïve rainbow trout could be infected with *T. bryosalmonae* by exposure to disrupted infected bryozoans or through cohabitation trials (Feist, Longshaw, Canning and Okamura 2001). A description of the organism from Arctic charr proposed the species name *Tetracapsula renicola* due to the target organ in fish (Kent, Khattra, Hedrick and Devlin 2000). However, as this paper was published subsequent to the description of Canning *et al.* (1999), the International Code of Zoological Nomenclature dictated that the first given name should have priority (Okamura, Anderson, Longshaw, Feist and Canning 2001). Therefore, *T. renicola* is deemed to be a junior synonym of *T. bryosalmonae*.

Subsequent ultrastructural and molecular analysis of *T. bryosalmonae* led to the suggestion that a radical reorganisation of the phylum Myxozoa was necessary to appropriately accommodate the newly designated genus, *Tetracapsula* (Canning *et al.* 2000). Due to striking developmental and structural features, it was argued that the family Saccosporidae (incorporating the single genus *Tetracapsula*) should be withdrawn from the class Myxosporea. The Malacosporea, a new third class of the Myxozoa, was described to accommodate the family, its name originating from the pliable nature of spores of the members. Subsequently, re-examination of a nematode-like parasite of freshwater bryozoans, *Buddenbrockia plumatellae* led to comparisons being made with *T. bryozoides*. On the basis of morphological findings and the fact that the rDNA sequences from *T. bryozoides* and *B. plumatellae* were almost identical (Monteiro, Okamura and Holland 2002), Canning *et al*. (2002) proposed that these malacosporeans were of the same species, with the name *B. plumatellae* taking historical precedence. *Tetracapsula bryozoides* was thus made a junior synonym of *B. plumatellae*, with the new genus *Tetracapsuloides* established to accommodate *T. bryosalmonae*.

Due to the ubiquity of myxozoan infections of bryozoan species (Canning *et al.* 2000), it seems likely that additional organisms will soon be classified in this new class of myxozoans. It should be noted, however, that future radical restructuring of the classification system of the myxozoans could occur, especially as further elucidation of the ancestral origins of the phylum is achieved via molecular studies.

1.6.2. Morphological characteristics of *T. bryosalmonae* in fish

Original studies of the Myxozoa led to the suggestion that direct transmission took place by sporogenesis. Upon host entry, small amoebuloid sporoplasms exit the valvular shell in the alimentary tract, cross the intestinal wall, travel towards target organs via the circulation and result in the formation of sporogonic plasmodia (Lom

and Dyková 1992, 1995). Subsequently, purely proliferative stages of Myxozoa were described and found to result in the production of very large numbers of parasitic stages which could then undergo sporogenesis. This mechanism allowed very heavy parasitic burdens to develop, which could only otherwise have been achieved by massive ingestion of spores or by autoinfection (*in situ* hatching of spores). This process was originally known as presporogonic proliferation, but as it was not ascertained if the process invariably preceded sporogenesis, the term extrasporogonic (denoting a process outwith the sporogonic phases) was felt to be more appropriate.

The original descriptions of *T. bryosalmonae* (PKX) in fish, observed the pathological changes surrounding extrasporogonic stages, located particularly in the renal interstitium (Ferguson and Adair 1977; Ferguson and Needham 1978). Later studies described the previously elusive sporogonic stages of the parasite in salmonid renal tubule lumina, and demonstrated their ability to remain long after resolution of clinical disease (Kent and Hedrick 1985a, 1986; Morris, Adams and Richards 1997). Subsequent transmission, immunological and molecular studies proved that both of these stages were part of the life cycle of one organism, *T. bryosalmonae* (Kent and Hedrick 1985a; Marìn de Mateo *et al.* 1996; Kent *et al.* 1998; Morris, Adams and Richards 1999, 2000b).

Extrasporogonic stages

Stages of PKX have been recognised in samples of salmonid gill at three days following exposure to endemic waters, with extrasporogonic stages recognised in the blood and kidney interstitium from 2-4 weeks post-exposure (Morris *et al.* 1997, 2000b). Initial reports of PKD described the manifestation of renal histopathological changes surrounding extrasporogonic cells. These stages became regarded as the typical form of PKX cells, having a diameter of approximately 10-20 µm (Seagrave *et*

al. 1980). Histologically, Ferguson and Adair (1977), and Ferguson and Needham (1978) reported a marked interstitial nephritis in rainbow trout, with regularly round multinucleated cells at the focal areas of cellular reaction. These parasitic cells stained weakly eosinophilic and were surrounded by a clear halo. Cytoplasmic inclusion bodies were commonly observed within each cell, being uniformly round and of approximate diameter 4 µm. The cells appeared to be invariably associated with a mononuclear cell reaction, with cells intimately attached to the convoluted membrane, and were not observed in clinically normal fish (Ferguson and Adair 1977; Ferguson and Needham 1978; Ellis *et al.* 1985). Parasites were also seen in the spleen, liver, gill vasculature, pancreas, muscle, and intestinal submucosa (Ferguson and Adair 1977; Ferguson and Needham 1978; Kent and Hedrick 1985a).

Upon ultrastructural examination of the PKX cells, although regularly round in outline, the very prominent plasmalemma (40-70 nm thick) was seen to be plicated with small pseudopodia evident (Ferguson and Adair 1977; Ferguson and Needham 1978; Seagrave *et al.* 1980). Each nucleus was seen to consist of one or two dense nucleoli, each surrounded by a lucent nucleoplasm. Cytoplasmic contents included mitochondria, rough endoplasmic reticula and phagosomes. Large electron-dense membrane-bound inclusion bodies were noted, thought to be secondary lysosomes (Kent and Hedrick 1986). Cytoplasmic granularity was noted due to the presence of many small (0.1-0.2 µm) spherical electron-dense bodies (EDBs) which were present throughout the cytoplasm, but most profuse close to the plasma membrane (Ferguson and Adair 1977; Ferguson and Needham 1978; Seagrave *et al.* 1980; Morris, Adams and Richards 2000c). A distinctive membrane demarcated each body, and an electron-lucent band, extending half to two-thirds across the diameter divided the electron-dense ground substance.

In the case of EDBs that were located adjacent to the plasma membrane, the electron-lucent bar was invariably orientated at 90° to the membrane. In some cases, the EDB seemed to be fusing with the plasma membrane, perhaps forming an invagination, as the outer membrane appeared continuous with this bar in some cases (Ferguson and Needham 1978; Seagrave *et al.* 1980). Other EDBs apparently showed tail-like projections (90-400 nm long) from their own delimiting membranes (Morris *et al.* 2000c). Seagrave *et al.* (1980) compared the EDBs of PKX to haplosporosomes seen in the haplosporean genus *Marteilia*, although, the distinctive electron-lucent bar had not been described in the latter organisms (Hoffmann and El-Matbouli 1994). Comparison with sporoplasmosomes – electron-dense cytoplasmic bodies in myxosporeans – had also demonstrated morphological discrepancies (Morris *et al.* 2000c). Smith *et al.* (1984) observed that some EDBs were apparently exocytosed, with secretory substances evident outside the PKX cell, possibly being detected by host cells. EDBs were found to be produced by both the Golgi apparatus and a vesicular system (Smith *et al.* 1984). Subsequently, it was suggested that the previously described vesicular bodies represented the *trans*-Golgi face of the Golgi apparatus, with production of EDBs being witnessed at this site by Morris *et al*. (2000c).

While Morris *et al*. (2000c) recognised active entrapment by structures resembling multivesicular bodies, these authors did not observe exocytosis of any EDB contents. Instead, interaction with the plasmalemma was observed, leading to the suggestion that the function of the contents might be related to avoidance of the host immune response or reinforcement of the cell membrane. Appearing morphologically distinct from haplosporosomes (of haplosporeans) and sporoplasmosomes (of myxosporeans), EDBs similar to those seen in PKX have currently only been reported in the malacosporean *T. bryozoides* and an unclassified intracellular organism detected in carp, proposed to be an undescribed life stage of

Sphaerospora renicola (Voronin and Chernysheva 1993; Canning *et al.* 1996; Morris *et al.* 2000c).

Ferguson and Needham (1978) observed by electron microscopy the presence of secondary cells within the primary PKX cells. These internalised cells appeared more regularly electron-dense, containing a nucleus, prominent Golgi apparatus, mitochondria, rough endoplasmic reticula (although less than the primary cells), double cell membranes, numerous cytoplasmic ribosomes, and vacuoles (Ferguson and Needham 1978; Feist and Bucke 1987; Morris *et al.* 2000c). Ferguson and Needham (1978) reported an absence of EDBs, although Morris *et al.* (2000c) observed occasional EDBs present in endocytotic vacuoles in secondary PKX cells. Although one secondary cell per mother cell was more common, up to seven internalised cells have been reported (Seagrave *et al.* 1980; Kent *et al.* 2000). Tertiary cells were seen within a limited number of the secondary cells, possessing a delimiting plasmalemma, nucleus, and mitochondria, but no EDBs. Feist and Bucke (1987) observed bundles of microtubules within secondary and tertiary cells, apparently consistent with myxosporean generative stages.

Kent and Hedrick (1985a, 1986) described primary cells (morphologically identical to interstitial stages) in the renal tubular epithelium. Degeneration of primary cells was seen in the epithelium (between epithelial cells), and their daughter cells – surrounded by primary cell debris – migrated into the lumen of tubule. As with the interstitial cells, PAS-positive granules were seen in these stages. Morris *et al.* (1997) first described the observation of ostensible endocytosis of the primary cell by secondary cells, disintegration of primary cells, and resultant phagocytosis of cellular debris by host cells. Intraluminal forms have also been noted, consisting of intact primary cells containing secondary and tertiary cells, some of which were observed in various stages of degeneration (Kent and Hedrick 1986). Whereas macrophages

usually encircled interstitial and blood stages of PKX, such an association was not seen with the intraluminal stages.

Later in the course of infection, degeneration of PKX cells has been linked with simultaneous enlargement of cytoplasmic vacuoles leading to rupture of the cells (Seagrave *et al.* 1980; Kent and Hedrick 1986; MacConnell *et al.* 1989). It has been suggested that the vacuoles were related to lysosomes, with the development and eventual release of secondary cells from degraded primary cells leading to autophagocytosis of the cytoplasmic contents, and possibly acting as a nutritional source for the cells (Morris *et al.* 1997). Large numbers of EDBs have been observed being released into the kidney interstitium, following discharge of a secondary cell from a ruptured primary cell (Morris *et al.* 2000c). It is thought that the released secondary cells become the sporogonic stages of development in the renal tubule lumen.

Sporogonic stages

More complex intraluminal forms of PKX have been observed 2-3 weeks following the appearance of extrasporogonic stages, including multinucleated enveloping cells with electron-dense multilaminate bodies (Kent and Hedrick 1986; Morris *et al.* 1997). Kent and Hedrick (1985a, 1986) described advanced intraluminal cells, including developing myxosporean spores in the renal tubules of American salmonids suffering from PKD. The spores measured approximately 12×7 µm, each possessing a pair of spherical polar capsules (2 µm in diameter), with four coils at the anterior end of each spore (Kent *et al.* 2000). It was suggested that these stages represented an equivalent of myxosporean trophozoites; spores developing from secondary cells released into the renal tubules. Only one spore was observed developing in each cell, unlike the multinucleated plasmodia reported in many myxosporeans (Lom and Dyková 1992, 1995). The monosporous development was

more akin to that previously described in *Sphaerospora* spp., resulting in the formation of pseudoplasmodia.

Upon initial ultrastructural examination of PKX spores, polar capsule primordia in the form of capsulogenic cells were observed posterior to the capsules, although other myxosporean features, including valvogenic cells and sporoplasms could not be differentiated (Kent and Hedrick 1985a). Subsequently, apparently immature valvogenic cells were recognised, surrounding capsulogenic cells (Kent and Hedrick 1986; Kent *et al.* 2000). Upon comparison with myxosporeans, it was originally considered that the spores did not complete their development in the kidney (Kent and Hedrick 1985a, 1986). This was assumed due to the absence of formation of hard shell valves, and the finding that the spores remained within their pseudoplasmodia throughout the course of infection, unlike known members of the class Myxosporea. It has since been proposed that the small spores with vestigial indistinct valves may be the final development stage of PKX in fish, the protection of hard shells not being required if they are being quickly ingested by filter-feeding bryozoans after release in fish urine (Kent *et al.* 1998, 2000). Intraluminal trophozoites and spores have been observed in the distal portions of renal tubules, and although not observed directly in collecting ducts, ureters or the urinary bladder, spores resembling those of PKX have been recognised in the urine of infected fish (Kent and Hedrick 1986). This has been interpreted as suggesting that normally the spores might be either retained in the fish tissue, or expelled into the environment in short bursts (Morris *et al.* 1997; Hedrick, Baxa, de Kinkelin and Okamura 2004).

1.6.3. Morphological characteristics of *T. bryosalmonae* in Bryozoa

The morphological characteristics of *T. bryosalmonae* life stages were observed to differ markedly between their piscine and bryozoan hosts (Canning *et al.* 1999, 2000). *Tetracapsuloides bryosalmonae* spores were observed developing in

spherical free floating sacs (up to 350 µm in diameter) in the coelomic cavities of bryozoans. The margin of the sac consisted of a single layer of proliferative flattened mural cells joined by gap junctions. Internalised individual mural cells became denser in nature, forming sporogonic cells. These cells differentiated into either large (12 µm in length) irregular sporoplasmogenic cells or denser smaller stellate cells. The ratio of sporoplasmogenic : stellate cells was in the order of 1:8, following nuclear meiotic division the former developed into two sporoplasms, while the latter divided into four capsulogenic and four valvogenic cells. The eight former stellate cells, linked by septate junctions encircled the two sporoplasms. The capsulogenic cells grouped at one pole of the spore (~19 µm diameter), forming four spherical polar capsules (~1.7 µm diameter), with the structure being encased by four valve cells formed from the valvogenic precursors.

The pliable valve cells stretched to cover the sporoplasms and capsulogenic cells, but did not seal the exit points of polar filaments within the capsules. The two irregular sporoplasms were occasionally observed to come in contact with each other or capsulogenic cells by simple membrane apposition. Standard cytoplasmic organelles were seen within the sporoplasms, lipid vacuoles and mitochondria being particularly evident. While *T. bryosalmonae* extrasporogonic stages in fish and *T. bryozoides* spores in bryozoans included many cytoplasmic electron-dense spheres (Ferguson and Needham 1978; Canning *et al.* 1996), only very few characteristic EDBs – membrane-bound with electron-lucent bars – were witnessed in the sporoplasms of *T. bryosalmonae* stages in bryozoans (Canning *et al.* 1999, 2000).

At least one *T. bryosalmonae* sporoplasm from each spore has been observed forming a secondary cell. Within each spore-containing sac, development of the myxozoan life stages was seen diffusely throughout the population. Even at the point of maturation, when the mural cells lining the sac had completely degraded, no *T.*

bryosalmonae spores were found with the characteristic hardened valves seen in members of the class Myxosporea. This morphological finding was consistent with the supposition that the soft-shelled forms seen in salmonid renal tubules were mature spores (Kent *et al.* 1998).

Thus, several striking morphological differences have been noted between stages of *T. bryosalmonae* seen in fish and bryozoan hosts (Canning *et al.* 2000). Whereas observed mitochondria appear plate-like in fish hosts, they are tubular in bryozoans. During development of the polar capsules, external tubes were seen in fish but not bryozoans. In fish, two polar capsules were seen in each spore, whereas four were seen in each spore in bryozoans. Sporal cellular differentiation was poorly developed in fish, but in bryozoans well-defined capsulogenic cells, valve cells and sporoplasms were seen. It has been hypothesised that bryozoans are the true hosts for the tetracapsulids, and fish are aberrant hosts, although this has not been proven (Kent *et al.* 2000).

1.7. Life cycle

Experimental transmission of the disease was successfully achieved by intraperitoneally injecting fingerling trout with either blood or homogenised renal or splenic tissue from infected fish, resulting in development of clinical PKD (Clifton-Hadley, Richards and Bucke 1984b; D'Silva, Mulcahy and de Kinkelin 1984; Kent and Hedrick 1985b). Initial suspicion of a direct life cycle was not substantiated by the failure of transmission trials involving cohabitation (of naïve and infected fish), the feeding of infected renal tissue to uninfected fish and the contact of non-diseased fish with the excreta of diseased stock (Ferguson and Ball 1979; D'Silva *et al.* 1984). The morphological association of PKX with the myxosporeans led to investigation of the possibility of similarities in life cycle strategies (Kent and Hedrick 1985a, 1986).

The radical discovery that an actinosporean (*Triactinomyxon*) in the worm *Tubifex tubifex* was a required alternate life cycle stage for *Myxobolus cerebralis*, subsequently led to similar life cycle strategies being identified in more than 25 myxozoan species (Markiw and Wolf 1983; Kent *et al.* 2001). It was not until the discovery of *T. bryosalmonae* in bryozoan colonies, that an invertebrate host for this parasite was found (Anderson *et al.* 1999a). *Tetracapsuloides bryosalmonae* has been described in a range of phylactolaemate bryozoans, possibly cycling between different hosts depending on availability (Okamura *et al.* 2001; de Kinkelin, Gay and Forman 2002).

Although Kent *et al.* (2001) designated the bryozoan stage of *T. bryosalmonae* as an actinosporean phase, striking morphological and developmental discrepancies make this classification controversial. It has been proposed that there are probably no other hosts of *T. bryosalmonae* apart from bryozoans and fish (Okamura *et al.* 2001). However, although transmission has been successfully achieved from infected bryozoans to fish (Canning *et al.* 1999; Feist *et al.* 2001), the converse route has not been conclusively demonstrated despite some circumstantial evidence (Okamura *et al*. 2001; Morris, Morris and Adams 2002a, 2002b). Therefore, the possibility of additional hosts of *T. bryosalmonae* has been seen as an important area of interest. The observation of infected colonies of bryozoans in waters thought to be completely free of salmonids and pike, together with the immature spore development and extreme host reaction seen in fish hosts, has led to the suggestion that, as accidental hosts of *T. bryosalmonae*, salmonids may not be essential components of the life cycle (Okamura *et al.* 2001).

It has been shown that fish may become infected with *T. bryosalmonae* through contact with spores released from bryozoans, or from eating infected colonies (Okamura *et al.* 2001). Morris *et al.* (2000b) demonstrated the presence of *T.*

bryosalmonae cells in the gill arch of fish exposed to endemic waters three days previously. These cells were thought to represent initial developmental stages, not yet containing the characteristic daughter cells of the extrasporogonic forms. It was suggested that, in common with some descriptions of actinosporean spores, the gill represented one, but not the only route of entry of the parasite. Within seven days of the gill stages being examined, extrasporogonic cells (complete with secondary cells) were found in the kidney interstitium, the circulation identified as the most probable route of transport (Kent and Hedrick 1986; Morris *et al.* 2000b). Through experimental bath exposure of rainbow trout to homogenised infected bryozoan material, *T. bryosalmonae* cells have been identified by *in situ* hybridisation within mucous cells of the epidermis (Longshaw, Le Deuff, Harris and Feist 2002), confirming the skin as an additional entry portal.

Sporogonic stages of *T. bryosalmonae* have been seen in the renal tubules of infected fish 2-3 weeks after the development of extrasporogonic forms (Morris *et al.* 2000b). The sporogonic stages shared antigenic determinants of the secondary cells contained within the extrasporogonic stages (Morris *et al.* 1997). While most extrasporogonic stages were seen to be absent from the interstitium after four months post-exposure, sporogonic stages were seen to persist in the tissue for more than 12 weeks beyond pathological resolution (Kent and Hedrick 1986). In the USA, the formation of *T. bryosalmonae* spores has been detected in some salmonid species – including rainbow trout and Arctic charr, while in the UK the most developed stages were reported in brown trout, grayling and Atlantic salmon (Clifton-Hadley and Feist 1989; Kent *et al.* 1998, 2000; Morris *et al.* 2000a). As the only reported non-salmonid fish infected with *T. bryosalmonae*, the route of infection of the northern pike is unknown (Morris *et al.* 2000a). As a predatory fish, it could be suspected that infection followed ingestion of diseased salmonids, but this mode of transmission has not been demonstrated in salmonids (Bucke *et al.* 1991). Klontz

(1984) concluded that the involvement of predatory waterfowl was not likely to be implicated in the life cycle. The presence of *T. bryosalmonae* in systems known to be absent of pike suggests that the species is not a vital component of the life cycle (Morris *et al.* 2000a). It is unclear on what scale the development of the parasite may continue beyond infection of fish hosts, although limited numbers of spores have been observed in the urine of infected rainbow trout (Kent and Hedrick 1986).

Kent *et al.* (1986) concluded that the parasite most probably undergoes vegetative reproduction in the fish host, supported by the observation of numerous daughter cells, and the high infection levels seen following an initially low parasite burden. Nuclear meiotic divisions were observed during the development of two sporoplasms from each sporoplasmogenic cell during infection of bryozoans (Canning *et al.* 2000). As myxozoan stages have been found to be diploid, nuclear fusion would have to occur to restore the status. It was suggested that following infection of a new host, secondary cells would be released from sporoplasms and undergo fusion. Whereas, in the open fluid-filled coelomic cavities of bryozoans cross-fertilisation may occur, in the densely packed tissues of fish, the fusion of gametes originating from the same organism may be the only option. It was hypothesised that this self-fertilisation (autogamy) in fish may result in increased homozygosity – leading to greater expression of recessive genes, compromising the viability of the parasite in fish hosts.

Of the phylactolaemate bryozoans, *Fredericella* Allman, 1844 is the only genus known to survive as living colonies during temperate winters (Hyman 1959; Gay *et al.* 2001). Most phylactolaemate species produce statoblasts – either buoyant floatoblasts or submerged sessoblasts – which remain dormant over the colder months, and germinate in the spring. Myxozoan infections have been observed in colonies of *Fredericella sultana* Blumenbach during the winter months (Okamura *et*

al. 2001). It is not known whether *T. bryosalmonae* obligately parasitises members of this genus on a seasonal basis, or if statoblasts could become infected – allowing wide distribution due to their chitinous shells allowing safe passage through digestive tracts. The discovery that infective stages of *T. bryosalmonae* were present throughout the year, confirmed that the seasonality of the disease was not simply due to an absence of infectious challenge throughout the colder months (Gay *et al.* 2001).

To date, the life cycle of *T. bryosalmonae* has not been fully elucidated, as despite concerted efforts, exposure of bryozoans to material released from fish has never elicited observed *T. bryosalmonae* infection (Morris *et al.* 2002a; Tops, Baxa, McDowell, Hedrick and Okamura 2004). Kent and Hedrick (1986) suggested that *T. bryosalmonae* sporoblasts may be released in the urine of salmonids, while Morris *et al*. (2002b) provided molecular evidence for the release of *T. bryosalmonae* from brown trout and possible bryozoan infection. Uninfected bryozoan colonies placed in PKD-endemic waters were found subsequently to be PCR-positive for *T. bryosalmonae*, although no morphological development was witnessed, suggesting that a latent infection may have resulted (Tops and Okamura 2003). The recent observation of malacosporean-like spores in the urine of rainbow trout has renewed interest in trying to close this aspect of the life cycle (Hedrick *et al.* 2004).

1.8. Immune response of fish to *T. bryosalmonae*

With most studied myxosporean species, a well-established equilibrium has been noted between the host and parasite (Lom and Dyková 1992, 1995). However, the presence of parasitic organisms either in atypical body tissues or exotic host species could result in the manifestation of a vigorous tissue response. Such a profound cellular response has been noted in the kidney interstitium, focusing on the

extrasporogonic stages of *T. bryosalmonae* in fish tissue (Ferguson and Needham 1978; Clifton-Hadley *et al.* 1987a). The teleost cellular response observed with PKD has been compared to epithelioid granuloma formation seen in mammals following a cell-mediated delayed-type hypersensitivity reaction of T-lymphocytes (Ellis *et al.* 1985; Foott and Hedrick 1987). Compared with the tissue response in rainbow trout, a more developed cellular response was observed in the kidneys of brown trout and Atlantic salmon, including the presence of epithelioid cells, activated macrophages, and giant cells (Ellis *et al.* 1985). It was suggested that the observed lower incidence of PKD in the latter two species could be due to the production of a stronger more effective immune response by these fish.

From the earliest reports of confirmed outbreaks of PKD, it has become apparent that fish that have recovered from the disease might demonstrate an acquired immunity, proving resistant to subsequent challenge (Ferguson and Needham 1978; Ferguson and Ball 1979; Klontz *et al.* 1986). Ferguson and Ball (1979) remarked that protection of 1+ fish was not age-related, but due to a history of previous exposure to the pathogen. However, a low level exposure did not seem to be capable of causing such a defensive response, instead recovery from an active clinical infection seemed to be required (Hedrick *et al.* 1985; Clifton-Hadley *et al.* 1986b; Foott and Hedrick 1987). Also, environmental temperature seemed to play a crucial role. The presence of PKX for prolonged periods in stock kept in water at 9°C did not elicit subsequent protection, while those held at 12°C did acquire apparent resistance (Clifton-Hadley *et al.* 1986b). Whereas the progression of clinical disease has been noted to be intimately associated with water temperature, it has been proposed that although the speed of recovery might be enhanced by falling temperature, resolution of signs was not dependent upon it (Ferguson 1981).

Tetracapsuloides bryosalmonae is thought to be highly antigenic, although the precise mode of action of immune protection against PKD has not yet been established, with both cellular and humoral components being implicated (Hedrick *et al.* 1985; Klontz *et al.* 1986; MacConnell *et al.* 1989). The strong association of predominately mononuclear cellular proliferation associated with *T. bryosalmonae* in the renal interstitium has led observers to postulate that the cellular response might be a key immune component (MacConnell *et al.* 1989). Macrophages have been intimately associated with extrasporogonic parasitic cells, with lymphocytes also thought to play a crucial role (Ferguson and Needham 1978; Klontz *et al.* 1986; MacConnell *et al.* 1989). MacConnell *et al.* (1989) proposed that destruction of *T. bryosalmonae* cells might be reduced by parasite-produced substances inhibiting macrophage killing mechanisms by preventing the fusion of lysosomes or inhibiting lysosome production. It has been postulated that the EDBs of primary *T. bryosalmonae* cells may play a role in a rescue mechanism of the parasite, allowing it to survive hostile host cell reaction (Angelidis, Baudin-Laurencin, Quentel and Youinou 1987; MacConnell *et al.* 1989). An active interaction with the cellular immune response could be supported by observations of intimate contact between phagocytes and parasites at areas of cellular surface interdigitation (Morris *et al.* 2000c).

Hedrick *et al.* (1985) suggested that a strong component of protection lay in the serum fraction (presumably including immunoglobulins), having observed that passive transfer of serum from convalescing fish to actively infected specimens led to reduced incidence and severity of disease. At seven weeks post-exposure, increased numbers of antibody-producing plasma cells have been observed, and PKD-infected trout have been shown to have immunoglobulin (IgM-like) levels of 34% of the serum total protein concentration (*cf.* 5-6% in normal fish) (Olesen and Vestergård Jørgensen 1986; MacConnell *et al.* 1989). Infected fish have shown a

progressive increase in the β-globulin portion of serum proteins (Klontz 1984; Klontz *et al.* 1986).

It has long been suspected that much of the mortality associated with PKD resulted from pathology following secondary infection by bacteria, viruses, fungi, protozoa, and metazoa (Alderman and Feist 1985; Klontz *et al.* 1986; Hoffmann and El-Matbouli 1994). A study involving an assessment of the response to vibriosis vaccine in PKD-infected fish demonstrated lowered phagocytic reactions, although the production of antibodies targeting the bacteria was not diminished (Angelidis *et al.* 1987). It was suggested that the parasite was not highly pathogenic, but that a possible rescue mechanism – comprising of immunosuppressive toxins inhibiting macrophage functions, and competition between parasite cells and phagocytes for glucose – could diminish cellular immune pathways. Conversely, Foott and Hedrick (1990) suggested that PKD-infected fish demonstrated greater resistance to bacterial challenge with heightened non-specific and specific defences. It was hypothesised that PKD-induced immunosuppression would only develop as a sequel to serious infections which resulted in the development of severe renal lesions. It was suggested that subclinical PKD infections could actually lead to an enhancement of non-specific immune responses, while advanced disease could lead to immunosuppression, as previously documented. However, Holland, Gould, Jones, Noble and Secombes (2003) demonstrated that specific immune responses were affected at a molecular level during infection of rainbow trout with PKD, resulting in cytokine down-regulation and immunosuppression.

Long-term exposure between pathogens and hosts has resulted in apparent evolutionary development of resistance of hosts. Evidence of resistant strains of salmonids to PKD is limited. Ferguson and Needham (1978) recorded that, despite sharing the same water supply as rainbow trout suffering over 75% mortalities from

PKD, native stocks of Atlantic salmon did not show signs of disease. Ellis *et al.* (1985) noted that although not entirely refractive to infection, apparent improved resistance of Scottish Atlantic salmon parr was seen compared with Norwegian imports. However, it could not be discerned if the differences were at a genetic level, or due to other factors such as history of exposure. Ostensible resistance of a population of indigenous American *Salmo salar ouananiche* to infectious stages which caused disease in Arctic charr, was suggested to be resultant of genetic resistance development following exposure of the strain to the pathogen (Brown *et al.* 1991). Currently, the use of resistant salmonid strains has been not been successfully implemented in routine prevention or control of PKD.

1.9. Diagnostic techniques

Initially, a putative diagnosis of PKD could be reached on the basis of the clinical history – especially the season of the year and previous outbreaks at the location, alongside characteristic clinical signs and gross pathological changes (Clifton-Hadley and Richards 1983). However, these factors are not pathognomonic for PKD; therefore further measures are necessary to confirm the diagnosis. A number of techniques, including histopathological preparations, immunological and molecular methods have been developed.

Comprehensive descriptions of the histopathological changes associated with PKD have allowed experienced operators examining standard haematoxylin and eosin stained sections to reach a positive diagnosis by identifying extrasporogonic stages in fish suffering from moderate to severe disease (Ferguson and Needham 1978; Clifton-Hadley *et al.* 1987a). The development of a diagnostic technique using kidney impression smears (stained by methylene blue, May-Grünwald-Giemsa or Leishman-Giemsa procedures) greatly reduced the time and labour involved in reaching a

diagnosis, also allowing the detection of individuals suffering from pre-clinical or mild disease (Klontz and Chacko 1983; Clifton-Hadley and Richards 1983). Extrasporogonic and sporogonic stages were identified from wet mounts made from preparations of fresh kidney, under light microscopy using bright field and phase contrast techniques (Klontz and Chacko 1983; Kent and Hedrick 1986). However, these techniques demanded a subjective interpretation on the part of an operator; therefore, more specific techniques were required for routine diagnostic use.

Although the well described ultrastructural features of *T. bryosalmonae*, in particular the characteristic cytoplasmic EDBs of the extrasporogonic primary cells, with their distinctive electron-lucent bands allowed confirmatory diagnosis (Ferguson and Needham 1978; Castagnaro, Marìn, Ghittino and Hedrick 1991; Morris *et al.* 2000c), the development of novel diagnostic techniques proved invaluable in both diagnostic and research applications. Castagnaro *et al.* (1991) first documented the use of lectin-based histochemistry in studies of PKD. Lectins, first described from plants, are sugar-binding proteins and glycoproteins that have been used in the study of various cell populations, having the ability to distinguish accurately a wide range of carbohydrate structures on the surface of cells (Hedrick *et al.* 1992). It was found that various lectins exhibited specific binding patterns to the parasite and surrounding cells, with GS-1 showing apparent specific staining of *T. bryosalmonae* interstitial and intraluminal stages, recognising carbohydrate residues of glycoconjugates. A rapid lectin-based diagnostic test was developed using fixed kidney imprints, allowing identification of early development stages in pre-clinical cases (Hedrick *et al.* 1992). Although initial reports suggested that GS-1 was specific for *T. bryosalmonae*, not cross-reacting with a concurrently present *Sphaerospora* sp. (Hedrick *et al.* 1992), subsequent studies of *Sphaerospora oncorhynchi* showed staining with GS-1 of these myxosporean spores in the renal tubules of sockeye salmon (Kent *et al.* 1993a, 1993b), although these findings were not later fully

replicated (Marìn de Mateo *et al.* 1996). More recently, multiple carbohydrate terminals on *T. bryosalmonae* have been identified using a panel of 21 lectins (Morris and Adams 2004).

Monoclonal antibodies (MAbs) have been used widely as diagnostic and investigative probes in the study of various fish diseases (Adams, Thompson, Morris, Farias and Chen 1995). The first successful attempt at producing MAbs to *T. bryosalmonae* resulted in two lines targeting the parasite: MAb 12 appeared specific, while MAb 18 also cross-reacted with host cell antigens in the kidney tubules (Adams, Richards and Marìn de Mateo 1992). The MAb 12 was later shown to bind to both extrasporogonic and sporogonic stages (Marìn de Mateo, Adams, Richards, Castagnaro and Hedrick 1993). Saulnier and de Kinkelin (1996) produced polyclonal antibodies and a panel of 11 MAbs showing apparent specificity for *T. bryosalmonae*, demonstrating varying degrees of assay restriction. Using these MAbs, an external parasitic antigen (13 kDa) of suspected immunological importance was detected. Morris *et al.* (1997) observed a changing antigenic profile of the parasite throughout its developmental stages, using a panel of four MAbs: A3, B4, C5 and D4. While B4 was specific for extrasporogonic secondary cells and sporogonic stages, the remaining three MAbs bound to vacuoles within the extrasporogonic parasites. The MAb B4 was shown not to be specific to *T. bryosalmonae*, targeting an antigen expressed on sporogonic stages from a range of *Sphaerospora* spp. (Marìn de Mateo *et al.* 1996). Two MAbs (A8 and B4) that bound to secondary *T. bryosalmonae* cells were found to cross-react with *Myxobolus* sp., from rohu, *Labeo rohita* Hamilton from Thailand (Petchsupa, Morris, O'Flynn and Adams 1999).

Molecular techniques have allowed the production of diagnostic probes of high sensitivity and specificity. Saulnier and de Kinkelin (1997) used four primers deduced

from the SSU RNA gene of *T. bryosalmonae* to conduct PCR analysis. It was shown that a nested PCR technique, involving an amplifying second round, afforded a high level of sensitivity, constituting an accurate rapid diagnostic assay for PKD. Kent, Khattra, Hervio and Devlin (1998) conducted rDNA sequence analysis of the parasite, demonstrating a homology between geographically isolated strains, but a contrast between *T. bryosalmonae* and the Myxosporea. Morris *et al.* (2000a) recommended the use of the primer sets 3F, 4R and 5F, 6R developed by Kent *et al*. (1998), stating that these facilitated easy rapid specific probes for diagnosis and life cycle investigations of *T. bryosalmonae*. Morris, Morris and Adams (2002c) described an optimised PCR procedure which reduced the incidence of false positive and negative results. Using *in situ* hybridisation, Morris *et al.* (2000b) examined 12 probes, finding four of them specific for *T. bryosalmonae* in tissue sections. A rapid *in situ* hybridisation technique was developed, dramatically lowering the processing time involved from two days to five hours. Using 18 SSU rDNA analyses, Anderson *et al.* (1999a) further elucidated the life cycle of *T. bryosalmonae* by demonstrating its presence in bryozoans. Therefore, molecular techniques have allowed increased specificity and sensitivity in the identification of *T. bryosalmonae*, although higher economic costs compared to traditional assays must be considered.

1.10. Treatment and control

Proliferative kidney disease has been found to be of great economic significance to the trout farming industry. In the UK alone, losses are estimated at £2.5 million per annum (Feist 2004). Apart from losses attributable to mortality, husbandry efficiency and management costs were noted to be compromised (Clifton-Hadley *et al.* 1986a). Poor food conversion ratios (~2.8:1 *cf.* target of 1.5:1), decreased feeding rates (to lower the oxygen demand of anaemic stock), reduced efficiency of labour and facilities (due to remedial measures), lowered carcass quality (affected product must

be gutted to remove kidney tissue, with severely affected carcasses condemned), would all lead to financial losses. Therefore, the development of effective control measures has been an area of intensive research.

The infectivity of *T. bryosalmonae* spores has been shown to be relatively short-lived in the water conditions common in field situations (de Kinkelin *et al.* 2002), leading to the suggestion that the impact of PKD on trout farms could be lowered by reducing the abundance of bryozoans in their inlet rivers. Previous to the aetiology of PKD being elucidated, a number of chemotherapeutants, such as antibiotic and anti-protozoal preparations were tested to no avail (Ferguson and Ball 1979). Preliminary investigations revealed that reducing the rate of feeding of stocks could decrease mortality rates, although not affecting morbidity (Ferguson and Needham 1978; Ferguson and Ball 1979). Ferguson and Ball (1979) also concluded that the season of the year and previous exposure to infective waters were of significance in the incidence of disease. Ferguson (1981) further concluded that the course of disease was significantly enhanced by increases in water temperature. It was shown experimentally that fish exposed to endemic water, subsequently kept at low temperature (5-7°C) failed to develop disease. On affected farms it was discovered that delaying exposure of first season stock to endemic water until July prevented development, presumably due to either a reduced infectious challenge or an insufficient time period at a permissive temperature (Ferguson and Ball 1979). Thus, a preliminary crude means of disease control was found.

Postponement of transfer of fish to infected water until July was implemented as a control measure in Ireland and England (Bucke *et al.* 1981). However, in the warmer English rivers, marked reductions in growth rate due to PKD were still encountered. It was also noted that mortality levels increased with the degree of intensification of production. During the peak season of disease, it was advocated that husbandry

stressors should be minimised, entailing reduced feeding and handling of stock (O'Flynn and Mulcahy 1995). Clifton-Hadley *et al.* (1986b) described apparent resistance following an experimental regime involving maintaining exposed fish in water at 12°C for one year, although this protocol was not feasible on commercial units. Modification of the method led to fish being exposed to endemic waters at 10-11°C, developing apparent resistance to PKD after four months (1300 degree-days) (de Kinkelin and Loriot 2001). It was suggested that further research into the minimum number of degree-days conferring immunity, could allow formulation of a practical control method for use in farms.

O'Hara (1985) proposed the use of saltwater (10–12‰) in the post-infective period, not only treating secondary pathogens, but also reducing osmotic stress on fish with compromised renal function. However, PKD outbreaks have been recorded in chinook salmon following transfer of infected smolts to sea water (Hedrick and Aronstien 1987; Kent *et al.* 1995b). Although secondary pathogen numbers were diminished, disease signs of PKD were seen for several weeks post-transfer (Hedrick and Aronstien 1987). A concurrent extremely low migratory return rate was seen in the salmon, possibly related to osmoregulatory imbalance and anaemia due to PKD-induced diminished renal function leading to marine mortalities (Kent *et al.* 1995b).

With such an economically important disease, much research focused on the potential of discovering an efficacious chemotherapeutant, initially with little success (Ferguson and Ball 1979; Bucke *et al.* 1981). Clifton-Hadley and Alderman (1987) described how the control of the external ciliate *I. multifiliis* – using a flush treatment of malachite green and formalin – coincidentally resulted in a slower development of PKD in a group of subclinically infected fish. It was shown that used individually, formalin could exacerbate PKD, while malachite green – an arylmethane dye –

ameliorated disease signs. However, in part due to the slow pH-dependent formation of equilibrium of malachite green with water, it proved difficult to measure uptake by fish, with tissue accumulation of dye, hepatic and gill toxicity resulting during clinical trials (Alderman and Clifton-Hadley 1988; Gerundo, Alderman, Clifton-Hadley and Feist 1991). It was shown that development of *T. bryosalmonae*, and consequent PKD, was diminished by treating exposed fish with malachite green once a week for a month following initial detection of kidney swelling (Alderman and Clifton-Hadley 1988; Clifton-Hadley and Alderman 1988). However, anxiety over environmental impacts due to the use of malachite green, with carcinogenic and embryotoxic concerns, have led to restriction of use of the treatment (le Gouvello, Pobel, Richards and Gould 1999).

Molnár, Baska and Székely (1987) noted a reduction in disease attributed to *Sphaerospora renicola* in common carp, *Cyprinus carpio* L. following oral treatment with fumagillin DCH. This antibiotic, produced by *Aspergillus fumigatus* Fresen, 1863 was known to be efficacious in the treatment of microsporidian diseases, including *Nosema apis* Zander, 1909 in honey bees, *Apis mellifica* L., and *Pleistophora* spp. Gurley, 1893 in eels, *Anguilla japonica* Temminck and Schlegel, and medical conditions including amoebiasis and cancer (Hedrick, Groff, Foley and McDowell 1988; Wishkovsky, Groff, Lauren, Toth and Hedrick 1990; le Gouvello *et al.* 1999). The mode of action has not been determined, although it is thought to preferentially affect DNA or RNA synthesis of parasites, perhaps affecting spore membranes. Hedrick *et al.* (1988) demonstrated effective elimination of *T. bryosalmonae*, using an oral preparation of fumagillin in experimentally infected chinook salmon. Toxicity at high doses was found to lead to anorexia or mortality due to splenic and renal haematopoietic tissue depletion (Wishkovsky *et al.* 1990).

However, regimes involving reduced doses of fumagillin and restricted feed intake were shown to lead to relatively successful control of disease (Hedrick *et al.* 1988; Wishkovsky *et al.* 1990). Due to apparent increased susceptibility to secondary pathogens following treatment with fumagillin, le Gouvello *et al.* (1999) emphasised the importance of minimising stressors post-treatment, and not treating fish affected by clinical intercurrent disease. An apparently successful protocol was described involving daily oral medication of rainbow trout at 3 mg kg BW^{-1} day^{-1}. Higgins and Kent (1998) described the use of TNP-470, a synthetic analogue of fumagillin, used in the control of an array of microsporidian infections in mammals and teleosts. Initial promising results were seen in using TNP-470 in sockeye salmon for control of PKD, with reduced parasitic burden and alleviation of clinicopathological signs. Morris *et al.* (2003a) demonstrated that rainbow trout treated with the drug for 14 consecutive days were immunocompromised in being unable to combat opportunistic bacterial pathogens. Although fish treated with TNP-470 were vulnerable to re-infection with *T. bryosalmonae*, it was postulated that delaying the onset of PKD until the later part of the season could allow natural immunity to develop, conferring future resistance to the stocks.

1.11. Aims and objectives

Past research into PKD has led to the accumulation of a wealth of knowledge regarding the aetiology, epidemiology, pathogenesis and pathology of the disease. Although much recent progress has been made in studying the life cycle of *T. bryosalmonae* and treatment of PKD, further investigation of these aspects could result in advances in control of the disease. The primary aim of the project was to assess the feasibility of laboratory maintenance of large numbers of bryozoan colonies infected with *T. bryosalmonae* from which a vaccine preparation could be

derived. It was also hoped to further knowledge of malacosporean biology and to test novel treatments for PKD. The main objectives are summarised below.

- Studies into optimising the laboratory culture of phylactolaemate bryozoans, leading to a potential reliable experimental source of *T. bryosalmonae*.

- Research into the biology of phylactolaemate colonies, with particular reference to malacosporean infections.

- Development of robust challenge methods for infecting rainbow trout with *T. bryosalmonae*.

- Assessment of the efficacy of crude vaccination preparations based on attenuated *T. bryosalmonae* material in conferring resistance to rainbow trout. Development of experimental vaccines expressing TF (Thomsen-Friedenreich) and Tn antigens, with experimental trials conducted to gauge the protective effect of these preparations on naïve rainbow trout subsequently exposed to *T. bryosalmonae*.

- Efficacy testing of potential chemotherapeutants against PKD.

Chapter 2: Collection and maintenance of freshwater bryozoans (Bryozoa: Phylactolaemata)

2.1. Introduction

The class Phylactolaemata within the phylum Bryozoa comprises freshwater microscopic sessile colonial coelomates known colloquially as moss animals (Hyman 1959). They are eurythermic - being distributed throughout the world. These filter-feeding creatures are ubiquitous, being found attached to submerged substrates in many waterways where excessive proliferation can lead to fouling of aquatic equipment and pipes (Jónasson 1963) and are particularly prevalent in lakes, ponds and streams (Wood 1989). Phylactolaemate bryozoans are hermaphrodites with gonads present attached to the peritoneum. Typically, bryozoans develop asexually from germination of a statoblast or from a progenitor which was produced from the metamorphosis of a sexually derived larva. The statoblasts represent asexual propagules which are produced in the autumn, survive the winter and germinate in the spring to result in formation of novel colonies (Mukai 1974). The colony comprises individuals known as zooids which consist of a ciliated tentacular crown (lophophore) and a trunk attached to the body wall (Figure 2.1). The lophophore is horseshoe-shaped, except in the case of the genus *Fredericella* in which it is almost circular. Individuals of phylactolaemate colonies are connected by a common fluid-filled hollow space known as the coelomic cavity or metacoel.

Phylactolaemates possess a well-defined digestive tract, with food material passing the protrusive epistome as a result of water currents produced by the ciliated tentacles (Figure 2.1) *en route* to entering the rounded or oval mouth. Distal to this is a short ciliated pharynx leading to a long non-ciliated oesophagus separated from the stomach by a stricture known as the cardiac valve. The stomach consists of the cardia, the blind-ended caecum and the pylorus leading to the intestine which tapers towards the anus. Retractor muscles of the lophophore are capable of withdrawing the tentacular crown to within the limits of the body wall.

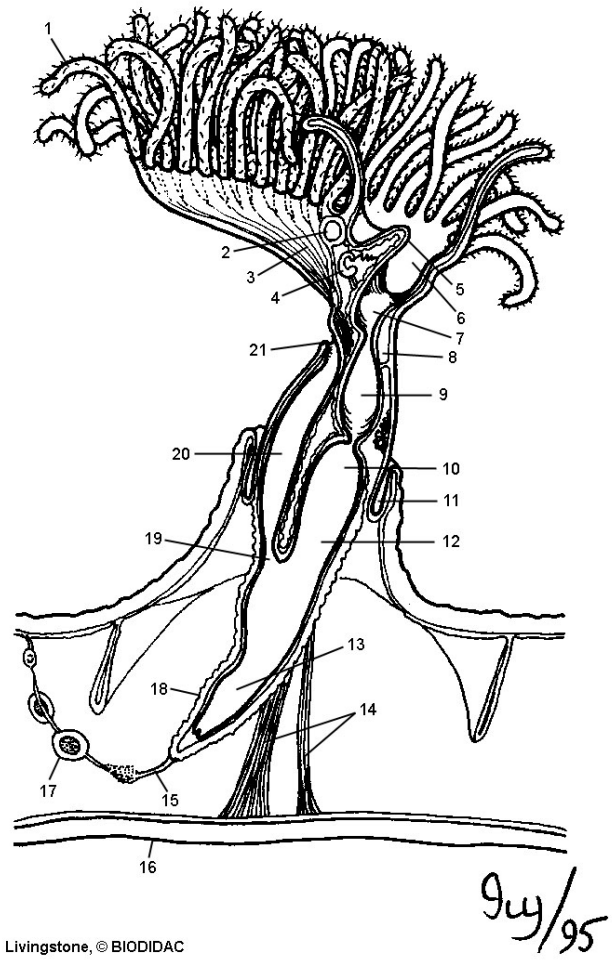

Figure 2.1: Representation of a sagittal section of a phylactolaemate zooid

(after Livingstone, used with permission)

1 = ciliated tentacle, 2 = sac of forked canal, 3 = ridge of lophophore, 4 = ganglion, 5 = epistome, 6 = mouth, 7 = pharynx, 8 = mesocoel, 9 = oesophagus, 10 = cardia, 11 = invaginated fold of body wall, 12 = stomach, 13 = caecum, 14 = retractor muscles of lophophore, 15 = funiculus, 16 = body wall, 17 = developing statoblast, 18 = peritoneum, 19 = pylorus, 20 = intestine, 21 = anus

Both an affinity for shaded unpolluted locations, and an aversion to swift-moving waters (due to waves or river currents) have been observed (Jónasson 1963). Populations have been seen to appear or disappear very rapidly, often with no obvious cause. Phytoplankton makes up their most important food source. Various parasites and predators have been associated with bryozoan colonies, including chironomid larvae, flat worms, naid annelids, snails, microsporidians, and myxozoans, some proving highly damaging to their hosts (Hyman 1959; Canning, Okamura and Curry 1997; Anderson *et al.* 1999a).

Despite the recognition of parasites of the Phylactolaemata for over 100 years (Allman 1856), relatively little research has been conducted into laboratory maintenance systems for these organisms (Morris *et al.* 2002a). The more recent discovery of the relationship between a malacosporean parasite of Bryozoa and the economically important salmonid PKD (Canning *et al.* 1999) has accentuated interest in developing robust controlled culture systems for freshwater bryozoans and thus potentially maintaining malacosporean parasites *in vivo* (Morris and Morris 2001).

The objectives of this study were:

- Collection of bryozoan statoblasts from a site known to be free of PKD and their germination in the laboratory, and collection of mature bryozoan colonies from a PKD-endemic site.

- Maintenance of the resultant bryozoan colonies in the laboratory and feeding trials using monocultures of algae and protozoa.

- To obtain a source of *T. bryosalmonae* infection by exposing naïve bryozoans to PKD-endemic waters.

- Development of a low maintenance bryozoan culture system which would potentially allow long-term culture of bryozoans without major inputs of labour.

Success with these objectives could potentially allow a source of *T. bryosalmonae* to be kept within a controlled research scenario indefinitely.

2.2. Materials and Methods

2.2.1. Collection and germination of bryozoan statoblasts

In March 2002, submerged branches and other material such as waste crisp packets were removed from a freshwater lake known as Airthrey Loch, Stirling, Scotland (56° 08' 52" North, 3° 55' 28" West). PKD has never been identified in any fish examined from this source (D.J. Morris, personal communication). In the laboratory, the surfaces of the material were disturbed by rubbing them by hand above a 500 ml beaker of distilled water. Buoyant statoblasts (floatoblasts) were released and observed on the surface of the bath. These were than pipetted under 9 cm plastic Petri dishes floating in a tank of Chalkley's medium (CM, Appendix 1). They were left undisturbed at 21°C for 3 days before being regularly observed using an inverted microscope (Olympus CK2). After hatching, the bryozoan colonies were maintained by suspending the Petri dishes vertically within test-tube racks (Nalgene) in well-aerated 8 L plastic aquaria filled with CM maintained thermostatically at 21°C. The bryozoans were recognised morphologically as belonging to the genus *Plumatella* Lamarck following published identification keys (Mundy and Thorpe 1980).

Subsequently, colonies of bryozoans were removed from the River Cerne in Dorset, England (50° 47' 22" North, 2° 28' 15" West), returned to the laboratory in sealed 5 L tupperware containers (Stewart) filled with river water aerated with battery-powered air pumps (Hagen). They were identified by colony and statoblast morphology as *Fredericella sultana*.

2.2.2. Initial laboratory maintenance of bryozoan colonies

Following germination of the statoblasts, the culture medium in the aquaria was changed twice weekly, being replenished with freshly made CM. Nutritional supplementation was provided daily using the protocol of Morris *et al*. (2002a).

Sterile six litre round-bottomed flasks were filled with Jaworski's medium (JM) and individually seeded with monocultures of *Synechococcus leopoliensis* Komárek, *Cryptomonas ovata* Ehrenberg, or *Pediastrum boryanum* Myen obtained from a commercial supplier (Sciento, Manchester, UK). The flasks were sealed with sterile rubber bungs penetrated with sterile 10 ml pipettes, connected to a compressed air supply allowing vigorous aeration. The flasks were kept adjacent to fluorescent strip lights which were lit for 16 hours daily. Approximately 200 ml of each algal culture was added to the bryozoan culture tanks on every second day. In addition, the tanks were initially seeded with 10 ml each of cultures of *Chilomonas paramecium* Ehrenberg and *Colpidium striatum* Stokes to allow protozoal proliferation in the culture medium.

To increase the number of Petri dishes with adhered *Plumatella* sp., areas of Petri dish surface including attached distinct colonies were excised from their original location using a scalpel blade. The resulting fragments of plastic were then individually attached to separate fresh Petri dishes using cyanoacrylate (Loctite 401 Instant Adhesive, Loctite Ltd., Welwyn Garden City, Herts.).

To optimise space utilisation within the culture tanks, specially designed rack systems were constructed to hold the maximum number of Petri dishes vertically orientated (Figure 2.2). Each rack measured 18 × 12 × 8 cm, being made of PVC sheets (Brett Martin) incorporating a stainless steel frame weight, with supporting stays made of bent stainless steel wire (028 welding wire) for eight Petri dishes.

2.2.3. Feeding trials of bryozoans

2.2.3.1. Ingestion of protozoa and algae by bryozoans

Colonies of *Plumatella sp.* and *F. sultana* as described in Section 2.2.1 were examined to assess their ingestion of different organisms. The monocultures of

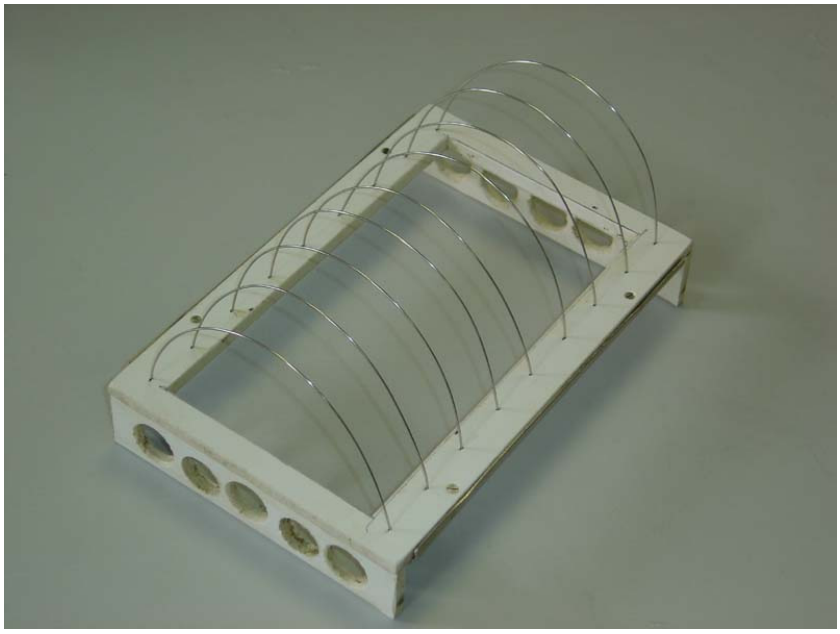

Figure 2.2: Rack for holding Petri dishes vertically in bryozoan culture systems

Figure 2.3 Apparatus for maintaining bryozoan colonies in waterways

different species of protozoa and algae listed in Table 2.1 and Table 2.2 were obtained from a commercial supplier (Sciento). In preparation for the trial, the bryozoans were starved for 12 hours to allow their intestines to be purged of previous ingesta. Individual monocultures were added drop-wise to the Petri dishes containing the bryozoans. Examination for signs of ingestion was conducted using an inverted microscope with a classification made according to the legend of Table 2.1. The Petri dishes containing *F. sultana* were then returned to their culture tanks, while those containing *Plumatella* sp. were covered and left undisturbed sitting flat on a laboratory bench at 21°C for 24-48 hours. As the feeding trial of *F. sultana* was conducted subsequent to that of *Plumatella* sp., cultures found to be entirely unsuitable as bryozoan food due to excessive size or filamentous morphology were excluded from the later trial.

2.2.3.2. Digestion of protozoa and algae by *Plumatella* sp.

Examination of faecal pellets produced by *Plumatella* sp. allowed information to be inferred on the nutritional value of those species which were ingested. The results were classified using the criteria of the legend of Table 2.1. It did not prove feasible to conduct this analysis on the colonies of *F. sultana* due to paucity of examinable bryozoan material and concerns about potential pathological effects due to restriction of the diet. Following the incubation period with the monocultures, bryozoan faecal pellets were pipetted from the media in the Petri dishes. Each was placed on to a microscope slide (Surgipath) and squashed under a glass cover slip to release the contents which were examined under an inverted microscope.

2.2.4. Long-term laboratory maintenance of bryozoans

Following the feeding trials, the bryozoans were maintained in 8 L aquaria filled with culture media supplemented with species of protozoa and algae of putative nutritional value. These included *Synechococcus leopoliensis*, *Cryptomonas ovata*,

Chlorococcum hypnosporum Starr, *Botrydium granulatum* (L.) Greville, *Eudorina elegans* Ehrenberg, *Gloeocapsa* sp. Kützing and *Pediastrum boryanum*. To each of the 8 L culture tanks, 250 ml of each of the monocultures was added on every second day; the pH of the media was measured and corrected to within the range pH 7-7.5 as necessary by drop-wise addition of 12M HCl or 5M NaOH. The bryozoan colonies were examined daily to monitor vitality. Bryozoan colonies were maintained for periods of one month each at either 15°C, 18°C or 21°C to determine the optimum temperature for growth of each species. Macroinvertebrates accidentally introduced with the bryozoans were removed with forceps from the Petri dishes twice weekly.

2.2.5. Exposure of laboratory-reared bryozoans to PKD-endemic waters

Colonies of *Plumatella* sp. which had developed from statoblasts collected from a location known to be free of PKD were maintained as described in Sections 2.2.1 and 2.2.2. The Petri dishes with the attached colonies were placed in sealed aerated 5 L tupperware containers filled with CM and transported at the end of June 2002 to sites in southern England known to be endemic for PKD. Upon arrival at each site, Petri dishes were attached by means of Velcro tabs (Heavy Duty Tabs, Velcro) to the specially designed apparatus described below to allow long-term placement in the water bodies.

Two devices were placed in the River Itchen, Hampshire, (51° 05' 33" North, 1° 13' 50" West), four were placed in the River Test, Hampshire, (51° 00' 11" North, 1° 30' 09" West) three in the River Avon, Wiltshire, (51° 00' 09" North, 1° 44' 41" West) and two in the River Cerne. Each device consisted of four sheets of 30 cm^2 6 mm thick PVC (Foam Lux, Brett Martin) attached by pipe clips to a central pole comprised of a one metre long PVC waste pipe (Polypipe, Paragon) of diameter 40 mm (Figure 2.3). Following attachment of four Petri dishes to each sheet, the

apparatus was initially submerged with the dishes orientated vertically to prevent trapping of air under the dishes and then was positioned with the bottom of the pipe being firmly pushed into the sediment in a rotary fashion to anchor the device. The top of the pole was left exposed to allow future location of the apparatus.

The devices were removed from the waterways six weeks after placement. The Petri dishes were removed, placed in sealed tupperware boxes filled with river water from their placement site and returned to the laboratory. They were placed in aquaria and maintained for two months as described in Section 2.2.4 with regular observation under an inverted microscope for signs of malacosporean parasitism.

2.2.6. Culture of algae from a PKD-endemic river

Water samples from the River Cerne were collected into sterile universal tubes. These were kept on ice until being returned to the laboratory. The samples were then added to 2 L conical flasks filled with sterile JM and incubated with constant aeration under fluorescent lighting. After 10 days, samples of the media were examined and any observed algae were speciated using a published identification key (Bellinger 1992).

2.2.7. Development of a low maintenance culture system for bryozoans

Due to the high inputs of labour necessary to rear bryozoan colonies as described in Section 2.2.4 it was decided to attempt to develop a system requiring less frequent and involved routine maintenance.

2.2.7.1. Version 1

A 150 L glass aquarium was filled with JM within a heated room of the Aquatic Research Facility (ARF), University of Stirling. The culture medium was vigorously aerated by an air stone and constantly mixed by means of a water pump (Blagden

Minipond, Sicce). Above the tank, a 90 cm long 30 Watt (W) fluorescent strip light (Freshwater light, Arcadia) was suspended and illuminated for 14 hours daily. At the opposite end of the tank from the water pump, to provide shading, a platform of dimensions 15 cm^2 of 6 mm thick PVC was placed over a rack (Figure 2.4) of Petri dishes with attached *Plumatella* sp. Before bryozoans were added to the system it was seeded with the species of protozoa and algae described in Section 2.2.4 and allowed to mature until there was an appreciable green hue. Periodically, depending on the condition of the media, a 50% media change was carried out using freshly made JM.

2.2.7.2. Version 2

Once again, the 150 L aquarium filled with JM, fluorescently lit, well aerated and agitated with a water pump (Figure 2.5). However, on this occasion the bryozoans were placed in a separate 8 L plastic tank alongside the main aquarium. Circulation between the two aquaria was provided by a water pump in the small tank which led via plastic tubing to the large aquarium, with return of fluid via a passive siphon line. The large aquarium was seeded and maintained as mentioned in Section 2.2.7.1 before *Plumatella* colonies were added to the smaller tank.

2.2.7.3. Version 3

On this occasion the 8 L plastic tank was aerated by an air stone and placed above the main 150 L aquarium (Figure 2.6). The main tank was once again filled with JM, fluorescently lit, aerated and fluid was pumped via tubing to the small tank where it was distributed through a spray bar. The fluid returned to the main tank via an overflow pipe embedded in the side wall of the smaller tank being released through a spray bar. Both tanks were now made impenetrable to winged insects by the addition of tight-fitting lids and sheets of 100 µm nylon mesh placed between the spray bars and the surfaces of the tanks. The large aquarium was seeded and maintained as

mentioned in Section 2.2.7.1 before *Plumatella* colonies were added to the smaller tank. Every two weeks, one third of the volume of the tank was drained and replaced with freshly made JM.

2.2.7.4. Version 4

The main tank was replaced in this design with a 25 L plastic aquarium featuring a central vertical overflow pipe (Figure 2.7), and the system was relocated to a section of the ARF aquarium system where there was a supply of chilled water. This main culture tank was filled with JM, aerated by an air stone and lit from the back by the 30 W fluorescent tube. An 80 L lidded drum was filled with JM and placed on the floor adjacent to the design. The JM was supplied to the main tank from the drum via a peristaltic pump modulated by a 24 hour timer at a rate of 1 L day^{-1}. Fluid was pumped from the lower to upper 8 L tank via plastic tubing, being released via a spray bar. Overflow from the upper tank returned to the lower tank via tubing and spray bar. The tanks were both fitted with lids, with any gaps being filled with either insulating tape or 100 µm nylon mesh. The upper tank was enclosed by a water sleeve comprising a 20 L polystyrene tank into which water at 15°C was constantly infused, with overflow water draining away passively.

The lower aquarium was seeded and maintained as described in Section 2.2.7.1 before *Plumatella* and *F. sultana* colonies were added to the upper tank. A duplicate 25 L tank was set up alongside the primary one and filled with JM. This tank was seeded as described in Section 2.2.7.1. This secondary tank was maintained in fluorescent light and constant aeration and acted as a reserve supply in case of the primary culture failing. Under such circumstances, the primary system could be drained and replenished with this reserve supply.

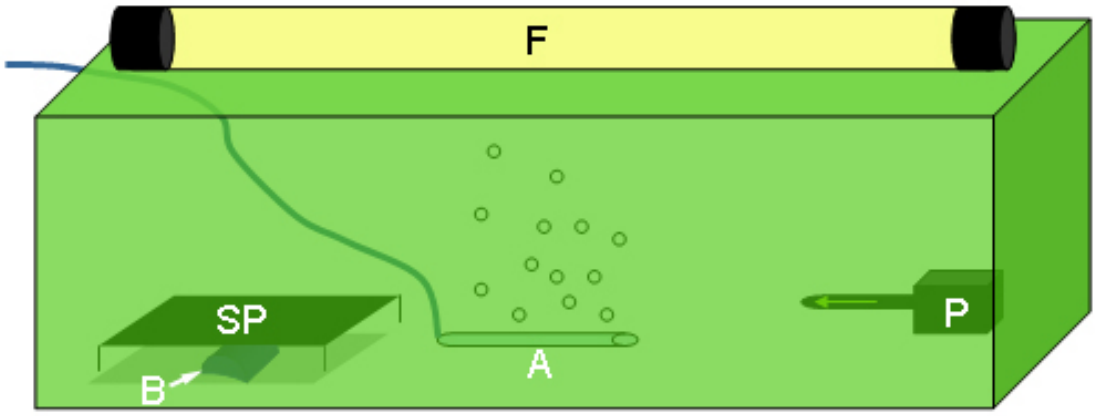

Figure 2.4: Schematic representation of bryozoan culture system version 1

A 150 L glass aquarium filled with JM.

Legend for Figure 2.4-Figure 2.8

"A" = air stone
"B" = rack of Petri dishes with attached bryozoans
"F" = fluorescent tube
"JM" = 80 L drum of Jaworski's medium
"L" = lid
"O" = vertical overflow pipe
"P" = water pump
"PP" = peristaltic pump
"SB" = spray bar
"SP" = shading platform
"W" = cool water inlet
"WS" = polystyrene water sleeve
Arrow = direction of flow of fluid through tubing

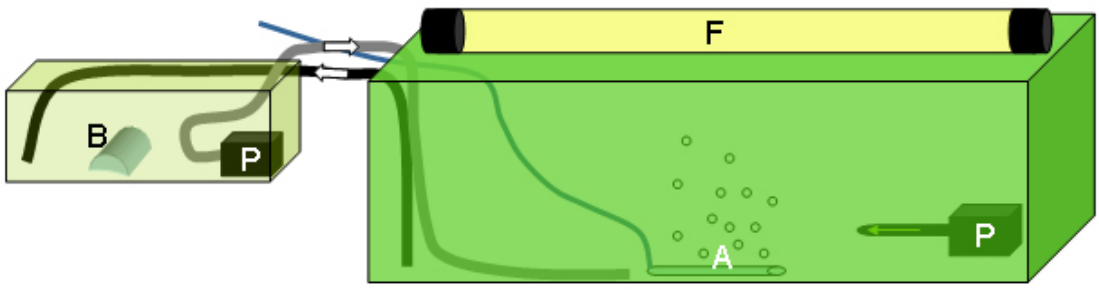

Figure 2.5: Schematic representation of bryozoan culture system version 2

Alongside the 150 L aquarium, an 8 L tank was placed.

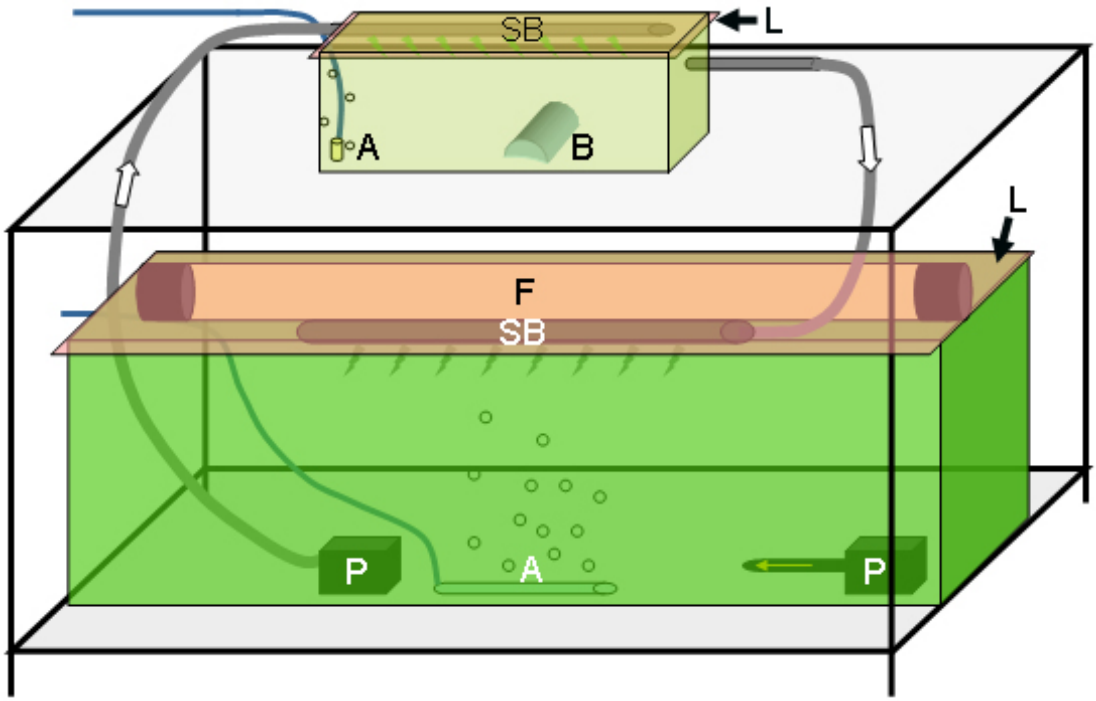

Figure 2.6: Schematic representation of bryozoan culture system version 3

8 L tank positioned above the 150 L aquarium.

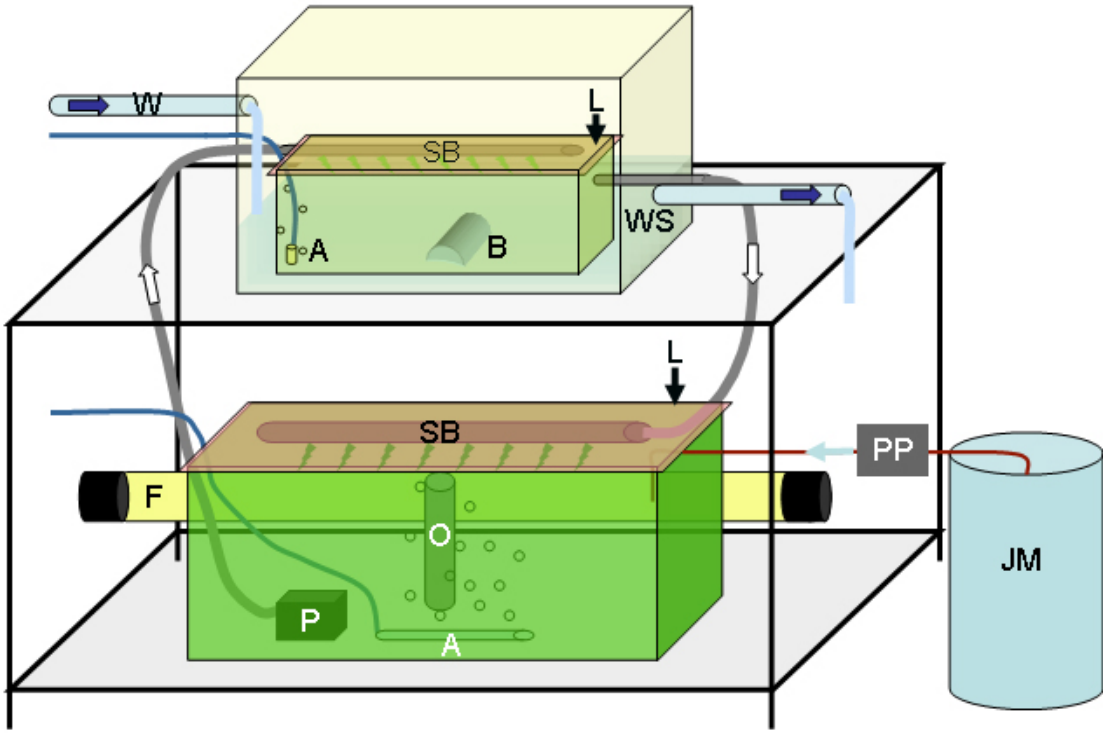

Figure 2.7: Schematic representation of bryozoan culture system version 4

8 L tank in water sleeve is positioned above a 25 L tank.

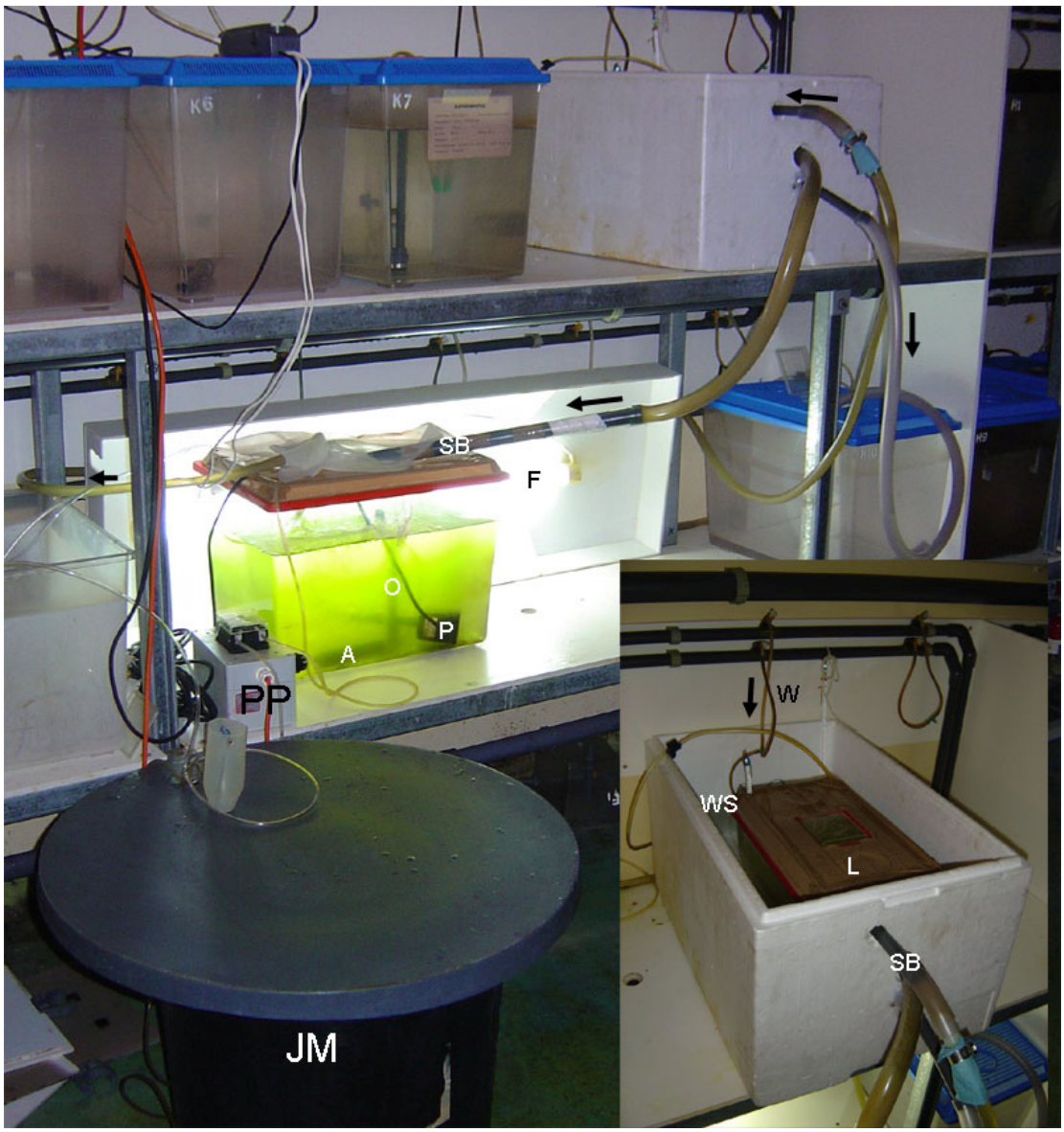

Figure 2.8: Photograph of bryozoan culture system version 4

Inset shows view from above of the upper 8 L tank containing bryozoan colonies.

2.3. Results

2.3.1. Initial laboratory maintenance of bryozoan colonies

Bryozoan colonies of the genera *Plumatella* and *Fredericella* were successfully maintained for up to six months using the method described. Following excision of fragments of Petri dishes and reattachment to new Petri dishes using adhesive, *Plumatella* sp. colonised the new dishes abundantly. Although there was a degree of attachment of *F. sultana* colonies to the surfaces of the Petri dishes, this was markedly rarer than seen with *Plumatella* sp. In general, the growth of *F. sultana* was less prolific than *Plumatella* sp. in this system.

2.3.2. Feeding trials of bryozoans

The observations made during the feeding experiments can be found in Table 2.1 and Table 2.2.

2.3.2.1. Ingestion of protozoa and algae by bryozoans

Currents of water were produced by the ciliated tentacles of each bryozoan lophophore resulting in material being directed towards the mouth of the zooid. Some highly motile protozoa such as *Chilomonas paramecium* and *Distigma proteus* Ehrenberg were witnessed to be able to actively evade ingestion. It was observed that the bryozoans ingested all species of protozoa and algae small enough to pass through their mouthparts. Fusiform algae such as *Synedra* sp. Ehrenberg were seen to be aligned longitudinally by the current before being ingested. However, filamentous algae were generally not ingested; although upon longitudinal alignment such material could on occasions pass through the mouthparts, all but the shortest fragments would subsequently be rejected. Some level of ingestion was witnessed in 36 of the 50 species exposed to *Plumatella* sp. and 29 of the 42 cultures exposed to *F. sultana*.

2.3.2.2. Digestion of protozoa and algae by *Plumatella* sp.

From examination of faecal pellets, some cultures which were readily ingested did not appear to have been digested by the bryozoan alimentary system. For example, the protozoa *Astasia longa* Pringsheim and *Bodo saltans* Ehrenberg were readily ingested by *Plumatella* sp. but examination of faecal pellets revealed the presence of intact motile organisms. *Stephanodiscus sp.* Ehrenberg (Figure 2.9a, b) and *Chlorella vulgaris* Beijerinck (Figure 2.9c, d) were also readily ingested but the faecal pellets produced contained intact algae. With other cultures examined, there was marginal digestion with evidence of incomplete degradation of the diet. For example, the faecal pellets resulting from ingestion of *Pediastrum boryanum* showed a combination of intact algae and algal casts (Figure 2.9e, f), while feeding with *Haematococcus lacustris* Rostafinski (Figure 2.9g, h), *Chlorococcum hypnosporum* (Figure 2.10a, b) and *Eudorina elegans* (Figure 2.10c, d) resulted in the production of faecal pellets containing recognisable but altered algal components. More complete digestion was seen of a number of the cultures including *Gloeocapsa* sp. (Figure 2.10e, f). Ingestion of *Cryptomonas ovata* resulted in efficient digestion leading to release of a purple pigment within the bryozoan intestine (Figure 2.10g, h).

Table 2.1: Species examined and results of feeding trial of *Plumatella*

description	ingestion	digestion	observations
Protozoa			
Astasia longa Pringsheim	+++	+	Faecal pellet (FP): living protozoa amongst amorphous material
Bodo saltans Ehrenberg	+++	-	Centrifuged and salt medium discarded before feeding. Live motile protozoa in faeces
Chilomonas paramecium Ehrenberg	++	++	Some actively avoid being trapped in pharynx
Colpoda steinii Maupas	+	+	Tiny ciliates not trapped by lophophore current. clumped detritus gather at mouth
Cyclidium sp. Müller	+++	++	Tiny. Many pass through lophophore
Distigma proteus Ehrenberg	++	+/++	Highly motile and contractile. Many evade current. Following ingestion: change from elongate to circular
Euglena gracilis Klebs	+++	-	FP: living organisms: initially circular, becoming elongate and motile
Euplotes viridis Ehrenberg	+++	++	Readily ingested
Paramecium aurelia Ehrenberg	+	-	Motile. very occasional ingestion if 'deliberately' swim into bryozoan mouth-part
Paramecium bursaria Focke	+	++	Only smaller individuals ingested – larger ones can escape current. Contain green symbionts
Trachelomonas sp. Ehrenberg	+++	++	Continuous ingestion: ~10 in pharynx before swallowed. After 1 day of feeding: many FPs produced
Chlorophyta			
Carteria sp. Diesing	+	+	Many settle out. Highly motile. Lophophores prone to retraction. FP: large irregular green mass
Chara sp. L.	-		'Stonewort'. large filamentous colonies
Chlamydomonas nivalis Bauer	+++	+	Mostly buoyant. Many pass through lophophore
Chlamydomonas reinhardtii Dangeard	+++	+	Many FPs produced: mixture of regular and irregular fragments
Chlorella vulgaris Beijerinck	+++	-	Pharynx fills with many algae before swallowing. FP: regular round algae (not motile)
Chlorococcum hypnosporum Starr	++	++	Swallowed rapidly (6-10 organisms in pharynx). Some particles too large: trapped in current, then released. Zooid mortality after 2 days of feeding
Cylindrocystis brebissonii Meneghini	-		Rapidly settle to bottom. those suspended seem too large to be ingested
Dictyosphaerium pulchellum Wood	+++	-	Tiny. recognisable algae in FP (amongst amorphous material)
Eudorina elegans Ehrenberg	+++	+	FP: irregular fragments
Haematococcus lacustris Rostafinski	+++	+	Red encysted stages digested: FPs contain small irregular fragments
Oedogonium foveolatum Zellfäden	-		Long filaments
Pandorina morum Bory	++	+	Many larger clumps settle. Clumps can become tangled in lophophore. FP: odd intact organism
Pediastrum boryanum Myen	+++	+	Some too large to be ingested. Live oval algae and algal casts in faecal pellets
Pleurococcus sp. Meneghini	+++	+	Many settle
Selenastrum gracile Reinsch	++	-	Crescent-shaped: many become entrapped @ base of lophophore. Many pass through lophophore
Stigeoclonium sp. Kützing	-		Long filaments. Some fragments aggregate into clumps
Trentepohlia aurea (L.) Martius	-		Long branching filaments
Ulothrix gigas Vischer	-		Filamentous (very few small fragments ingested)

description	ingestion	digestion	observations
Chlorophyta (contd.)			
Volvox tertuis Meyer	-		Large spherical colonies. Some become lodged in lophophore
Zygnema cylindricum Transeau	-		Long filaments
Bacillariophyta			
Asterionella formosa Hassal	+	+	Stellate. Only some fragments ingested
Navicula sp. Bory	+++	-	FP: intact algae amongst a brown matrix
Nitzschia sp. Hassall	+	+	Many settle. Often taken into pharynx but then expelled at swallowing
Stephanodiscus sp. Ehrenberg	++	-	Centric diatom. Many pass through lophophore. FP: entire algae seen
Synedra sp. Ehrenberg	++	-	Pennate diatom. Ingested following axial alignment
Xantophyta			
Botrydium granulatum (L.) Greville	++	++	FP: amorphous. After 2 days of feeding: guts fairly empty and few FPs
Vaucheria sessilis (Vaucher) de Candolle	-		Filamentous. Very occasional ingestion of fragments
Chrysophyta			
Ochromonas danica Pringsheim	+	+	Initially rapid ingestion, subsequently slowing down
Synura sp. Ehrenberg	++	+	
Cryptophyta			
Cryptomonas ovata Ehrenberg	+++	+++	Guts appear purple following digestion
Cyanophyta			
Chroococcus prescottii Drouet and Daily	+++	-/+	FP: undigested algae amongst amorphous material
Gloeocapsa sp. Kützing	+++	++	
Gloeotrichia longiarticula West	-		Long filaments
Oscillatoria animalis Agardht	+		Only very few short filaments ingested. Many axially-lined up but then rejected
Phormidium foveolarum (Montagne) Gomont	-		Some filaments lined up in lophophore parallel to body axis, but released after ~30-60 sec
Synechococcus leopoliensis Komárek	+++	++	Tiny cyanobacteria very readily ingested
Tolypothrix distorta Kützing ex Bornet and Flahault	-		Long branching filaments
Rhodophyta			
Batrachospermum sirodotia Skuja	-		Filamentous clumps with coccoid bodies akin to 'frog-spawn'. Only very limited ingestion of fragments/debris
Porphyridium purpureum (Bory) Drew and Ross	-		

- no ingestion / unchanged components in faecal pellets
+ variable ingestion / some digestion of particles
++ moderate ingestion / few undigested articles seen
+++ high level of ingestion / very few undigested particles seen

Table 2.2: Species examined and results of feeding trial of *F. sultana*

description	ingestion	observations
Protozoa		
Astasia longa Pringsheim	++	
Blepharisma japonicum Suzuki	++	
Chilomonas paramecium Ehrenberg	+	
Climacostomum virens Ehrenberg	-	Cocci: too large to be ingested
Colpidium striatum Stokes	-	
Cyclidium sp. Müller	++	
Distigma proteus Ehrenberg	-	
Euglena gracilis Klebs	+++	Very readily ingested
Euplotes viridis Ehrenberg	++	Very small
Nuclearia deliculata Cienkowski	-	Filamentous
Paramecium aurelia Ehrenberg	-	
Paramecium bursaria Focke	-	Too large to be ingested
Trachelomonas sp. Ehrenberg	++	
Chlorophyta		
Carteria sp. Diesing	+++	Very rapidly ingested, particularly efficiently filtered
Chlamydomonas nivalis Bauer	+++	Rapid ingestion
Chlamydomonas reinhardtii Dangeard	++	
Chlorella vulgaris Beijerinck	++	
Chlorococcum hypnosporum Starr	+++	Rapid ingestion
Closterium sp. Ralfs	-	Too large to be ingested
Dictyosphaerium pulchellum Wood	++	Small coccoid bodies
Eudorina elegans Ehrenberg	++	Some large clumps not ingested
Haematococcus lacustris Rostafinski	+	Red encysted stages ingested; green stages not
Pandorina morum Bory	+	A few single bodies ingested, but many clumps rejected
Pediastrum boryanum Myen	-	Became caught in the turbulence of lophophores
Pleurococcus sp. Meneghini	++	
Scenedesmus quadricaudus (Turpin) Brébisson	+	Limited ingestion: many reach mouth but then apparently actively rejected
Selenastrum gracile Reinsch	++	
Staurastrum gracile Ralfs	+++	Very readily ingested

description	ingestion	observations
Chlorophyta (contd.)		
Stephanosphaera pluvialis Cohn	++	
Bacillariophyta		
Asterionella formosa Hassal	-	
Navicula sp. Bory	-	
Nitzschia sp. Hassall	+	Limited ingestion, with some apparent rejection
Stephanodiscus sp. Ehrenberg	++	Diatoms: readily ingested
Synedra sp. Ehrenberg	+++	Very readily ingested
Xantophyta		
Botrydium granulatum (L.) Greville	-	Appear too large to be ingested
Chrysophyta		
Ochromonas danica Pringsheim	++	Readily ingested
Synura sp. Ehrenberg	-	Too large & actively motile: could escape lophophore currents
Cryptophyta		
Cryptomonas ovata Ehrenberg	+++	Very readily ingested
Cyanophyta		
Chroococcus prescottii Drouet and Daily	++	Ingested readily
Gloeocapsa sp. Kützing	+++	Small, readily ingested
Gloeotrichia longiarticula West	-	Filamentous
Synechococcus leopoliensis Komárek	++	Very rapid ingestion

- no ingestion
+ variable ingestion
++ moderate ingestion
+++ high level of ingestion

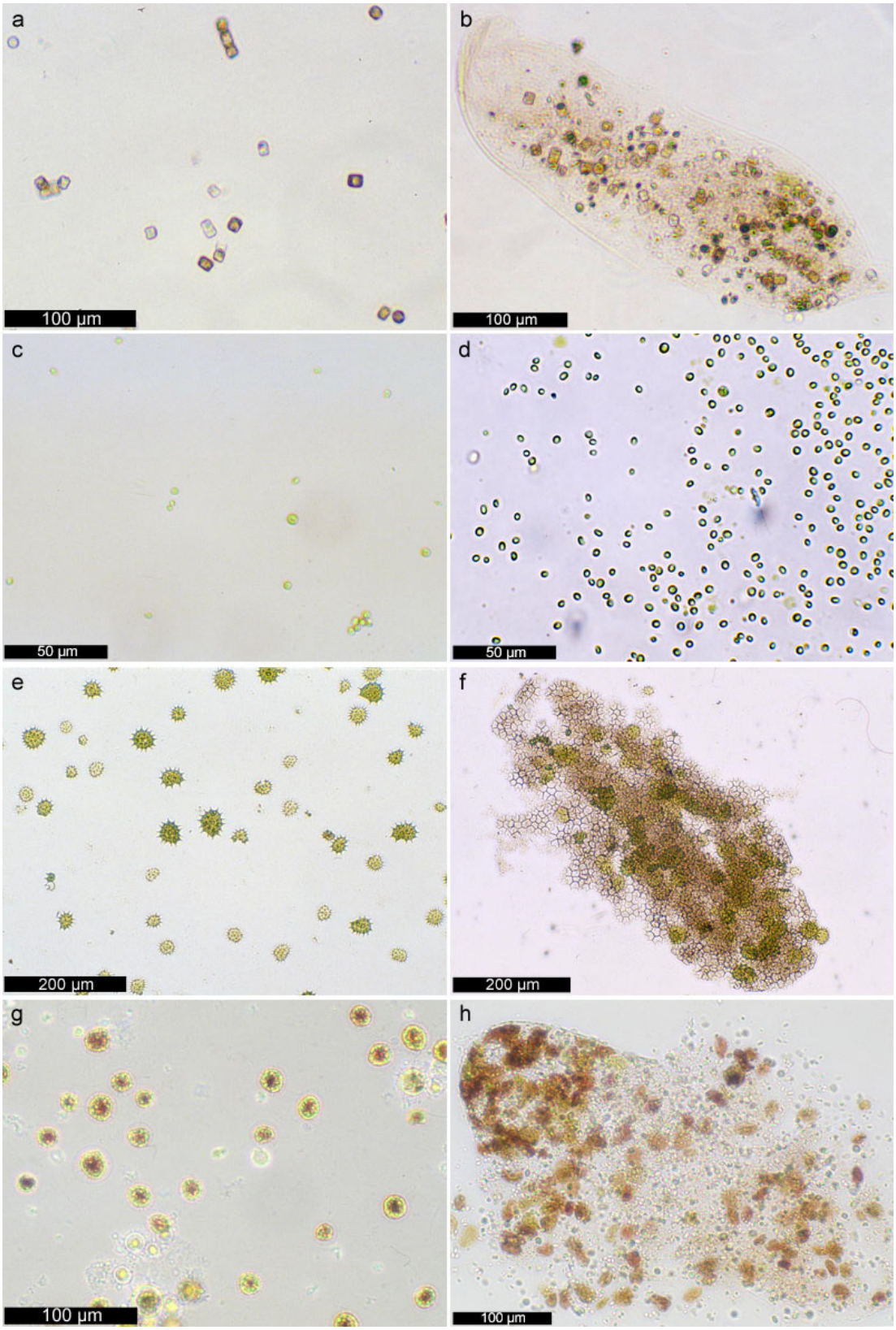

Figure 2.9: Images of algae before and after ingestion by *Plumatella* sp.

Left column = algae before ingestion. Right column = faecal pellet contents following ingestion of same species. (a, b) *Stephanodiscus* sp., (c, d) *Chlorella vulgaris*, (e, f) *Pediastrum boryanum*, (g, h) *Haematococcus lacustris*.

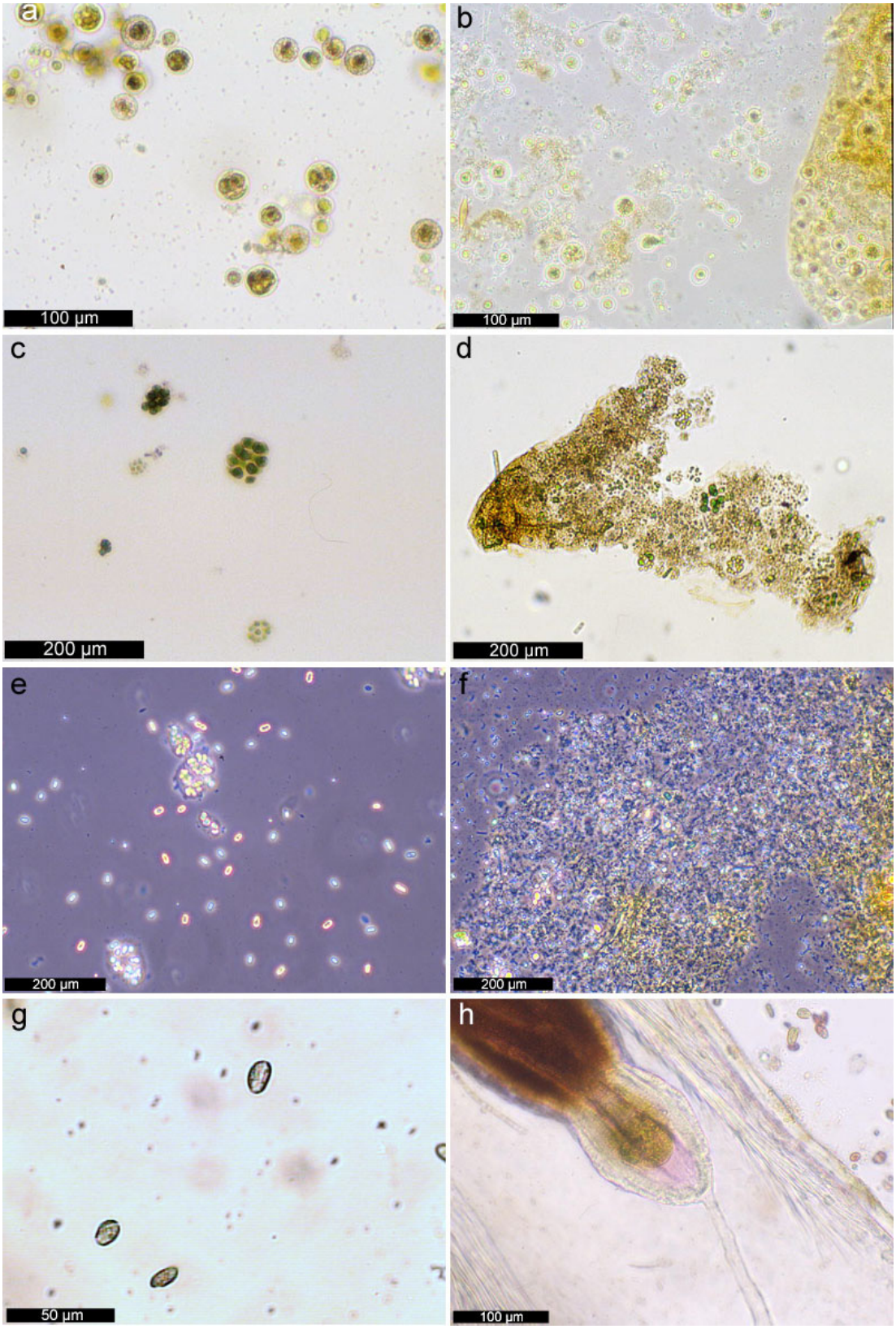

Figure 2.10: Images of algae before and after ingestion by *Plumatella* sp.

Left column = algae before ingestion. Right column = faecal pellet contents [except (h)] following ingestion of the same species. (a, b) *Chlorococcum hypnosporum*, (c, d) *Eudorina elegans*, fragments of other previously ingested species can also be seen in (d), (e, f) *Gloeocapsa* sp. (viewed with phase contrast), (g) *Cryptomonas ovata* before ingestion, (h) caecum of bryozoan intestine after ingestion of *C. ovata* showing the presence of purple pigment.

2.3.3. Long-term laboratory maintenance of bryozoans

Bryozoan colonies were successfully maintained for periods in excess of six months using this methodology. It was found that the colonies of *Plumatella* sp. thrived best in aquaria thermostatically regulated at 21°C. Conversely, colonies of *F. sultana* exhibited signs of degradation at 21°C, but were found to proliferate under maintenance in an incubator at 18°C with less growth seen at 15°C. It was found that a large number of macroinvertebrates colonised the Petri dishes, some of which – including midge larvae (Diptera: Chironomidae) – were observed to feed on bryozoan material. In addition, many oligochaetes including those belonging to the families Naididae Müller, 1773 and Tubificidae Müller, 1774 as identified by the key of Brinkhurst (1963) were seen to be associated with the bryozoans.

2.3.4. Exposure of laboratory-reared bryozoans to PKD-endemic waters

Of the 176 plates placed in the waterways, only three Petri dishes contained living bryozoan colonies upon retrieval. These consisted of two from the River Test and one from the River Itchen. Some of the other plates were seen to have dead bryozoan remnants attached to them. No characteristic signs of malacosporean infection were observed during the period of maintenance of the colonies.

2.3.5. Culture of algae from a PKD-endemic river

Following culture of water collected from the River Cerne, the following algae were identified: *Chlamydomonas* sp. Ehrenberg, *Monoraphidium contortum* (Thuret) Komárkova-Legnerová, *Actinastrum hantzschii* Lagerheim, *Scenedesmus dimorphus* (Turpin) Kützing and *Selenastrum* sp. Kützing.

2.3.6. Development of a low maintenance culture system for bryozoans

2.3.6.1. Version 1

Three weeks after seeding the tank, it was found to be of turbid green appearance. The Petri dishes with adhered *Plumatella* sp. colonies survived for several days within the system. Although successful in preventing the sedimentation of algae and protozoa, there was some difficulty with the current from the water pump leading to dislodging of some of the Petri dishes from their rack. One week following addition of the bryozoans, the tank was found to contain vast numbers of chironomid larvae. At this stage, no living bryozoan material was present and many chironomid larvae were intimately associated with the dead bryozoan remnants.

2.3.6.2. Version 2

Following seeding, the 150 L tank became turbid as described above with some proliferation of macroinvertebrates noted, especially chironomid larvae. At this point the water pump was activated and bryozoan colonies placed within the smaller tank. As the return flow from the larger to smaller tank depended upon a siphon system, it was necessary to meticulously extricate any air bubbles from the tubing before activating the pump. Despite such efforts, it was found that the tubing invariably became air-locked within 2-3 days of flow. This would result in the media being unable to return from the larger to smaller the tank, leading to drainage of this tank and potential desiccation of the bryozoans.

2.3.6.3. Version 3

As previously seen, the larger tank became turbid within two weeks following seeding with protozoa and algae. Few macroinvertebrates were observed within the system, with no population explosion of chironomid larvae witnessed. As this system incorporated an overflow mechanism to allow return of the fluid to the lower tank, there was no problem with air-locks in the tubing. Initially, bryozoans were

successfully kept in the system, although limited growth was witnessed. The turbidity of the tank was seen to be reduced after about 30 days with subsequent demise of the bryozoan colonies being witnessed. When the system was active, the mesh under the spray bars became clogged with algae within one to two weeks. The temperature of the media in the system was measured as 22 ± 2°C.

2.3.6.4. Version 4

Turbidity of the larger tank was successfully achieved as described above. Regulated infusion of JM via the peristaltic pump resulted in a fairly constant level of turbidity. The media of the bryozoan culture tank remained at 15 ± 2°C, with little proliferation of chironomid larvae seen. Colonies of *Plumatella* sp. and *F. sultana* remained viable within the system for 30 days and 150 days respectively. The main labour inputs involved in the maintenance were periodic replenishment of the JM drum, occasional swapping over to the secondary algal tank and scraping algal deposits from the surface of the tanks adjacent to the light source. Some of the bryozoan colonies added exhibited signs of malacosporean parasitism with *Buddenbrockia plumatellae* and *Tetracapsuloides bryosalmonae* as subsequently described in Chapters 3 and 4.

2.4. Discussion

Because the primary aim of this study was to develop culture methods for phylactolaemate bryozoans, it was initially necessary to obtain some bryozoan specimens. As this work was undertaken in the winter, statoblasts were collected as no viable colonies were growing at suitable sites at that time. The successful germination of statoblasts of *Plumatella* sp. was accomplished without the need for conditioning of the statoblasts or the use of artificial photoperiod regimes as previously suggested for freshwater bryozoans (Mukai 1974). Following germination, the requirements for nutritional supplementation of the resulting zooids were found to be adequately supplied using a previously devised method involving the culture of monocultures of algae and polycultures of protozoa (Morris *et al.* 2002a).

However, the complexity and high level of labour involved with such a system led to the decision to conduct a re-examination of the nutritional utilisation of colonies of *Plumatella* sp. and *F. sultana*. In common with previous findings (Kamiński 1984), it appeared that size was a crucial factor in the ingestibility of prey, although it was found that some organisms of a size suitable for ingestion such as the diatom *Nitzschia* sp. were taken into the mouth but apparently rejected before being able to pass through the pharynx. As members of the genus *Nitzschia* produce the neurotoxin domoic acid (Lundholm, Hansen and Kotaki 2004), the rejection by bryozoans could represent a rudimentary defence mechanism. Bryozoans have previously been described as exhibiting responses attributed to chemical stimulation (Marcus 1941). The dynamics of particle capture and water pumping by the bryozoan lophophore has been shown to be a complex mechanism (Riisgård and Manríquez 1997). The lophophore has been shown in experiments using Indian ink to be able to generate currents capable of drawing in water from several millimetres around the zooid (Brooks 1929). Gilmour (1978) suggested that *Plumatella* spp.

were able to discriminate between particles of different weight, with heavier objects being rejected via the protrusive epistome (Figure 2.1), implying that size is not the only criterion involved in successful ingestion. This mechanism could explain the reaction by *Plumatella* sp. to *Selenastrum gracile* Reinsch and by *F. sultana* to *Scenedesmus quadricaudus* (Turpin) Brébisson which both accumulated proximate to the mouthparts but were subsequently rejected. In addition, due to their marked motility, some species including *Chilomonas paramecium* and *Synura* sp. Ehrenberg could escape the currents generated by the lophophore and thus avoid ingestion. It was found that filamentous algal species were apparently unsuitable as potential food, as the bryozoans were unable to ingest all but the shortest of fragments. Filamentous algal accumulations often gathered around the lophophores, impeding potential ingestion of other more suitable prey.

The examination of faecal pellets following feeding of individual species to *Plumatella* sp. revealed a wide divergence in digestion levels. Despite starvation of a period of 12 hours before administration of the test diet, it was found upon examination of some faecal pellets that a mixed population was present (Figure 2.10d). This suggested that some organisms took longer than 12 hours to be purged from the bryozoan intestine, but it was felt that starvation in excess of this period might compromise the viability of the subjects. There did not appear to be any predictable relationship between taxonomical grouping of potential prey and resulting digestibility, with related members exhibiting contrasting results. For example, ingestion of some protozoa such as *Trachelomonas* sp. Ehrenberg resulted in well-digested faecal constituents, while exposure to other protozoa such as *Astasia longa* produced living faecal organisms that actively swam away upon disruption of the pellet. This demonstrated that the observation alone of marked ingestion of organisms by bryozoans presents insufficient data to gauge nutritional value. Consistent with previous findings, no single species examined seemed to fully satisfy

the nutritional requirements of *Plumatella* sp. (Jebram 1980). Some colonies apparently suffered pathological effects after 48 hours of being offered a single species such as *Chlorococcum hypnosporum* or *Botrydium granulatum* despite relatively vigorous ingestion. It appeared that only those species which were planktonic were highly suitable as bryozoan food, with benthic or adherent species proving unavailable for ingestion. It appears from previous findings that bryozoan growth depends not only on the constituents of the diet, but also the concentration (Toriumi 1972). The production of individual flasks of algae in high yielding culture media allowed a high concentration of algae to be fed to the bryozoans throughout the current study.

Kamiński (1984) described the food composition of phylactolaemates in a mesotrophic lake and found in the gut contents large numbers of diatoms, shown to be indigestible by *Plumatella* sp. in the current study. The presence of non-digestible components in the diet appeared to compromise neither wild bryozoans reported by Kamiński (1984, 1991) nor our laboratory-reared specimens. It seems possible that they may confer positive characteristics to the diet by acting as bulking agents aiding in peristaltic contractions, thus degrading other concurrently ingested digestible organisms. It would have been desirable to carry out digestion trials with *F. sultana* in addition to *Plumatella* sp., but difficulties in maintaining abundant colonies of the former species using the established methodologies led to insufficient viable material being available for such an experiment. However, the ingestion trials using *F. sultana* demonstrated marked similarities to the results from *Plumatella* sp., implying that digestibility of prey between the genera may also show consistencies. The fact that *F. sultana* was successfully maintained on the same initial and modified diets as used for *Plumatella* sp. supports such a suggestion.

Plumatella colonies were successfully maintained for extended periods using the modified culture methods developed following the feeding trials. However, in the systems, colonies of *F. sultana* proved more fastidious, with increased viability noted only at temperatures below 20°C. The stream from which these colonies had been collected is known to be cooler than other local sources (A. Mercer, personal communication), implying that water temperature may be a factor in the proliferation of *Fredericella* in preference to other genera at the site. Despite the successful long-term laboratory culture, it was found that occasionally there would be marked poor growth rates and deterioration of the bryozoan colonies. It was ascertained that the pH of the algal cultures could become markedly alkaline following increased growth periods. This was corrected by careful titration of the media with HCl before addition to the bryozoan systems, but it is unknown if such algal blooms could also have led to the production of toxins by dietary constituents including cyanobacteria which may have proved pathogenic to the phylactolaemates.

Following placement in PKD-endemic waters, few of the laboratory-reared bryozoans survived the six week period before being re-examined. Throughout the laboratory culture of Bryozoa it has become apparent that proliferation can be unpredictable – a finding also described in wild populations (Okamura and Hatton-Ellis 1995) – suggesting that they have very specific culture requirements. As some sites – such as in the River Cerne – harbour a predominance of one species of Bryozoa, it could be that the local ecosystem determines the suitability for maintenance for phylactolaemates. The bryozoans used in this trial belonging to the genus *Plumatella* may not have been ideally suited to the environments to which they were translocated, with possible differences in water quality parameters. Mr. W. Struthers, Water Quality Laboratory, Institute of Aquaculture, University of Stirling assayed the water hardness (with reference to $CaCO_3$) of samples from bryozoan environments. That of the laboratory culture system was 38 mg L^{-1}, the River Avon was 190 mg L^{-1}

and the River Cerne was 197 mg L^{-1}. Thus, the transfer from the soft water of the laboratory to the hard water of the rivers may have detrimentally affected the bryozoans. If the experiment was to be replicated, it would be advisable to either obtain uninfected bryozoans from a hard water source or to gradually alter the hardness of the culture media to avoid such a potential shock to the cultures.

Of the few specimens that survived the transfer, no sign of malacosporean infection was visualised. A similar study was carried out using laboratory-reared *F. sultana* and *Plumatella fungosa* Pallas placed in PKD-endemic waters for two weeks (Tops and Okamura 2003). No sign of malacosporean infection was seen in their samples upon examination for a period of 28 days, although positive identification of *T. bryosalmonae* by molecular techniques led the authors to propose the presence of rudimentary cryptic parasitic stages within the bryozoans. Therefore, in the current study, molecular analysis of the bryozoans successfully retrieved from the rivers might have yielded similar results. The failure to obtain infected bryozoan colonies by this method was redressed by the sampling of established *F. sultana* colonies at a site known to be infected with *T. bryosalmonae* as described further in Chapter 4.

The aim of developing a low maintenance culture system for phylactolaemate bryozoans led to various designs being implemented. A previous design shown to be useful for culturing certain sessile aquatic organisms and proposed for use with Bryozoa seemed unnecessarily elaborate with potential for failure of multiple moving parts (Emschermann 1987). Another laboratory culture method relied upon supplementing Bryozoa with pond water (Mukai, Fukushima and Jinbo 1987), which could potentially result in the introduction of unknown contaminants into the system. Therefore, it was decided to base the premise of the design upon the relatively simple systems described by Wood (1971, 1996). However, those designs also relied upon periodic seeding of the system with pond water and the continued

presence of goldfish to aid algal colonisation which could potentially complicate life cycle studies of myxozoans. Therefore, to promote algal growth in the main tank, it was filled with JM, which from Sections 2.3.1 and 2.3.3 was known to be successful for rearing of algae and apparently not harmful to bryozoans. This method allowed control over the inputs to the system and standardisation of culture conditions between different batches of bryozoans. As colonies are often found in shaded environments (Jónasson 1963), they would not thrive in the bright conditions required for algal growth, thus entailing segregation of the two culture vessels. The designs relied upon constant movement of media within the system; such conditions of water flow having been shown to be capable of encouraging phylactolaemate feeding (Okamura and Doolan 1993). However, the initial systems suffered from mechanical problems with the circulation of media around the system and vast proliferation of chironomid larvae. Although the larvae could not conclusively be shown to cause the demise of the bryozoans at that time, their presence coincided with such. Indeed they were observed to scavenge upon dead colonies, and it has been suggested that they are capable of predating bryozoans (Hyman 1959). The initial strategy of placing mesh sheets below the spray bars successfully reduced chironomid numbers but also led to accumulations of algae on the mesh eventually reducing the turbidity of the system.

The final version of the culture system seemed to address the issues raised in the previous designs. The regular infusion of fresh JM prevented excessive algal blooms occurring which potentially would elevate the pH of the system and might release toxins from degrading algae. The reduction in the water temperature of the bryozoan tank by the water sleeve allowed the specimens of *F. sultana* to survive for extended periods of time. Indeed, colonies of *F. sultana* were successfully reared in this system for longer than was achieved in the previously established laboratory system. *Plumatella repens* L. has been shown to thrive at water temperatures of 23°C and

28°C, but less well at 13°C (Toriumi 1972), perhaps explaining the shortened lifespan of *Plumatella* colonies within the final version of the system (15 ± 2°C).

The new system entailed minimal maintenance, requiring only occasional refilling of the JM drum every 2-3 weeks and checking of the integrity of the pipes. The original method had required daily examination and twice weekly replenishment of the supplemented media, with all the entailing algal maintenance issues. Despite the introduction of chironomid larvae with the bryozoans, no vast increase in larvae population was noted. This could be attributed to the sealing of the lids of the tanks preventing the adult midges from being able to successfully mate and then return to the system to lay their eggs. Thus, it did not prove necessary to carry out the laborious removal by hand of chironomid larvae from the Petri dishes. As the bryozoans introduced to the system would also have had algae associated with them, some of these species would potentially colonise the culture tank in addition to those added during the original seeding. The algae successfully cultured from a PKD-endemic river included members of genera found to be ingested during the feeding trials. Previously, *F. sultana* has been shown to grow in waters harbouring a wide diversity of phytoplankton with suggestion that it has a very poor digestive efficiency requiring a high turnover of appropriate nutrition (Raddum and Johnsen 1983). Bacteria of multiple genera have also been shown experimentally to be ingested by phylactolaemates (Richelle, Moureau and van de Vyver 1994). Bacterial colonies were not specifically added to the media during the current study, but as the systems were not maintained in a sterile fashion there would have been development of commensal populations of various microorganisms – including bacteria – which may have supplemented the bryozoan diet.

Within each of the laboratory systems deployed, the bryozoans were seen to develop translucent areas of new growth. Thus, unlike their wild equivalents which

possess almost opaque thick cuticles, it was possible to observe the internal contents of the colonies. In addition to allowing study of their feeding habits, this facilitated careful and repeated study of the bryozoans for signs of malacosporean parasitic development within the coelomic fluids of the hosts. The development of a successful low maintenance bryozoan culture system has allowed the long-term culture of potential hosts of *T. bryosalmonae*, potentially safeguarding a year round reliable source of the parasite.

Chapter 3: The development of *Buddenbrockia plumatellae* (Myxozoa: Malacosporea) in *Plumatella repens* (Bryozoa: Phylactolaemata)

3.1. Introduction

Whereas much of the primary interest in the class Malacosporea has stemmed from damaging parasitic effects on captive fish stocks, the developmental cycles and pathological consequences in invertebrate hosts are equally intriguing. Schröder (1910) first named a myxozoan parasite of Bryozoa in his description of *Buddenbrockia plumatellae*. In this account, worm-like stages were described in *Plumatella fungosa* and *Plumatella repens*, with the parasite originally being assigned as a mesozoan. Later consideration of the behavioural characteristics and histological structure of the organism, including respectively writhing motility and the recognition of four longitudinal blocks of muscle fibres, led to the inference of affinities between *B. plumatellae* and nematodes, thus allying the parasite with the Metazoa (Schröder 1912).

Canning, Okamura and Curry (1996) described a myxozoan parasite in *Cristatella mucedo* Cuvier, which formed free-floating coelomic spore sacs within the bryozoan host. This parasite was originally assigned to the newly formed genus *Tetracapsula*, being named as the type species, *T. bryozoides* (Canning *et al.* 1996). Canning *et al.* (2002) assigned the genus *Tetracapsula* to the newly established class Malacosporea – denoting the formation of soft spores – within the phylum Myxozoa. Morris *et al*. (2002a) described the development of vermiform parasites that upon maturation released malacospores within colonies of *P. repens* that had been previously exposed to cell suspensions of kidney material taken from brown trout infected with *T. bryosalmonae*. However, the authors noted morphological and developmental differences between the observed parasites and the descriptions by Schröder (1910, 1912) of *B. plumatellae* and thus concluded the two to be related but separate species. A later description centred on the development of *B. plumatellae* in populations of *Plumatella fungosa*, *Hyalinella punctata* Hancock and

Fredericella sultana (Canning *et al.* 2002). In light of the proposed synonymy of *B. plumatellae* and *T. bryozoides* (Monteiro *et al.* 2002) (which produce vermiform or spheroid spore sacs respectively), the previous study of Morris *et al*. (2002a) opens up the intriguing possibility of vermiform alternate spore sac stages also existing for *T. bryosalmonae*.

The objectives of this study included observation of developmental cycles and examination of potential pathological effects of malacosporean parasites within Bryozoa. In the anticipation of developing protocols for the long-term laboratory maintenance of malacosporeans, it was hoped to further examine the potential longevity of the parasites within their invertebrate hosts and to investigate the possibilities of horizontal transmission of infection from bryozoans either to other bryozoans or to fish.

3.2. Materials and Methods

3.2.1. Collection of bryozoan colonies

The freshwater fish farm on the River Avon (mentioned in Section 2.2.5), was visited on two occasions in June 2002. The site was mainly stocked with rainbow trout with some additional coarse fish, and was known to be endemic for PKD. Submerged and floating objects were removed from the water courses at various points in the farm and examined grossly using a ×10 hand lens for signs of bryozoan proliferation. On the first visit, bryozoans were found colonising the bark of branches within the water. Using a knife, the colonised areas of bark were carefully excised from the branches and placed in sealed aerated five litre tupperware containers filled with river water and returned to Stirling. On the second visit, in addition to the collection of more colonies from bark, multiple bryozoan populations were observed on the underside of sheets of black plastic "bubble-wrap" which had been placed on the inlet channels as a management procedure in an attempt at limiting weed proliferation. Sections of the plastic sheeting were removed and placed in the containers with the bark samples for return to the laboratory.

3.2.2. Maintenance of the bryozoans in the laboratory

The substrata with attached bryozoan colonies were fixed to plastic Petri dishes of nine centimetre diameter with cyanoacrylate adhesive. The Petri dishes were suspended vertically using a rack system (as described in Section 2.2.2) in five litre tupperware tanks containing CM and maintained with constant aeration at room temperature (21°C). The specimens were maintained by the addition of four litres of CM to one litre of a mixture of live cultures as described in Section 2.2.4, the culture media being removed and replaced every two days. The bryozoan colonies were examined daily, using a dissecting microscope (Olympus SZ30) and an inverted microscope (Olympus CK2). Any observed chironomid larvae or other predatory

invertebrates putatively deleterious to Bryozoa were removed with forceps and discarded. Later, when it was decided to feed bryozoan colonies with higher concentrations of algae and protozoa, 800 ml of the mixture of live cultures was centrifuged (Sigma Laboratory Centrifuges 4-15) at 2,600 × *g* for 20 min, the pellet was then resuspended in 100 ml CM and added to 4.9 L of CM in the tupperware container. Statoblasts were collected from parasitised colonies and placed under Petri dishes containing CM as described in Section 2.2.1 in an attempt to promote their germination.

3.2.3. Study of bryozoans by light microscopy

The bryozoans were examined daily for characteristic signs of myxozoan parasitism using dissecting and inverted light microscopes. A 35 mm camera (Olympus SC35) was attached to the Olympus CK2 inverted microscope for the capture of still images; while for capture of moving images, a Panasonic F15 video camera was attached. The video camera was connected either to a video cassette recorder or directly to a personal computer (PC). ATI video capture software (version 7.5, Windows 98) was used to digitise and store the images as either video files (.mpeg) or to allow frame capture producing still image files (.bmp). Alternatively, an Olympus LH50A inverted microscope was used, with an attached Zeiss AxioCam MRc digital camera, employing Zeiss MRGrab (version 1.0.0.4, Windows 2000) image capture software to produce still image files (.tiff).

3.2.4. Ultrastructural examination of Bryozoa

Infected bryozoan zooids were dissected and the resultant 0.5 mm^3 cubes of tissue were fixed in 2.5% glutaraldehyde fixative for 2-4 hours at 4°C. They were rinsed in cacodylate rinse buffer (0.1 M, pH 7.2) overnight at 4°C, and post-fixed in 1% (w/v) osmium tetroxide in cacodylate buffer for one hour at room temperature. The remaining procedures were conducted at room temperature. The samples were then

placed *en bloc* in 2% (v/v) uranyl acetate in 30% (v/v) acetone and kept in darkness for one hour. The material was dehydrated through an acetone series: 40 min in 60% (v/v), 40 min in 90%, two soaks of one hour in 100%. The material was then placed in 50% acetone / 50% Spurr's resin (Spurr 1969) overnight, then rotated for 24 hours in 100% Spurr's resin before being embedded in Beem capsules and polymerised for 48 hours at 60°C. Semi-thin sections of the resin blocks were sectioned at 0.5 µm and then stained with toluidine blue. Following examination of the semi-thin sections by light microscopy for evidence of myxozoan infection, ultra-thin gold sections of thickness 90 nm were cut, placed on 200 mesh formvar-coated copper grids and stained with 4% (v/v) uranyl acetate in 50% (v/v) ethanol for four minutes and in lead citrate (Reynolds 1963) for seven minutes. The grids were examined using a Philips 301 transmission electron microscope at 80 kV. Photographs were taken with an integral flat plate camera using black and white Kodak 4489 EM film.

3.2.5. Examination of bryozoan statoblasts by scanning electron microscopy

Statoblasts were dissected from myxozoan-infected bryozoan colonies, fixed in 1% (v/v) glutaraldehyde in 0.1 M sodium cacodylate for one hour at 4°C and transferred to 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate for 3 days at 4°C. The remaining procedures were conducted at room temperature. The material was washed in cacodylate rinse buffer before being post-fixed in 1% (w/v) osmium tetroxide in cacodylate buffer for two hours. Then followed dehydration through an ethanol series: 30 min each in 30% (v/v), 60%, 90% and two bathes in 100%. The material was then placed in 50% (v/v) hexamethyldisilazine (HMDS) in absolute ethanol for two immersions of 30 min, followed by two incubations of 30 min in 100% HMDS. The statoblasts were then air dried, before being mounted on aluminium stubs, coated with gold for 90 sec at 40 mA using an Edwards S150B sputter coater and examined using a Philips 500 scanning electron microscope at 15 kV.

Photographs were taken using an integral camera with Ilford FP4-125 black and white roll film.

3.2.6. Attempted horizontal transmission of *B. plumatellae* between bryozoans

3.2.6.1. Direct injection of coelomic contents from *B. plumatellae*-infected to uninfected bryozoans

A Petri dish with adhered colonies of *P. repens* showing characteristic signs of *Buddenbrockia* infection was placed on the stage of an inverted microscope (Olympus LH50A). A microinjector (Narishige IM-6, Tokyo, Japan) was connected to an oil-hydraulic micromanipulator (Narishige MO-155) attached to the microscope stage. Glass capillary tubes (Narishige GD-1) of length 90 mm and bore 1 mm – which had been previously stretched to a fine point (< 100 µm) using a heated capillary stretching apparatus (Research Instruments Ltd.) – were attached to the microinjector. Coelomic contents were removed from an infected colony of *P. repens* including various stages of *B. plumatellae* development (Figure 3.1). Both the outer wall and peritoneum of the bryozoans proved resilient to puncture, requiring sustained pressure with the micropipette to breach them. The withdrawn material was immediately injected into uninfected bryozoan colonies (Figure 3.2 and Figure 3.3) which had been observed for 3 months without showing any sign of infection, and the recipient colonies observed daily for 30 days. Coelomic contents were withdrawn from an uninfected *P. repens* colony and injected into another colony as a negative control method. Following initial failure of transmission of infection, the entire procedure was subsequently replicated.

3.2.6.2. Exposure of uninfected *P. repens* to *B. plumatellae* material

Three ml of culture medium surrounding heavily infected colonies of *P. repens* was removed with a Pasteur pipette and flushed onto a Petri dish containing adhered uninfected colonies of *P. repens*. The medium was left in place for 60 min before the

plate was added to a tub containing standard bryozoan culture medium (as described in Section 3.2.2) and kept for 30 days, being observed regularly.

Cohabitation trials of uninfected and infected colonies were also facilitated by the natural presence of populations of each status on single Petri dishes. These colonies were maintained in common media for 3 months.

3.2.7. Exposure of *B. plumatellae* spores to trout mucus

Three rainbow trout from the ARF of known *T. bryosalmonae* exposure status were scraped with microscope slides, leaving deposits of mucus on the slides. One fish was taken from each of the following groups: pre-clinical PKD-affected (injected with PKD-affected kidney four weeks previously), PKD-recovered (injected with PKD-affected kidney 12 weeks previously) and PKD-naïve rainbow trout. The slides were kept moist in sealed Petri dishes during transfer from the ARF to the laboratory. Spores of *B. plumatellae* dissected from infected *P. repens* under a dissecting microscope were pipetted onto each slide. An additional blank slide was also coated with spores to act as a control. The slides were observed under an inverted microscope for 60 min.

3.2.8. Experimental exposure of rainbow trout to *B. plumatellae* spores

Twelve rainbow trout of mean weight 90.2 g, from a source known to be free of PKD, were anaesthetised in 4 mg L^{-1} benzocaine in the ARF. For identification, the adipose fins of six of the fish were clipped and the two groups were placed for recovery in separate 20 L tanks. After three hours of recovery time, the water inflow to each tank was closed, and the water level reduced to nine litres while maintaining vigorous aeration. To the unclipped group's tank, one litre of CM containing media from *B. plumatellae*-infected bryozoans within which malacosporean spores were observed was added; while to the clipped group one litre of freshly-made CM was

added as a control. After 60 min, the fish were allocated to a single 100 L tank, and maintained at 18°C. Fifty days later, the fish were overdosed in 10 mg L^{-1} benzocaine and euthanased by severing the spinal cord. For fixation, samples of kidney, spleen and liver were placed in universal tubes containing 10% neutral buffered formalin for 24 hours.

3.2.8.1. Processing of samples for histology

Formalin-fixed samples of tissue were processed overnight in a Shandon Citadel automatic tissue processor. After being embedded in paraffin wax (Tissue Tek No. 2, Bayer), the blocks were trimmed and soaked in distilled water for 60 min prior to cutting. Sections of 5 µm were cut on a Reichert-Jung Biocut microtome, and then stretched on a water-bath at 45°C before being floated onto glass slides. The slides were then dried in an oven at 60°C for 60 min.

3.2.8.2. Examination of tissue sections by immunohistochemistry

The paraffin-embedded tissue sections were examined by immunohistochemistry based on the method described by Adams and Marìn de Mateo (1994). Positive control slides of known *T. bryosalmonae*-infected kidney were included to verify the assay.

The incubation stages of the immunoassay were carried out at room temperature (21°C), using slide racks and wash sleeves (Hybaid Omnislide) to prevent drying of the slides. Firstly, slides were dewaxed by immersing in two changes of xylene for five minutes each. The slides were then rehydrated through an alcohol series comprising five minutes in 100% ethanol, three minutes in 70% (v/v) ethanol and three minutes in distilled water. The sections were then encircled on the slides using a liquid blocker (Super PAP Pen, Agar Scientific Ltd., Essex, UK). Endogenous

peroxidase activity was blocked by incubating the ringed sections for 10 min with 10% (v/v) hydrogen peroxide in methanol.

The slides were washed three times in tris buffered saline (TBS, Appendix 2), before 10% (v/v) goat serum in TBS was added for 10 min to block non-specific binding sites. The slides were then tapped dry on to a paper towel, and the anti-*T. bryosalmonae* monoclonal antibody P01 (Aquatic Diagnostics Ltd., Stirling, UK) diluted to 5 µg ml^{-1} in phosphate buffered saline (PBS, Appendix 2) was incubated on the slides for 60 min. The slides were washed as before, and then incubated with biotinylated anti-mouse immunoglobulin G (Sigma, Missouri, USA) diluted 1/100 in TBS for 60 min. Next followed washing as before, and then incubation for 60 min with streptavidin peroxidase (Sigma) diluted 1/100 in TBS, followed by further washes.

The slides were then incubated with 3,3'-diaminobenzidinetetrahydrochloride (DAB) substrate (Appendix 2) for 10 min, followed by rinsing in tap water for three min to halt the reaction. Counterstaining was then achieved by placing the slides in Mayer's haematoxylin (3 mM haematoxylin, 1 mM sodium iodate, 0.1 M aluminium potassium sulphate dodecahydrate, 5 mM citric acid, 30 mM trichloroacetaldehyde hydrate) for three minutes, before flushing the slides in running tap water until the purple colour was lost. The slides were then dehydrated in alcohol series: three minutes in 70% ethanol, five minutes in 100% ethanol, and two bathes of five minutes in xylene, before being cover slipped with Pertex (Sigma). Sections were examined using light microscopy with *T. bryosalmonae* stages appearing brown in colour.

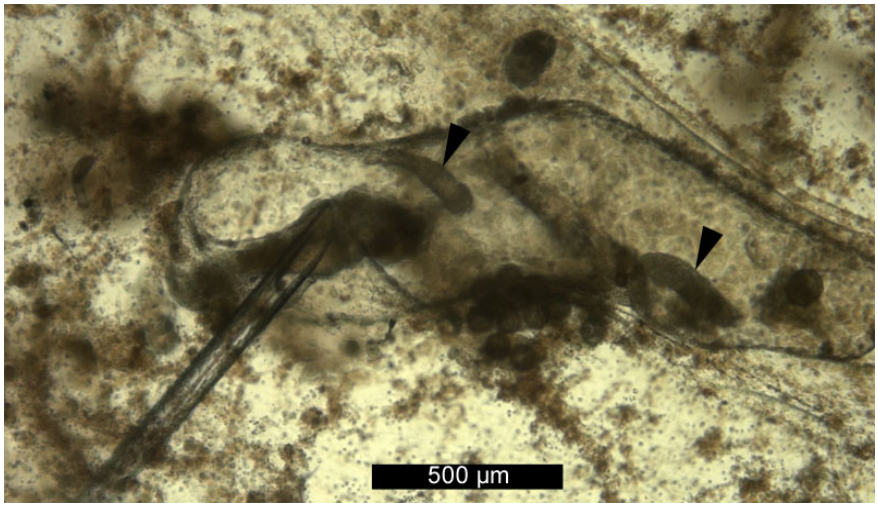

Figure 3.1: Micropipette impinging on peritoneum of donor infected zooid

B. plumatellae 'worms' (arrowheads) can be seen in the metacoel

Figure 3.2: Micropipette impinging on peritoneum of recipient uninfected zooid

"i" = bryozoan intestine

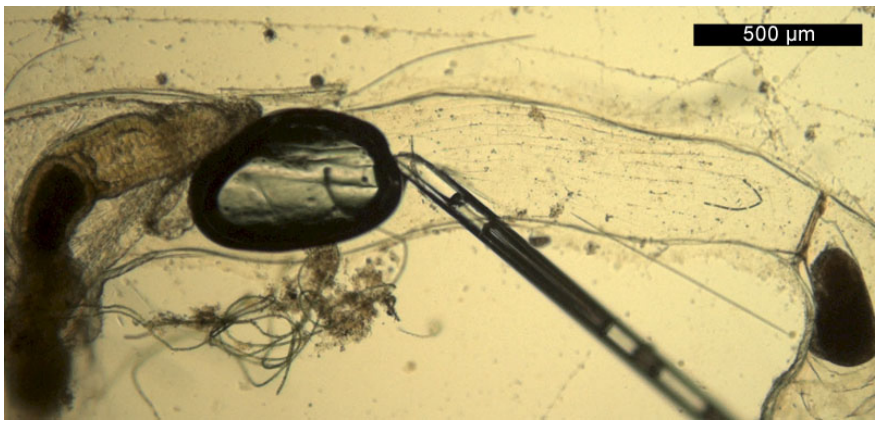

Figure 3.3: Following injection of infected material into recipient zooid

3.3. Results

3.3.1. Collection of Bryozoa

Multiple bryozoan colonies were identified by gross examination of the bark of branches of wood and black plastic “bubble-wrap” sheets removed from the inlet water of the fish farm on the River Avon (Figure 3.4). Following transportation to the laboratory, microscopic confirmation was made of the presence of living bryozoans on the collected material. Many other invertebrates were also seen within the water samples and intimately associated with the bryozoan colonies.

3.3.2. Observations of the bryozoan colonies

The bryozoan colonies spread from their original substrata (bark or black plastic “bubble-wrap” sheeting) to colonise the surface of Petri dishes. Fourteen days after collection from the field site, areas of the bryozoan colonies displayed signs of malacosporean parasitism consistent with previous description (Morris *et al.* 2002a). Over the next three months, the development of myxozoan parasites within multiple bryozoan colonies was observed. Statoblasts were collected from infected areas of the colonies and examined by scanning electron microscopy (Figure 3.5), which together with analysis of colony morphology, determined the identity of the bryozoans as *Plumatella repens* using established identification keys (Mundy and Thorpe 1980; Ricciardi and Reiswig 1994). Attempts at germinating statoblasts from infected colonies proved unsuccessful.

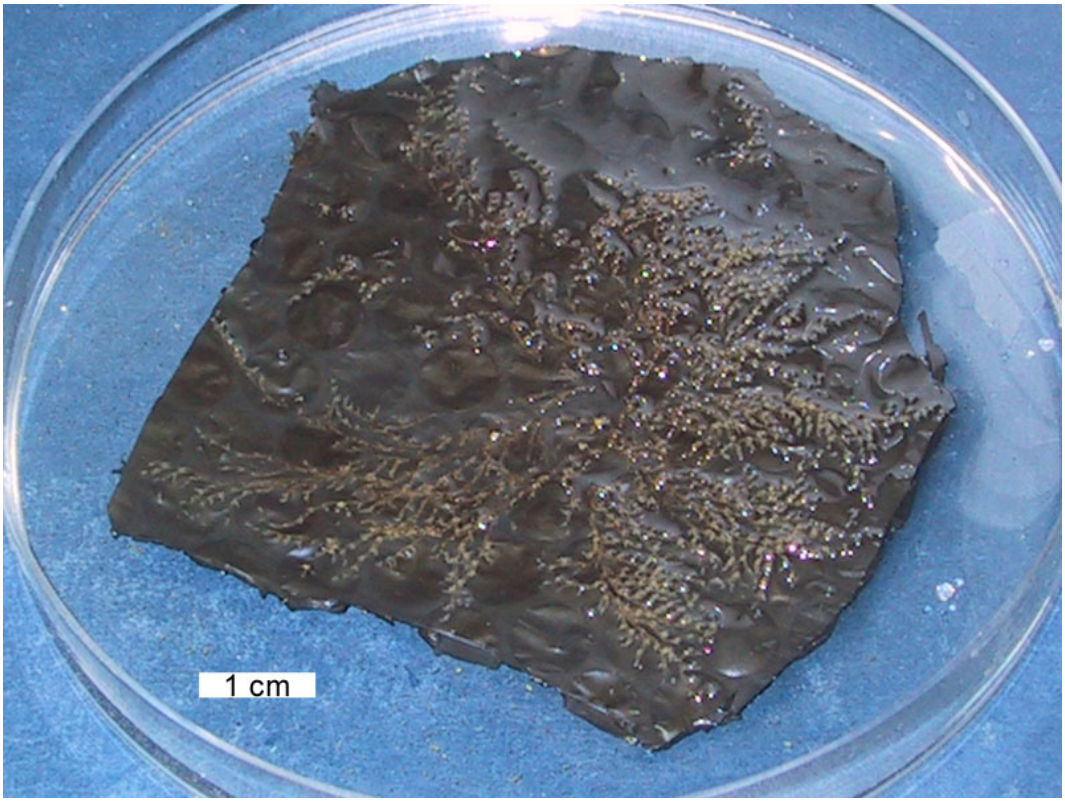

Figure 3.4: Bryozoa adhered to black plastic sheeting attached to a Petri dish

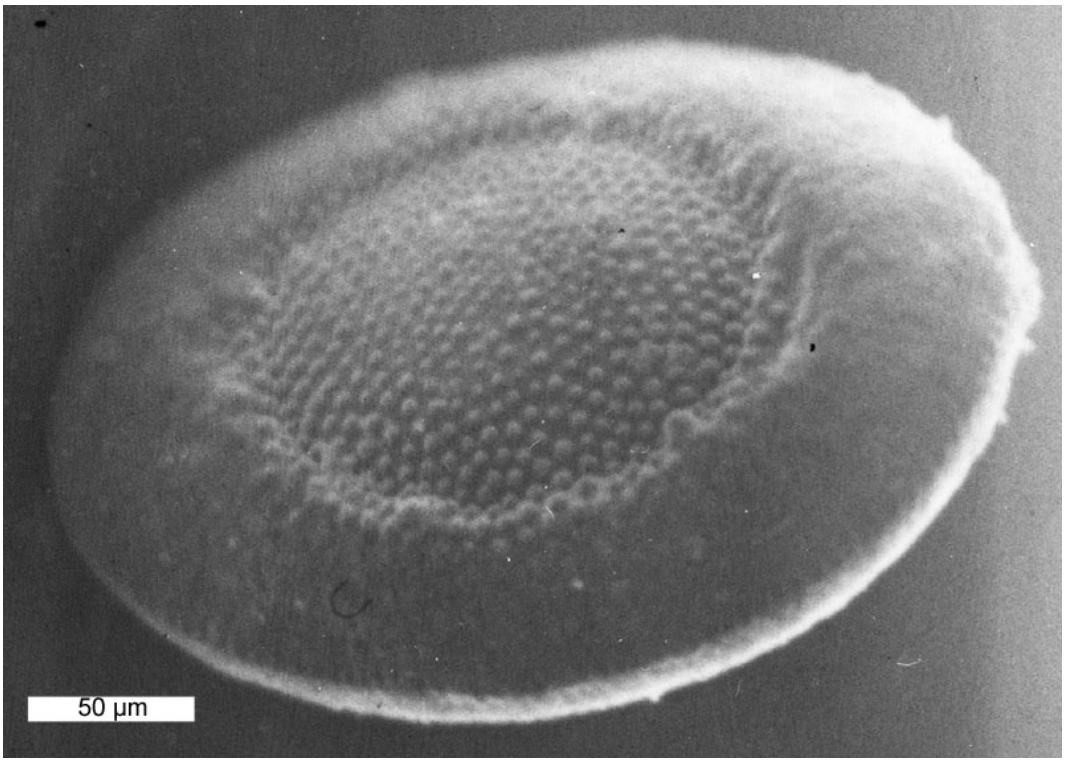

Figure 3.5: Scanning electron micrograph of a statoblast from a bryozoan colony infected with *Buddenbrockia plumatellae*

3.3.3. Examination of myxozoan development by examination of live bryozoan colonies using light microscopy

3.3.3.1. Initial recognition of malacosporean infection

Initially, vermiform stages of up to 2 mm in length of the malacosporean parasite *Buddenbrockia plumatellae* as previously described (Canning *et al.* 2002) were recognised within the bryozoans (Figure 3.6). Several areas of the colony were found to be infected with multiple translucent immature and opaque mature spore sacs present within the same unit (Figure 3.7). Whereas the former possessed empty internal lumina, the latter were packed with granular refractive bodies, presumably maturing myxozoan spores. Eleven days following the first observation of vermiform parasitic infestation, myxozoan spores with four polar capsules were visualised within the metacoel of parts of the colony. Infected bryozoan material was dissected and the mean diameter of the released spores was 17.67 μm (n = 29; s.d. = 0.75). Each spherical spore possessed an outer limiting membrane, four spherical polar capsules (Figure 3.8) and two cytoplasmic sporoplasms (Figure 3.9). Continuous waves of infection were witnessed, with various developmental stages being evident within individual colonies. These stages included small swirling particles (Figure 3.10), stages adherent to the peritoneal wall of the host (Figure 3.11 and Figure 3.12), and developing stages free within the coelom which matured into elongated spore sacs (Figure 3.13) becoming filled with spores.

Following observation of myxozoan infection, the growth rate of the infected colonies was noticeably reduced. In response to this, a higher concentration of algal and protozoal food components was prepared by centrifugation of cultures and added to the culture tank in an attempt at maximising the nutrition of the diseased bryozoans. Following this change in the culture conditions, an improvement in growth rate was witnessed, with the formation of new zooids evident even in heavily infected colonies.

3.3.3.2. Sequential development of *B. plumatellae* within a single bryozoan colony

Infection was observed in multiple independent colonies within the bryozoan material. Two weeks following the initial recognition of infection in the bryozoan population, early signs of parasitism were recognised in an individual zooid. This zooid was linked to another zooid which appeared to have degenerated but within which an immature elongated stage of *B. plumatellae* could be seen (Figure 3.14). The resulting colony which developed from the single zooid that was attached to the degenerate unit was studied for the next 77 days.

Days 0–8: Early swirling and budding peritoneal stages

On day zero, the first sign indicative of infection was the presence of numerous swirling particles in the coelom. Due to continual movement caused by the internal currents in the metacoel of the host, close examination of the cells proved problematic. However, these cells appeared irregular in shape and of approximately 2-7 µm in diameter (Figure 3.10). Additionally, at this stage circular buds of diameter 30-50 µm could be seen attached to the internal peritoneal body wall of the bryozoans (Figure 3.11 and Figure 3.14). On day four, irregularly round bodies of diameter 50-60 µm could be seen swirling in the metacoel. On the following day, oblong structures could be seen attached to the internal bryozoan body wall by irregularly shaped tips; these bodies passively swayed from side to side due to the flow of coelomic fluid within the bryozoan (Figure 3.12). These structures were subsequently seen to become increasingly elongated with a worm-like structure of length 300 µm being seen unattached within the metacoel on day eight.

Days 9–19: Vermiform stages including internal masses

At this stage the ‘worms’ appeared to comprise a defined body wall encapsulating an empty lumen. On day nine, bodies could be seen within the lumina of several

parasites (Figure 3.15). These irregularly round stages of diameter 15-40 µm were seen to be moved freely within the 'worm' with no attachments evident (Figure 3.16). Aggregates of these internalised bodies were evident on day 10 within parasitic 'worms' of up to 700 µm in length. The number of internalised cells – of increasing uniformity and regularity in size and shape – continued to increase to almost completely fill the lumina by day 14. The internal structures appeared to coalesce, forming an aggregate with a 'corrugated' appearance (Figure 3.17). The parasitised bryozoan colony consisted of two zooids at this time. On day 17, 'worms' of up to one mm in length were seen to be packed full of material (Figure 3.18 and Figure 3.19). An irregular projection with a roughened bulbous appearance was seen at the proximal end of one of the 'worms' within the bryozoan colony now comprising three zooids (Figure 3.20). Examination of the tip of this worm allowed visualisation of the outer membrane which was scalloped on its external surface.

Days 20–24: Spore formation

Twenty days after the first signs of infection, numerous round bodies of approximate diameter 18 µm were seen swirling within the colony now comprising four zooids (Figure 3.21). These bodies were seen to have distinctive refractive components, recognised as the polar capsules of myxozoan spores (Figure 3.22). The spores were seen to be present in all parts of the bryozoan coelom including some in close proximity to the lophophore. Upon retraction of the lophophore, it appeared that some of these were ejected from the bryozoan metacoel into the surrounding media, although the rapid speed of lophophore retraction made this difficult to visualise. Synchronous to the presence of spores, various early developmental stages could be seen, ranging from immature sacs containing only a few internalised stages, to fully developed spore sacs containing recognisable spores (Figure 3.23). Intact spore sacs were never observed being expelled from bryozoans despite extensive observation; instead it appeared that upon maturity of the sacs, spores were

released within the coelomic cavity of the host. Spores were seen to be lost from the bryozoans within 12 hours of appearance.

Days 25–31: Fragmentation of the bryozoan colony

On day 25, pinching of the peritoneum was seen between two adjacent zooids in the colony. By the following day, the stenosis of the coelomic cavity between the zooids was more pronounced, leaving a connecting channel of approximately 60 μm in diameter (Figure 3.24). This connection remained patent, with numerous spores disseminating from the established infected part of the colony to the distal extremities of new bryozoan growth. By day 28, the communication channel had been sealed resulting in fragmentation of the bryozoan colony. However, signs of ongoing parasitism were evident in each of the two newly demarcated colonies. Despite the high parasitic burden and segregation, the resulting bryozoan colonies continued to grow, with 10 zooids being present on day 31.

Days 32–77: Statoblast formation and ongoing waves of infection

On day 32, early stages of statoblast production were seen on the funiculus of one of the zooids. At this time, cells of approximate diameter 10-20 μm, presumed to be developmental stages of *B. plumatellae* could be seen attached to the peritoneal wall of parts of the bryozoan. Multiple stages of parasitic development, ranging from the presence of swirling particles to maturing spore sacs could be seen concurrently within the infected areas. By day 34, there was conspicuous pinching of the bryozoan peritoneum at various locations.

Mature statoblasts were seen in several parts of the infected bryozoan colonies 45 days after initial detection of parasitism. Several statoblasts were seen in conjunction with overt signs of infection, including the presence of small swirling particles of diameter 2-7 μm and irregularly round and elongated stages of length 50-100 μm

(Figure 3.25-Figure 3.27). On day 62, clear differences could be seen grossly between the infected and uninfected colonies on the Petri dish. The infected area appeared much more heavily fragmented, with fewer statoblasts evident, and looked relatively devitalised in comparison with an uninfected population of *P. repens* adhered to the same Petri dish (Figure 3.28). The infected colony became increasingly fragmented over the following 14 days, with no vital signs of bryozoan life being evident in any of the material after day 77. Statoblasts were removed from the dead colonies, but not successfully propagated.

3.3.4. Examination of semi-thin sections of *P. repens* infected with *B. plumatellae*

Upon examination of semi-thin sections of infected *P. repens*, multiple 'worms' of *B. plumatellae* could be seen, possessing characteristics as described in previous reports (Canning *et al.* 2002; Okamura *et al.* 2002; Morris *et al.* 2002a). Within the metacoel of individual bryozoans, multiple 'worms' of varying maturity could be seen, ranging from immature stages possessing empty lumina to mature sacs filled with spores containing conspicuous twin sporoplasms (Figure 3.29-Figure 3.31). The walls of the immature sacs were composed of three defined layers: the outermost a fine sinuous membrane, the middle composed of muscle blocks interspersed by connective tissue, and the innermost constituting a layer of cuboidal cells (Figure 3.32). In some, presumably more developed sacs, accumulations of cells similar to those of the innermost layer could be seen unattached within the lumen (Figure 3.33).

Further developed sacs of diameter greater than 60 µm contained irregularly round solidly-stained rudimentary spores of diameter 6-9 µm (Figure 3.34). While the distance between muscle blocks was increased, no internal layer of mural cells could be discerned. Within the same bryozoan, multiple larger 'worms' could be seen

containing distinctive mature spores (Figure 3.32 and Figure 3.33). While the morphology of the sacs had changed little, the spores now appeared hollow as opposed to solid. Within the irregularly shaped spores of diameter 10-15 μm, dark-stained bodies could be seen resembling myxozoan sporoplasms. Within some single 'worms', both solidly-stained and hollow spores could be seen suggesting that the latter was a sequel to the former (Figure 3.35).

Within one part of the bryozoan, a circular encapsulated mass of possible parasitic origin of diameter 27 μm was observed (Figure 3.36). This area was further examined using transmission electron microscopy (TEM).

3.3.5. Ultrastructural examination of *P. repens* infected with *B. plumatellae*

Ultra-thin (90 nm) sections of the blocks described in Section 3.3.4 were stained with uranyl acetate and lead citrate, and examined by TEM. A range of developmental stages of *B. plumatellae* was examined.

3.3.5.1. Morphology of spores

Present within mature 'worms' were multiple spores in which up to four distinctive polar capsules and two sporoplasms could be seen (Figure 3.37). Each mature spherical polar capsule (diameter 1.4 μm) was encompassed with an electron-lucent outer halo and consisted of a dense granular central matrix containing a coiled polar filament of thickness 50 nm (Figure 3.38). Up to four turns of the filament could be seen within the spores examined, each turn appearing as paired sigmoid flexures of the filament as it passed through the plane of section. In one polar capsule, the distal termination of the filament could be seen contacting and traversing the electron-lucent halo (Figure 3.39). At this point, the filament was contiguous with two prongs of a mushroom-shaped plug of material sited between the filament and the exterior. The electron-dense outer layer of the spore was discontinuous over this area,

leaving a fine layer of material between the polar filament plug and the metacoel of the host. The polar capsules were contained within capsulogenic cells comprising multiple organelles including nuclei and mitochondria. The gap junctions between adjacent capsulogenic cells appeared to be regularly delineated, demarcating four evenly spaced capsules, suggesting that two possible axes of symmetry might exist in the spores (Figure 3.40 and Figure 3.41).

3.3.5.2. Morphology of mature spore sacs

The mature spores were contained within thinly walled vermiform sacs (Figure 3.37), with occasional spores seen in the bryozoan metacoel outwith the spore sacs (Figure 3.42). The mural layers of the sacs mentioned in Section 3.3.4 were further examined. The outer layer was composed of vacuolated cells possessing many finger-like external protrusions. An inner layer of longitudinal muscle blocks contained multiple mitochondria with distinctive tubular cristae (Figure 3.43), while between the two layers was a matrix forming a basal lamina. Occasional pale cells were interspersed between the more abundant denser cells of the external mural layer (Figure 3.44). These former cells possessed less prominent external projections, although they contained numerous electron-dense bodies and vacuoles including a cytoplasm similar in appearance to the adjacent basal lamina.

3.3.5.3. Putative malacosporean cells within bryozoan tissue

In one area of bryozoan tissue, an encapsulated mass of cells was observed (Figure 3.45, *cf.* Figure 3.36). This mass of cells was poorly differentiated, but the outer layer comprised vacuolated cells possessing external projections. The internal content of the mass was composed of an irregular network of undifferentiated material, vacuolated cells, occasional pale-stained cells (consistent with those observed interspersed in the wall of maturing 'worms', *cf.* Figure 3.44), and discrete accumulations of material similar to the basal lamina. The mass was surrounded by

fine strands of material – presumably of host origin – forming a pseudocapsule. Examination of the adjacent bryozoan peritoneum showed a layer of vacuolated cells, well-organised basal lamina and muscle cells: apparently demonstrating morphological similarities between the mural structures of host and parasite. These similarities could also be observed in an area where a spore sac containing immature spores was sited close to bryozoan peritoneum – which could be identified by the presence of numerous adjacent cilia (Figure 3.46). Although the bryozoan tissue did possess external projections they were neither as well defined nor distinctive as in the myxozoan tissue. However, the general mural architecture was consistent between the two organisms, featuring layers of basal lamina and discrete muscle blocks.

Throughout the bryozoan body wall, occasional areas were noted where electron-lucent pale cells were visible (Figure 3.47). These cells appeared consistent with those seen in the wall of developing 'worms' (*cf.* Figure 3.44), each containing a single granular nucleus with a dense nucleolus, multiple vacuoles, mitochondria, and numerous electron-dense bodies (many of which appeared crescent-shaped) (Figure 3.48). These pale cells were intimately associated with the host basal lamina, the vacuolar contents staining similarly to the basal lamina (Figure 3.49 and Figure 3.50). Figure 3.51 shows an area of apparent communication between a cellular vacuole and the surrounding basal lamina, possibly representing parasitic engulfment of host material.

3.3.5.4. Early developmental stages of spore sacs

Immature spore sacs were observed with their lumina packed full of undifferentiated cells (Figure 3.52). The external wall of these developing sacs was readily recognisable – featuring vacuolated cells with copious external dactylate projections – however, the muscle layer, basal lamina and internal layer of cells were not

evident. Some of the vacuoles appeared electron-lucent, while others contained homogenous material. Some organised accumulations of undifferentiated cells were noted with no apparent outer lining of vacuolated cells (Figure 3.53). In some of these immature spore sacs, a layer of basal lamina could be seen aggregated around the internal periphery of the developing stages (Figure 3.54 and Figure 3.55). There were large accumulations of material within the numerous vacuoles of the outer cellular layer of the sac.

3.3.5.5. Immature spore sacs

In further developed spore sacs, no undifferentiated cells were evident within the lumina, however, defined mural cell layers could be discerned (Figure 3.56). The outer layer featured the characteristic external projections, although fewer vacuoles were recognised than in more primitive stages. A layer of basal lamina lay between the outer layer and muscle blocks enclosing a well defined internal layer of polygonal cells. Within some sections, accumulations of cells could be seen within the otherwise empty lumina of immature spore sacs (Figure 3.57 *cf.* Figure 3.33). The majority of these intraluminal cells appeared morphologically similar to the cells comprising the inner layer of the wall of the 'worm', with discrete zones of basal lamina also apparent in the mass. In other sections, multiple intraluminal cells of diameter 0.9-6 µm could be seen, some apparently budding from inner mural cells (Figure 3.58). Subsequently, the inner mural layer of cells was lost, and an apparent division of cells into uniformly sized (6 µm) irregularly round cells could be seen (Figure 3.59). In more developed sacs, larger cells of diameter 7 µm (presumably immature spores) became distinct while smaller unattached round cells of diameter 1.5 µm could also be seen within the lumen (Figure 3.42). In transverse section, four discrete muscle blocks were noted in the wall of the sac. In all of the developmental stages, the outer wall of the 'worm' remained consistent in structure showing external dactylate projections and vacuolation, although the vacuoles appeared more

electron-lucent in later stages. In some maturing spore sacs, recognisable myxozoan spores (featuring polar capsules) were present in tandem with immature spore stages suggesting that these stages were sequential and that the developmental cycle culminated at this point (Figure 3.37 *cf.* Figure 3.35).

3.3.6. Attempted horizontal transmission of *B. plumatellae* between bryozoans

3.3.6.1. Direct injection of coelomic contents from *B. plumatellae*-infected to uninfected bryozoans

No recognisable sign of *Buddenbrockia* infection was seen in any of the injected colonies which remained viable despite the invasive procedure. The method was repeated and again no sign of infection was recognised in the recipient colonies.

3.3.6.2. Exposure of *B. plumatellae* material to uninfected *P. repens*

As a controlled exposure method, culture medium surrounding infected bryozoans was flushed onto a Petri dish containing uninfected bryozoan colonies. No sign of *B. plumatellae* infection was noted after 30 days culture.

Several of the Petri dishes were found to have both uninfected and infected populations of *P. repens* adhered. Despite this constant cohabitation for up to 97 days, none of the uninfected colonies developed characteristic signs of infection. In fact, greater vitality was evident in the uninfected than infected specimens (Figure 3.28).

3.3.7. Exposure of *B. plumatellae* spores to trout mucus

Spores exposed to slides coated with mucus from pre-clinical PKD-affected or PKD-recovered or PKD-naïve rainbow trout were all seen to alter morphologically, resulting in recognisable firing of polar filaments (Figure 3.60). The firing was observed in a large number of the spores 10-15 min after exposure. The mean

diameter of the spores following release of the filaments was 17.63 µm (n = 9, s.d. = 1.43) with mean length of the extruded polar filaments measuring 11.98 µm (n = 6, s.d. = 0.77). None of the spores exposed to the control slide were seen to fire their polar filaments within the period of examination.

3.3.8. Experimental exposure of rainbow trout to *B. plumatellae* spores

No sign of infection was seen upon examination by immunohistochemistry of kidney, spleen and liver samples from all six rainbow trout exposed to *B. plumatellae* spores or the six fish in the control group.

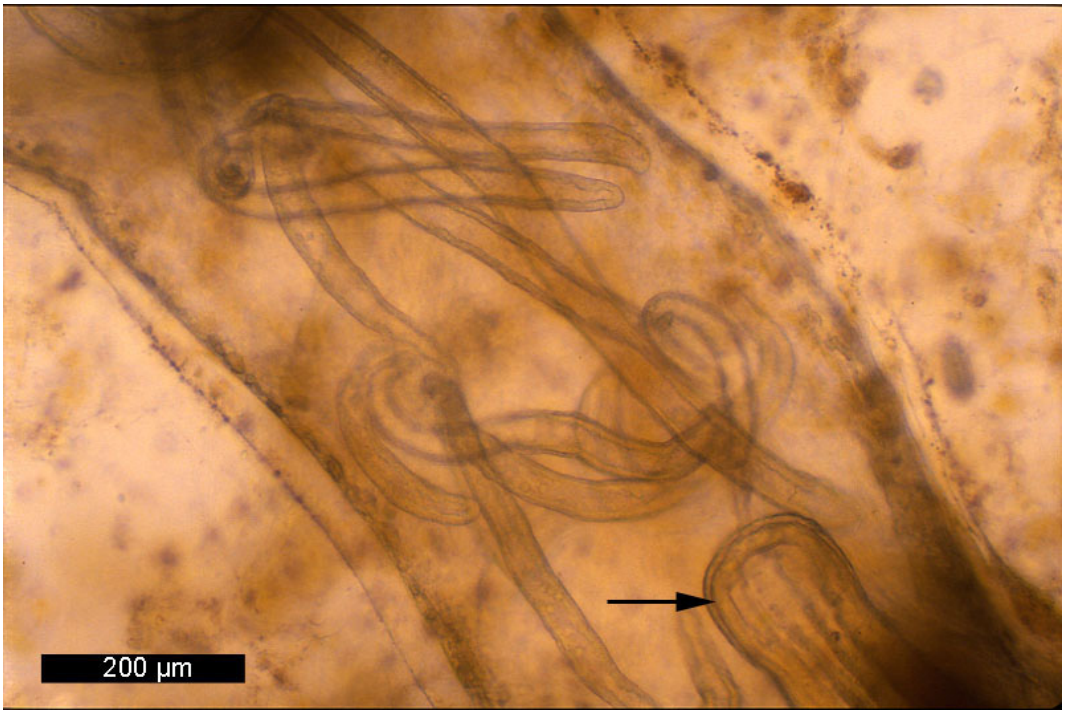

Figure 3.6: Multiple vermiform stages of *B. plumatellae* within a zooid of the bryozoan *Plumatella repens*

Arrow = caecum of bryozoan intestine.

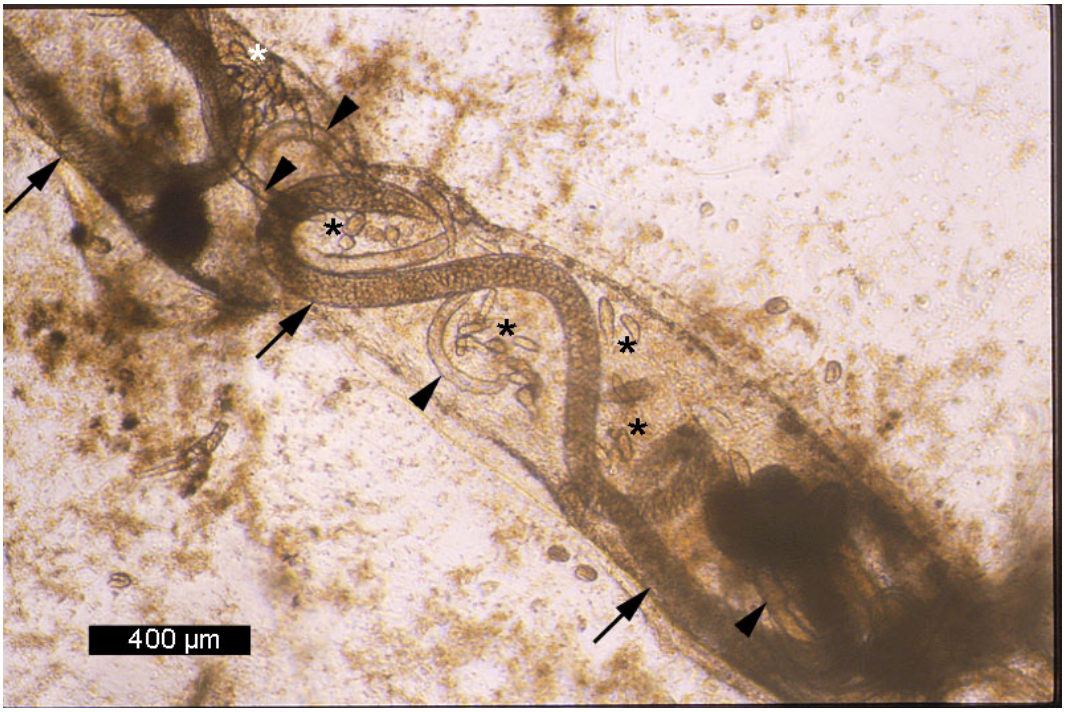

Figure 3.7: Immature and mature parasitic stages within a single zooid

Immature stages, including rudimentary sacs ("*") and undeveloped 'worms' (arrowheads) appear translucent, whereas mature spore-filled 'worms' appear relatively opaque (arrows).

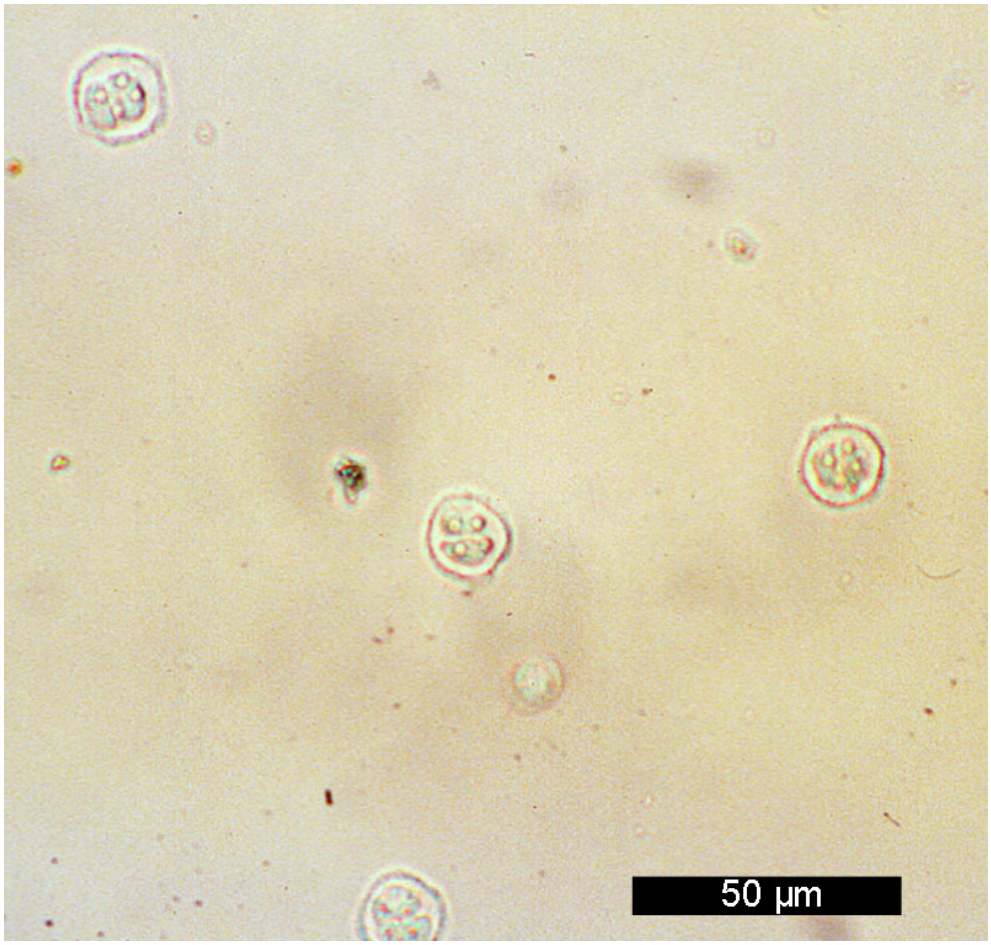

Figure 3.8: Dissected spores of *B. plumatellae* from *P. repens*

Each spore contains four refractive spherical polar capsules.

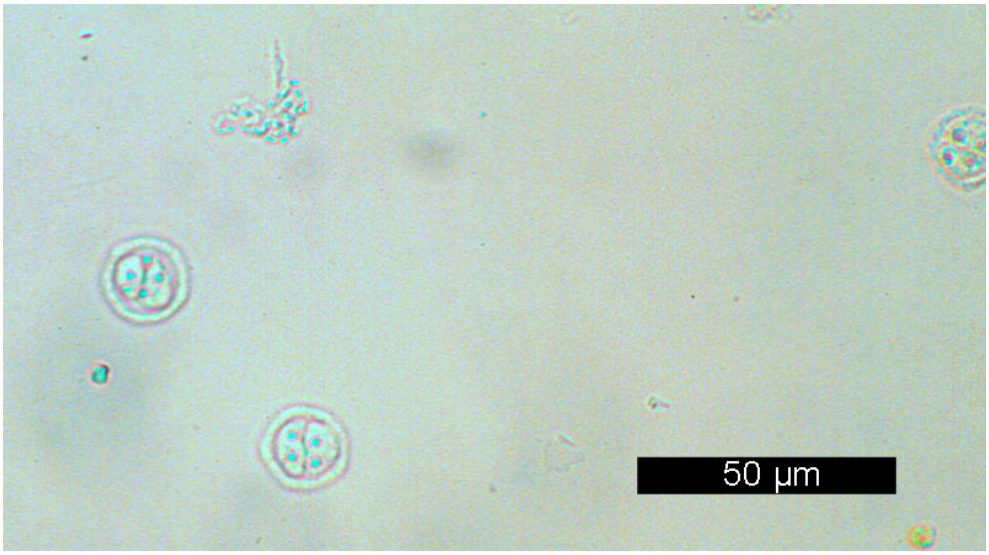

Figure 3.9: Dissected spores of *B. plumatellae* from *P. repens*

Contained within well-defined outer membranes, each spore possesses two germinative sporoplasms.

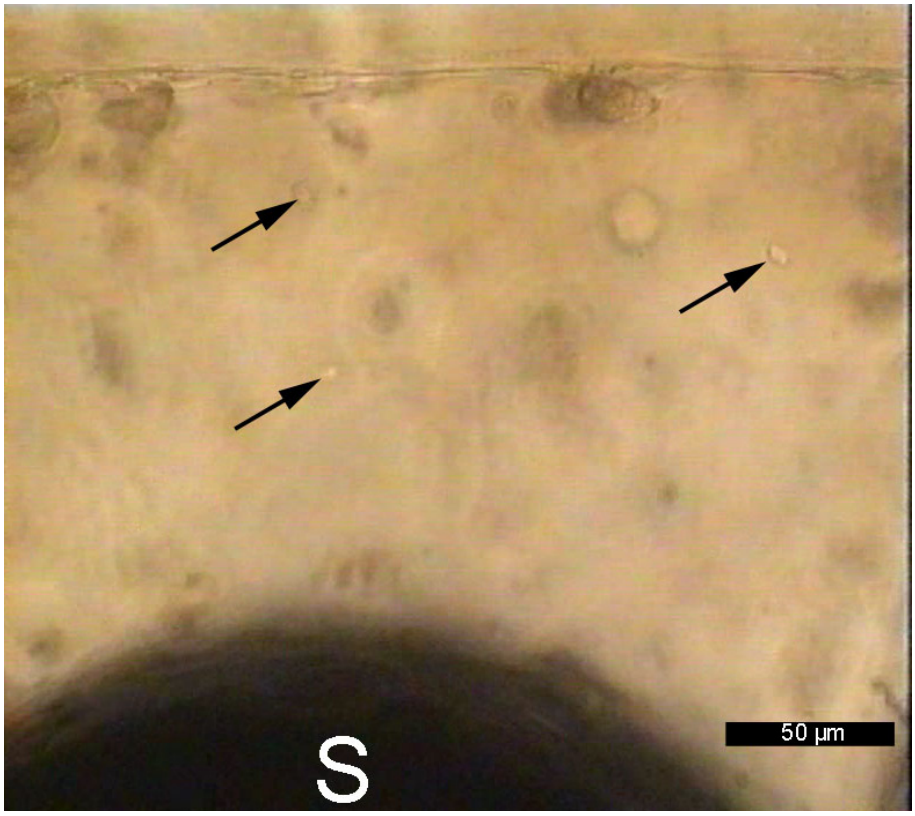

Figure 3.10: Early development of *B. plumatellae* in *P. repens*

Swirling particles are denoted by arrows; “s” = bryozoan statoblast.

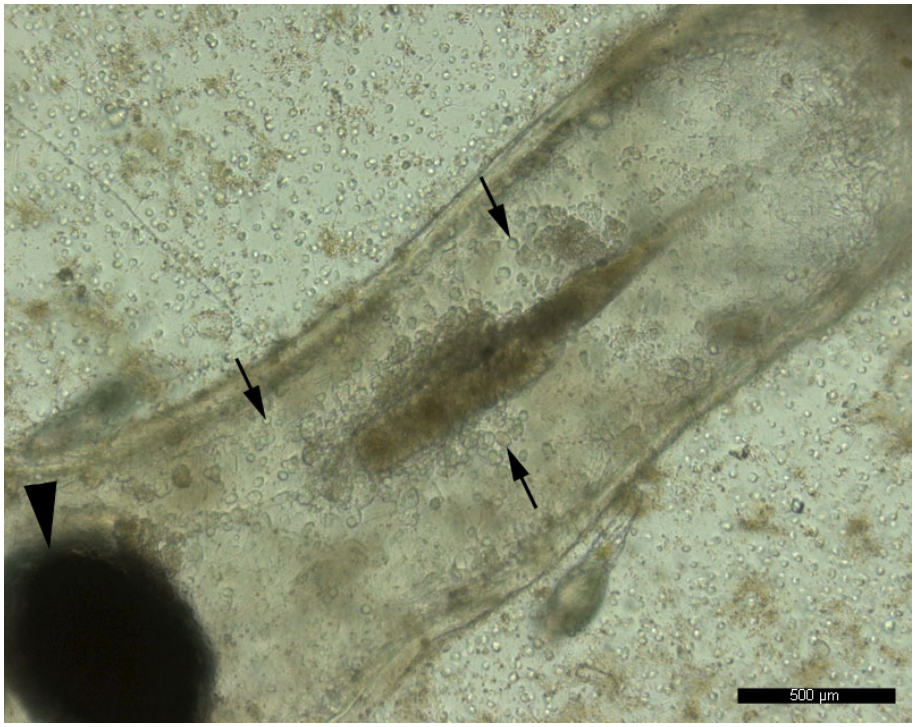

Figure 3.11: Early stages of *B. plumatellae* attached to bryozoan peritoneum

Arrows = budding stages attached to peritoneum; arrowhead = statoblast;

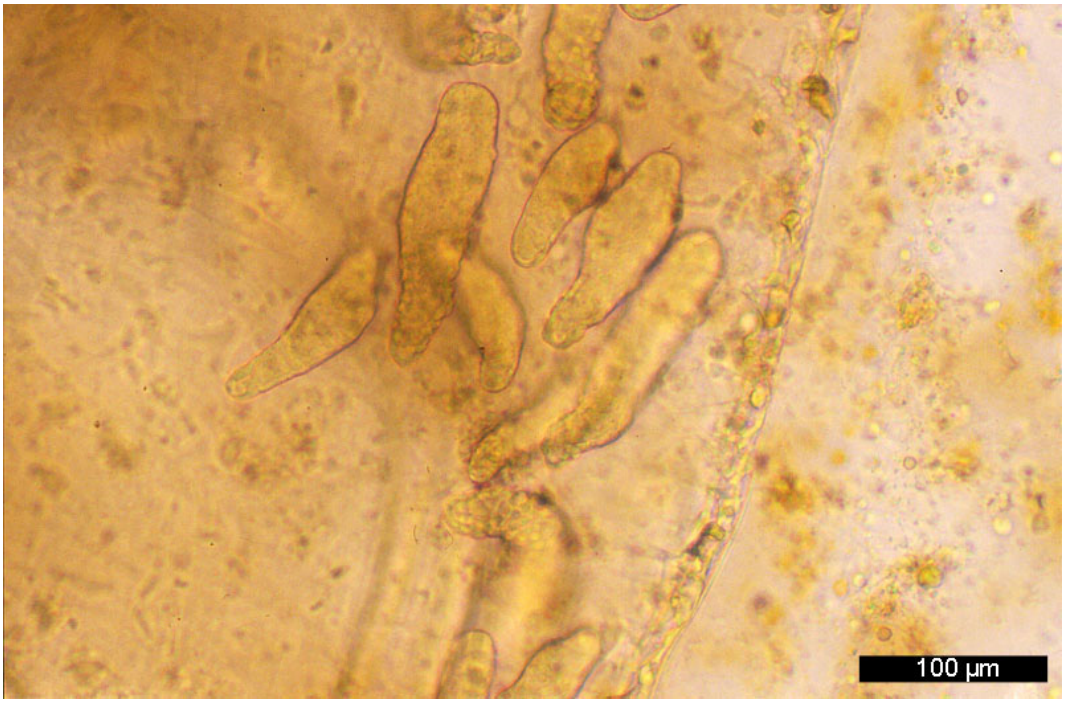

Figure 3.12: Immature attached stages of *B. plumatellae*

These elongated pyriform stages were still attached to the bryozoan peritoneum by irregularly surfaced areas at their thinner proximal ends. The sacs were uniformly orientated upwards due to the current of the metacoel swaying the unattached distal ends.

Figure 3.13: Multiple immature spore sacs within *P. repens*

Some of these stages swirled freely within the metacoel, while others appeared tethered to the bryozoan peritoneum.

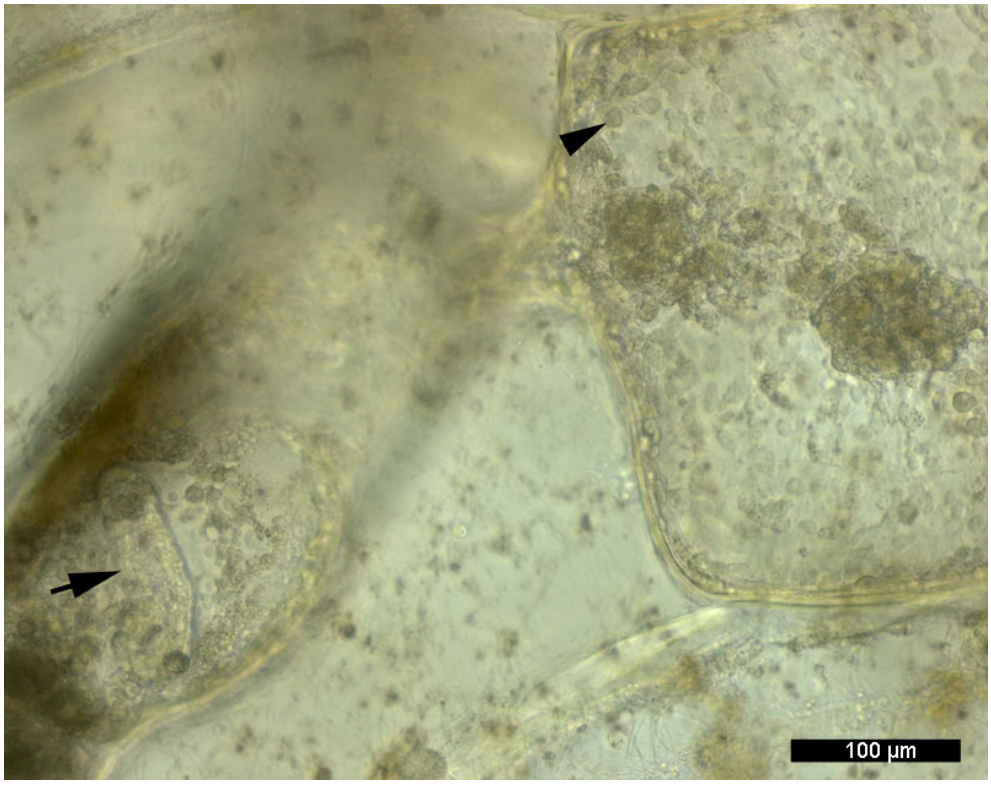

Figure 3.14: Single zooid connected to degenerated infected zooid

Many small round cells (arrowhead) could be seen attached to the peritoneal wall of a zooid linked to another degenerate zooid containing an elongated stage of *B. plumatellae* (arrow); numerous swirling particles could also be seen in the zooid on the right (not visible in this picture).

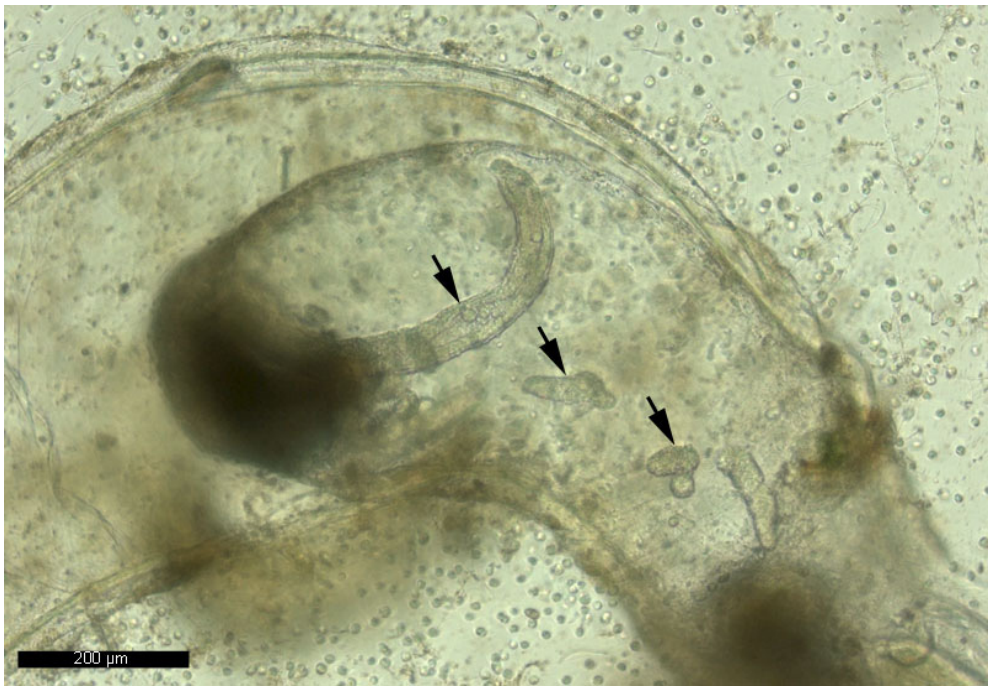

Figure 3.15: Multiple developmental stages of *B. plumatellae* within *P. repens*

Arrows = *B. plumatellae*; top arrow = vermiform sac containing internal bodies.

Figure 3.16: Internal spherical masses within 'worm' of *B. plumatellae*

Arrow = internal stages which move freely during writhing movement of the 'worm'.

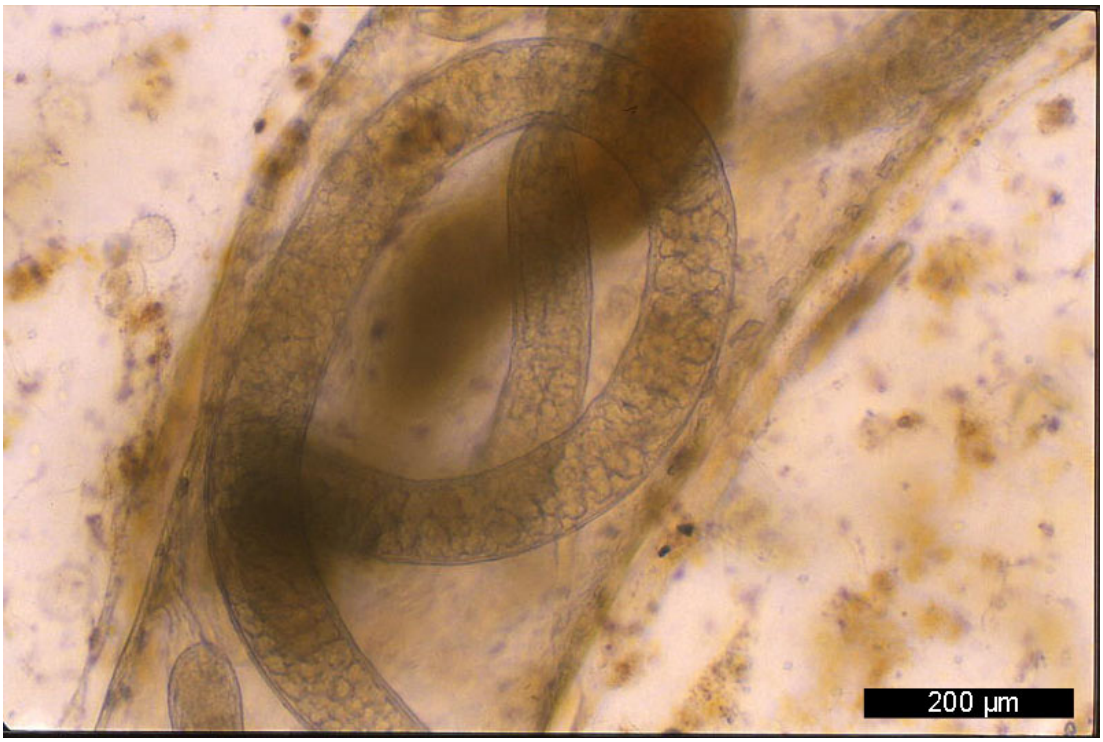

Figure 3.17: Further differentiation of vermiform stages

Internal contents of 'worm' further differentiated to show a 'corrugated' appearance.

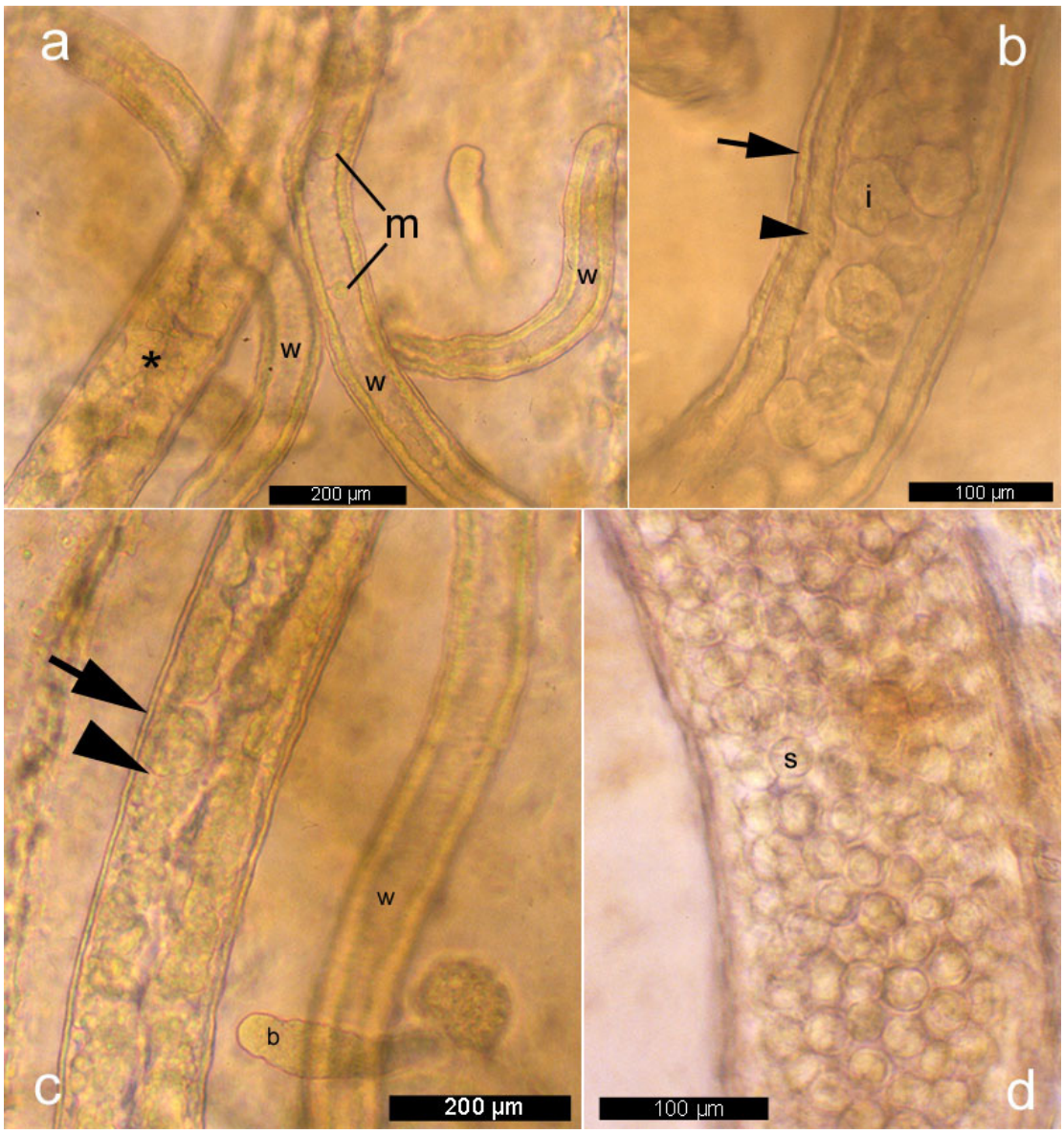

Figure 3.18: *B. plumatellae* 'worms' at various stages of development

(a) Immature 'worms' ("w") are seen with lumina, one of which contains internal masses ("m"), and a more developed 'worm' can also be seen ("*").
(b) Well-defined outer (arrow) & inner (arrowhead) mural layers with irregular luminal bodies ("i").
(c) Outer layer (arrow) is still defined, inner layer (arrowhead) is thickened, the lumen is diminished, a formative bud ("b") and immature 'worm' ("w") can also be seen.
(d) Almost mature spores ("s") are observed packing the lumen.

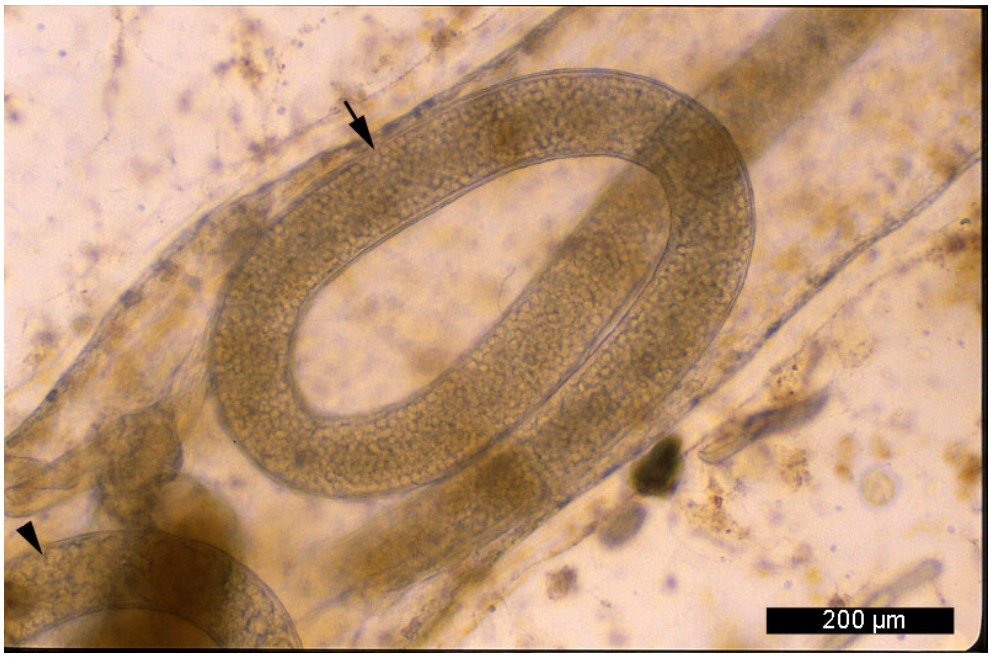

Figure 3.19: Coiled maturing spore sac

Arrow = almost mature sac containing discernible spores;
arrowhead = immature spore sac showing corrugated appearance as in Figure 3.17.

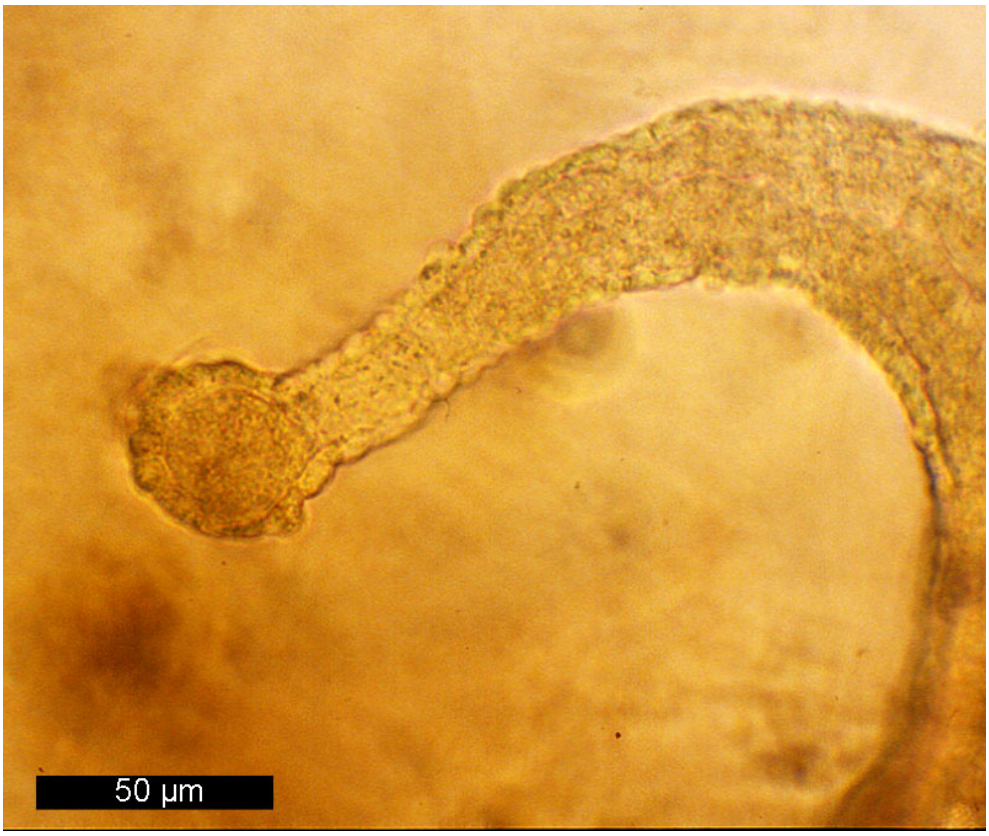

Figure 3.20: Bulbous tip of developing vermiform spore sac

The outer membrane of the worm can be clearly discerned near the tip.

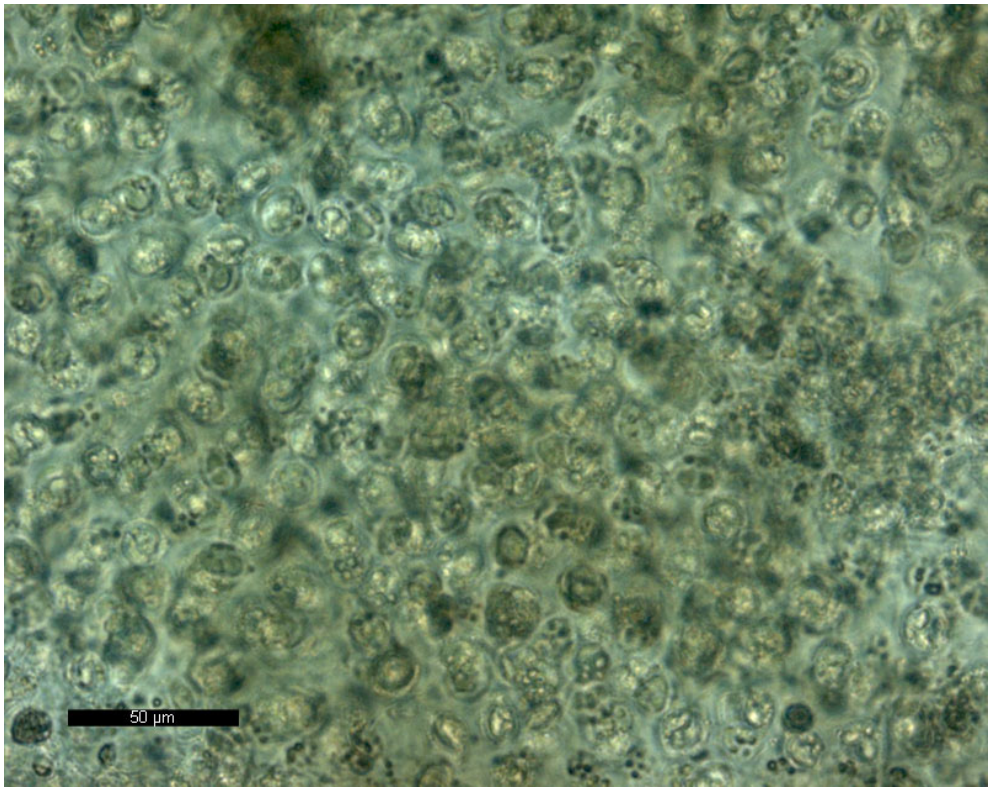

Figure 3.21: Released *B. plumatellae* spores packed within bryozoan metacoel

Figure 3.22: Released *B. plumatellae* spores within bryozoan

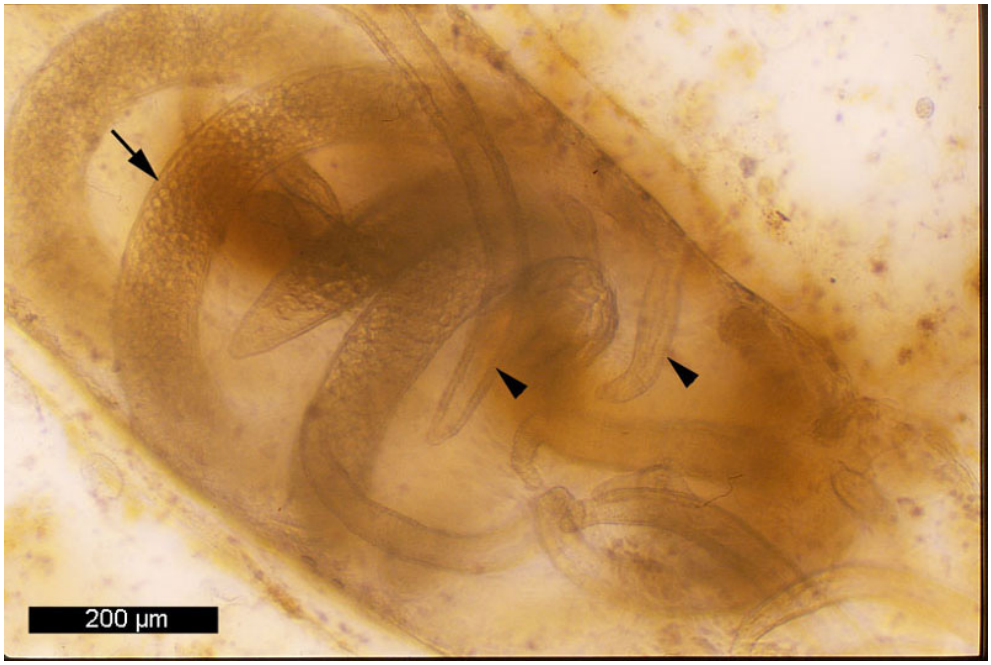

Figure 3.23: Various stages of development within one zooid

Arrow = mature spore sac; arrowheads = immature empty sacs.

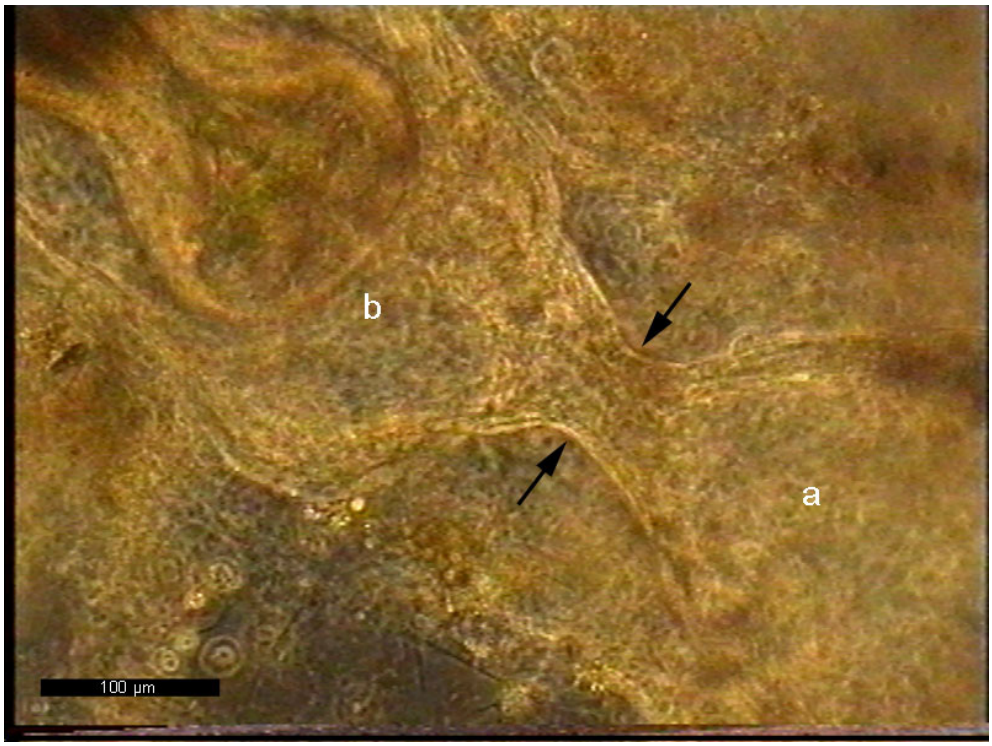

Figure 3.24: Stenosis of the connection between the coelomic cavities of two adjacent zooids in an infected colony of *P. repens*

Although the peritoneum was pinched (arrows), spores travelled freely from the part of the colony with established infection ("a") to a more recently formed zooid ("b").

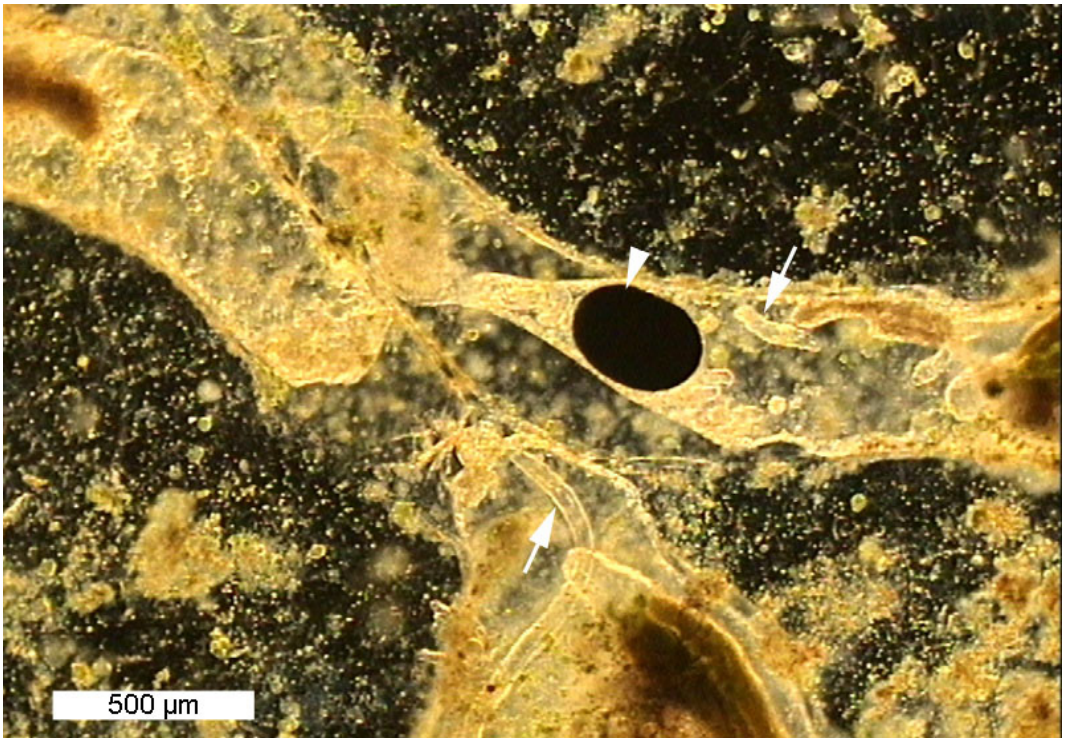

Figure 3.25: *B. plumatellae* infection in parallel with statoblast production

Arrows = parasitic stages; arrowhead = statoblast.

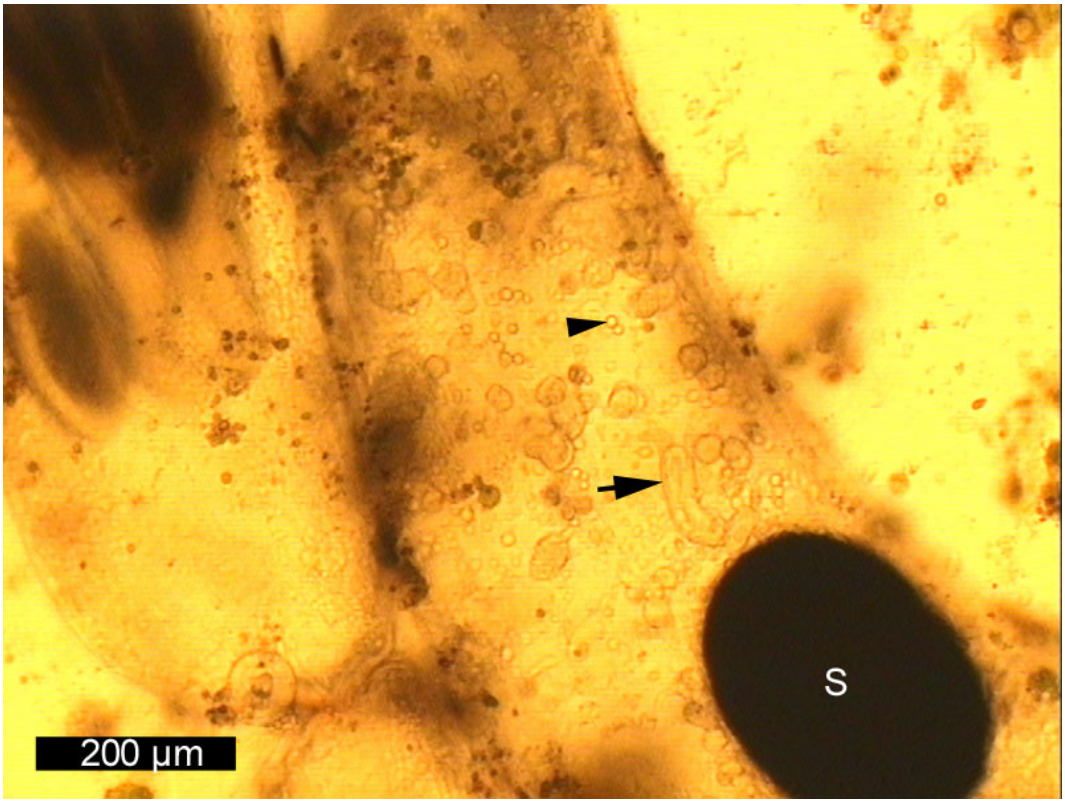

Figure 3.26: Various stages of development alongside statoblast formation

Small round cells (arrowhead) can be seen attached to the host peritoneum, while larger round and elongated stages (arrow) were also seen in this zooid exhibiting statoblast ("s") production.

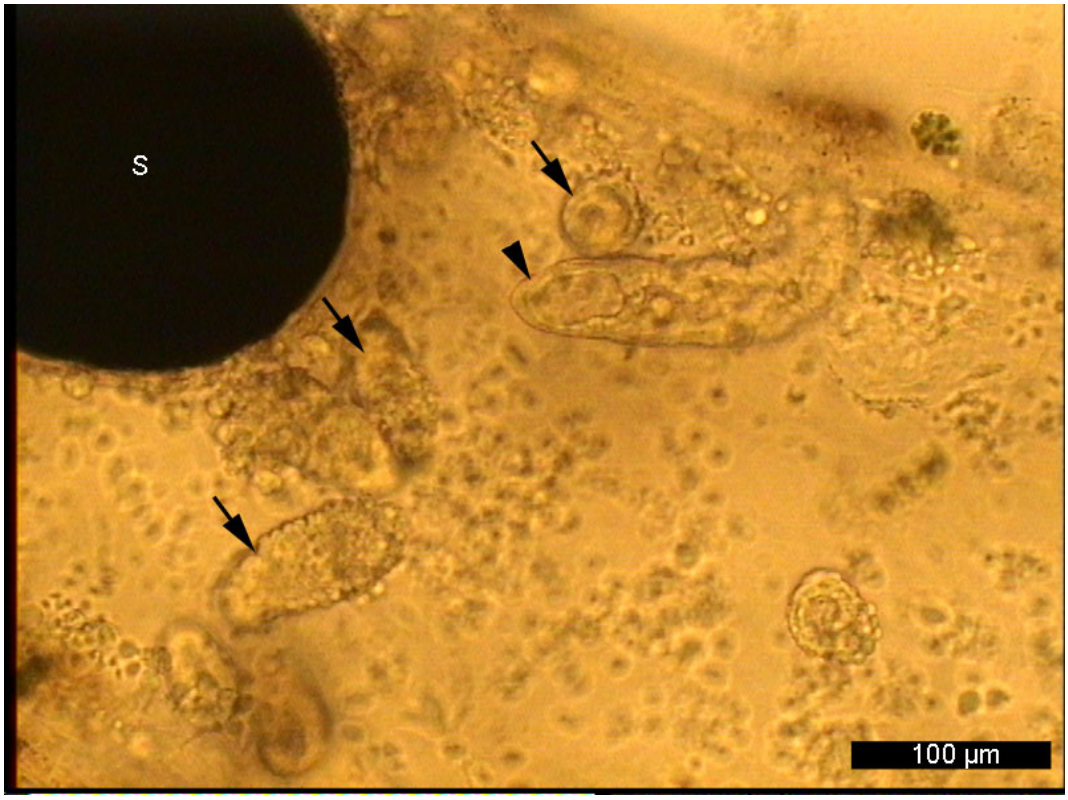

Figure 3.27: Parasitic stages in a zooid containing a mature statoblast

Arrows = immature parasites; arrowhead = further developed sac with hollow proximal end; "s" = statoblast. There are multiple small stages attached to the bryozoan peritoneum.

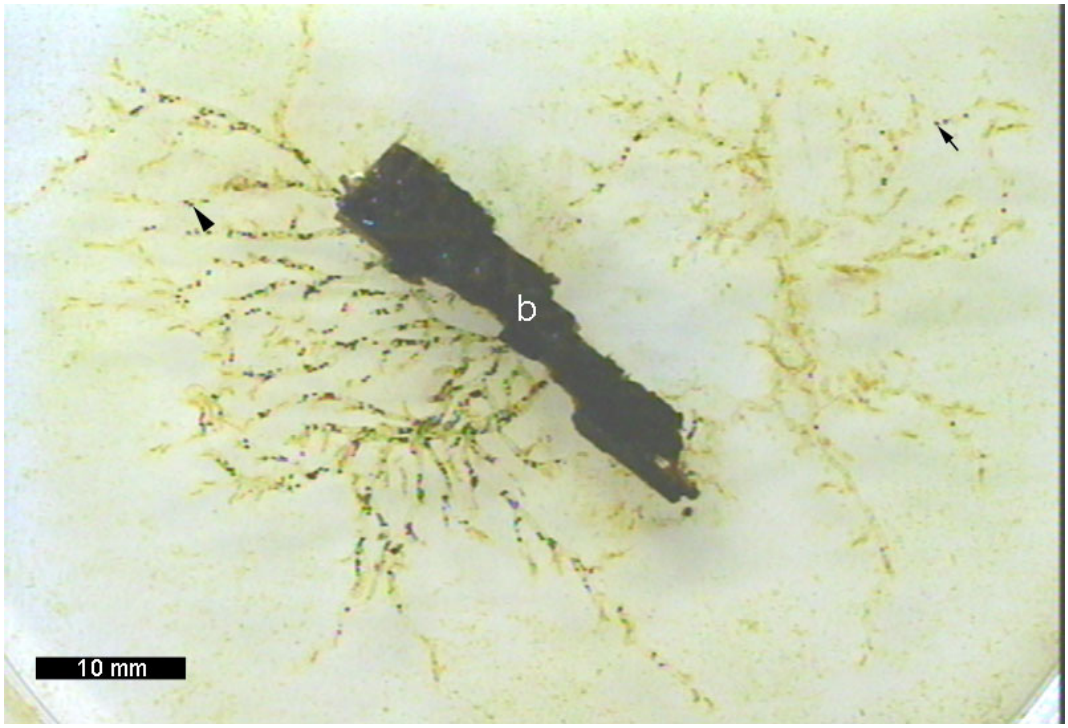

Figure 3.28: Surface of Petri dish with adhered colonies of *P. repens*

Colonies on left are uninfected, while those on the right are infected with *B. plumatellae*; "b" = bark of wood from which bryozoan colonies originated; arrowhead = statoblasts within uninfected healthy bryozoan colonies; arrow = statoblasts within infected bryozoan colonies showing signs of fragmentation and diminished statoblast formation.

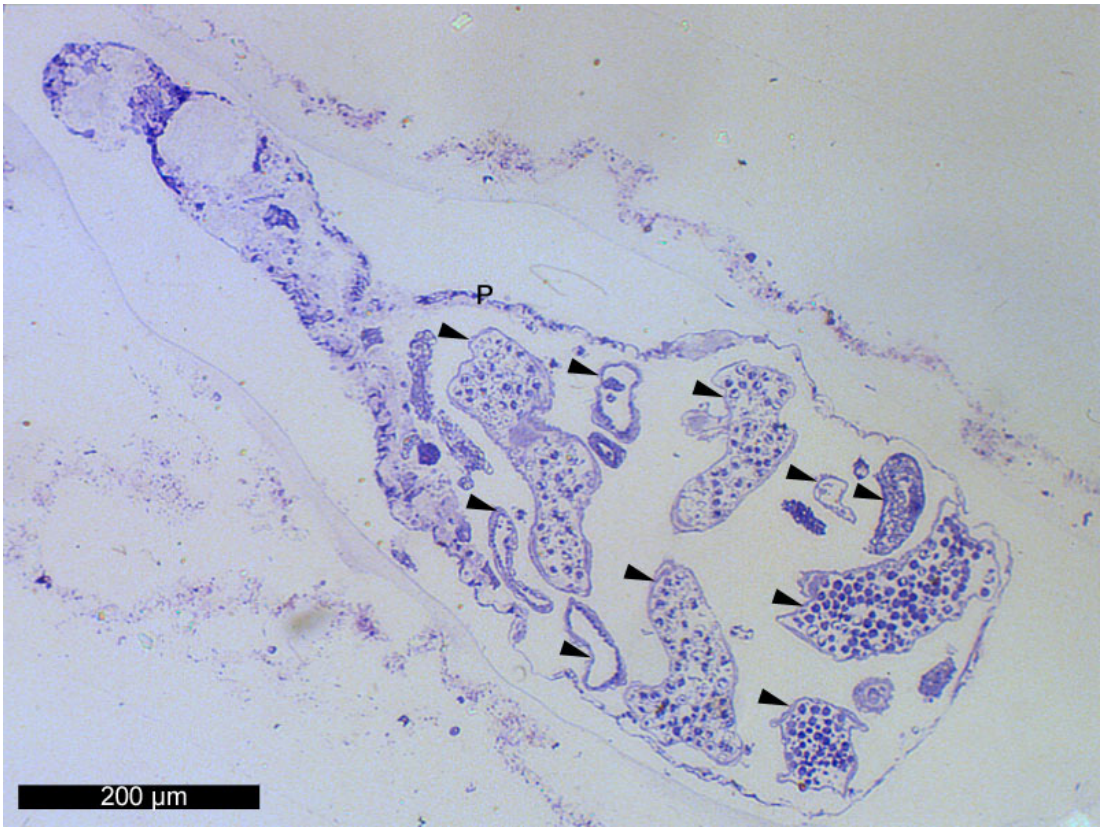

Figure 3.29: Semi-thin section of *P. repens* infected with *B. plumatellae*

Arrowheads = sacs of *B. plumatellae* at various stages of development; "p" = bryozoan peritoneum. (toluidine blue)

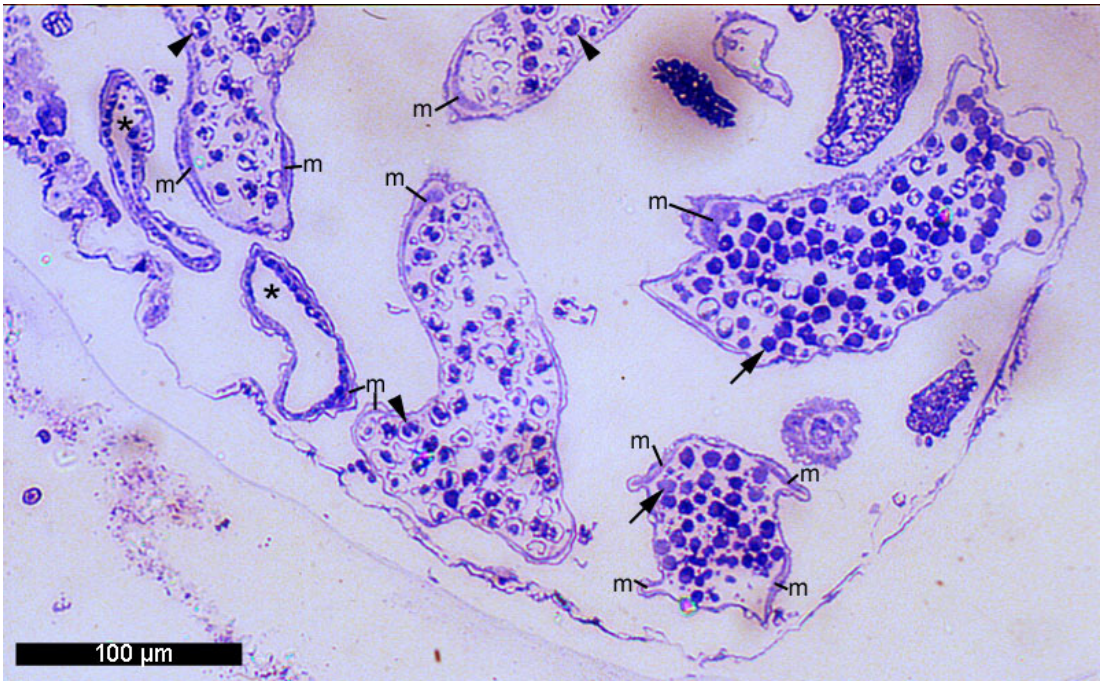

Figure 3.30: Immature and mature sacs of *B. plumatellae* within one zooid

"*" = immature empty sacs; arrows = immature densely-stained spores; arrowheads = mature spores possessing two sporoplasms; "m" = muscle blocks. (toluidine blue)

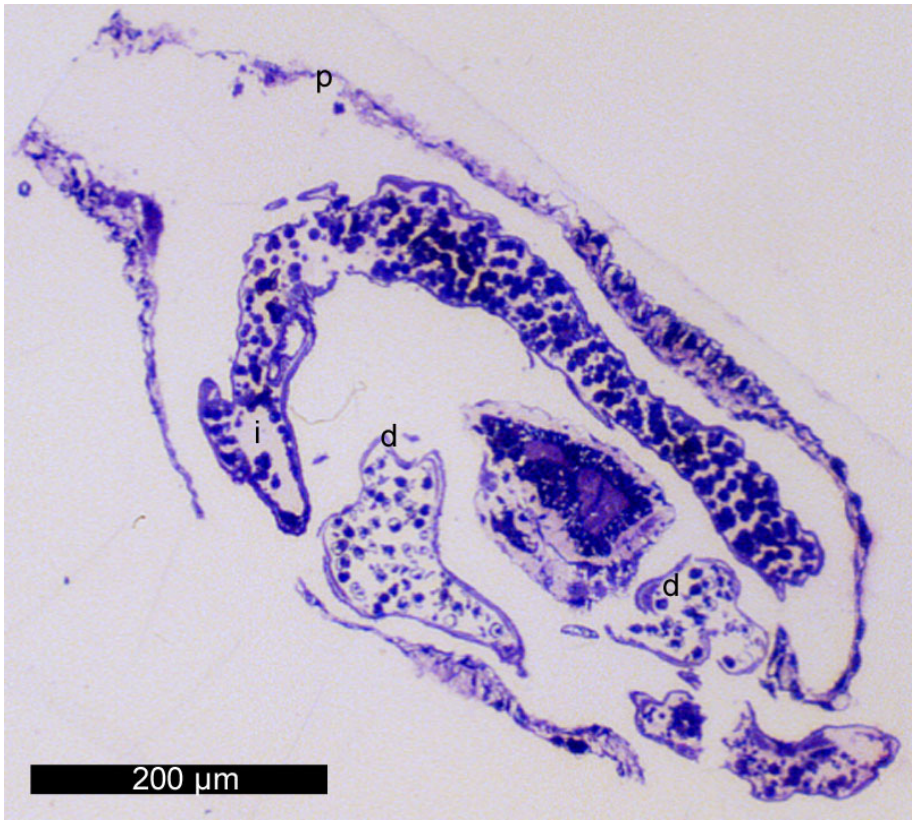

Figure 3.31: Immature and differentiated spore sacs of *B. plumatellae*

"i" = immature 'worm'; "d" = differentiated spore sacs; "p" = bryozoan peritoneum. (toluidine blue)

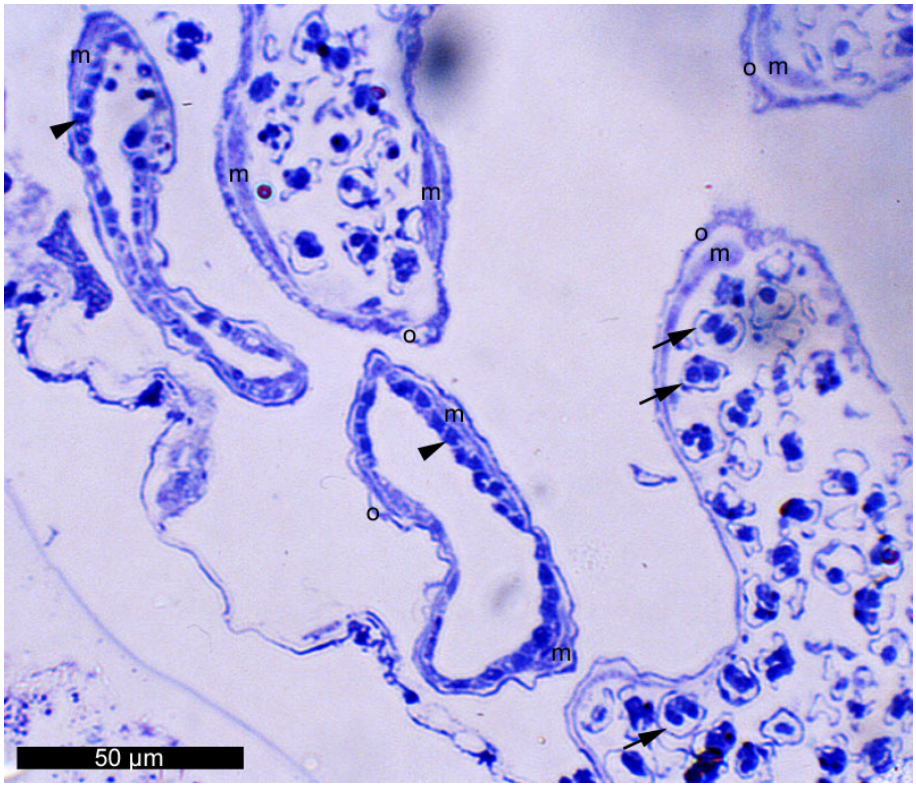

Figure 3.32: Multiple immature and mature spore sacs

Arrowheads = cuboidal cells of the internal mural layer of empty immature sacs; "m" = muscle blocks; "o" = outer mural layer; arrows = mature spores within differentiated sacs. (toluidine blue)

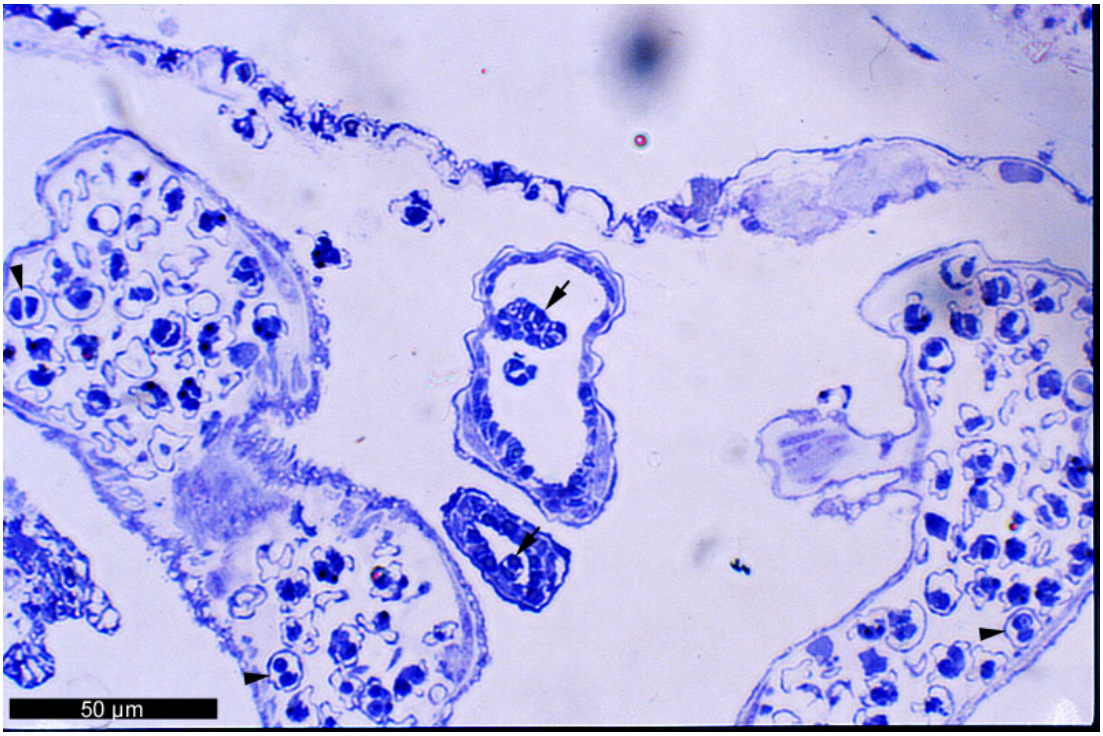

Figure 3.33: Internal masses within cross sections of immature spore sacs

Arrows = internal accumulations of cells within 'worms'; arrowheads = mature spores. (toluidine blue)

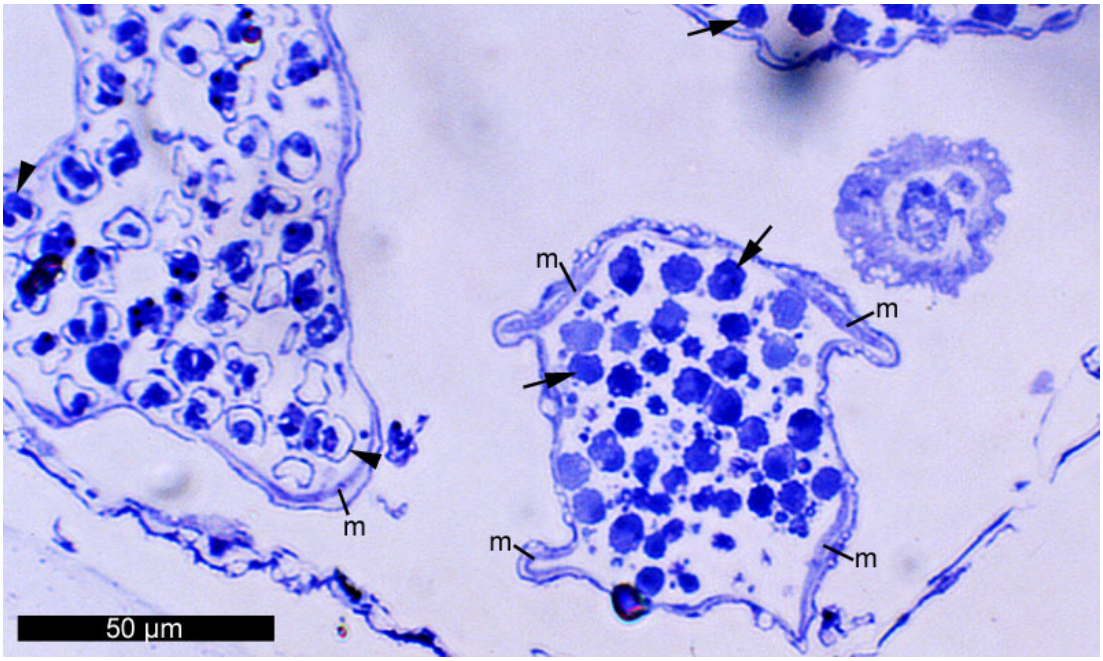

Figure 3.34: Cross section of immature spore sac and adjacent mature sac

Arrows = immature spores; "m" = muscle blocks; arrowheads = mature spores. (toluidine blue)

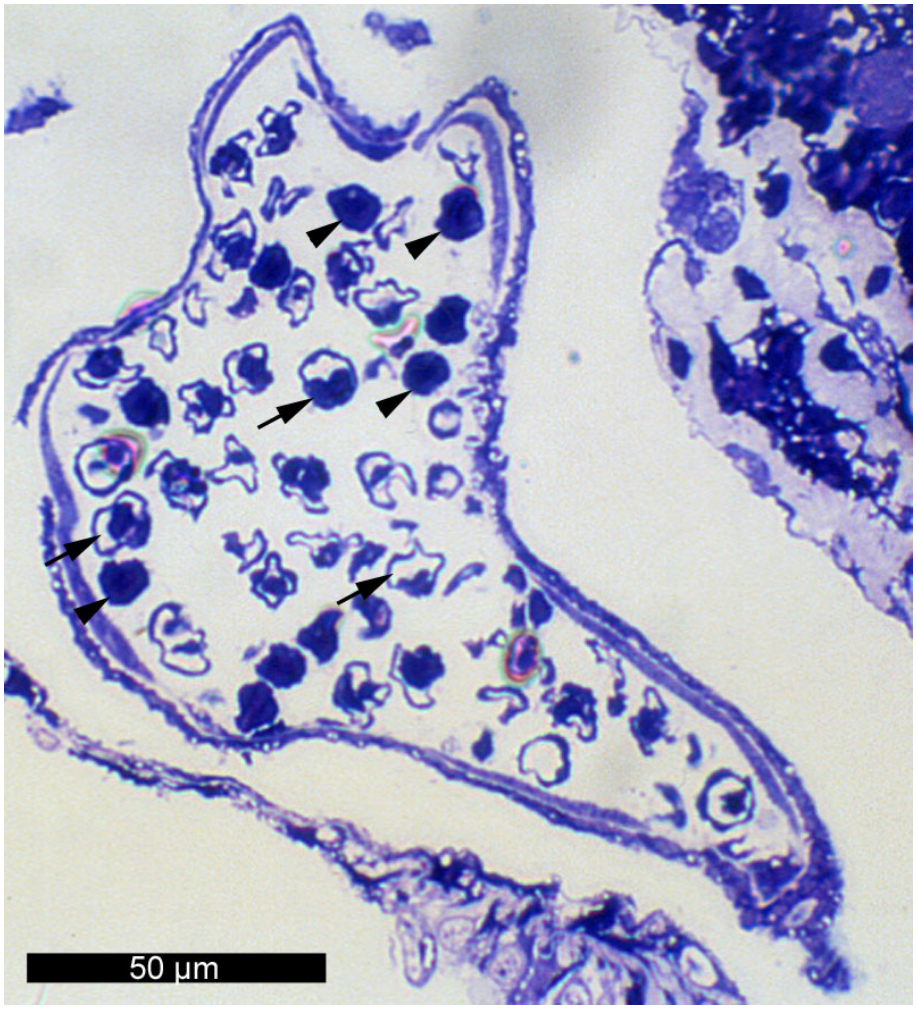

Figure 3.35: Immature and mature spores within a single spore sac

Arrowheads = immature densely-stained spores; arrows = differentiated spores. (toluidine blue)

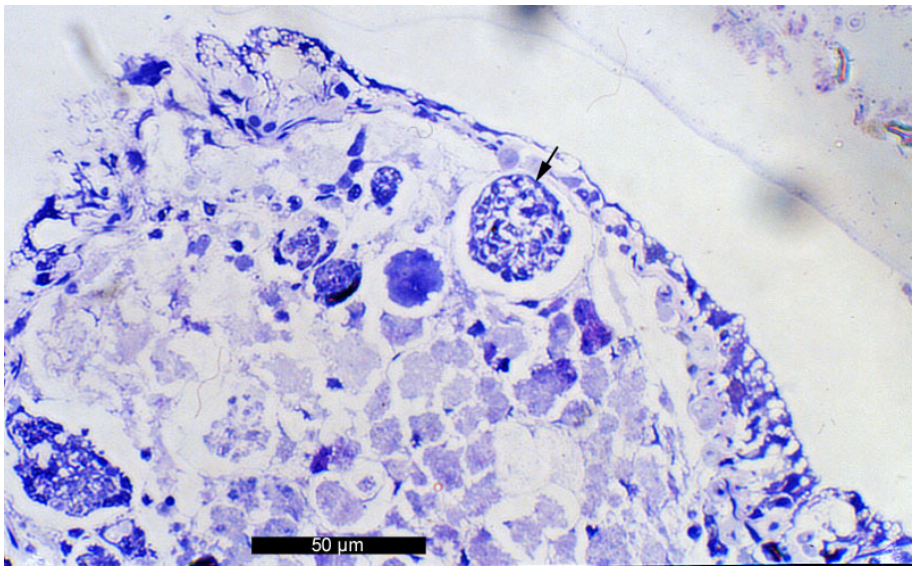

Figure 3.36: Encapsulated mass within infected bryozoan

Arrow = encapsulated mass of putative parasitic origin. (toluidine blue)

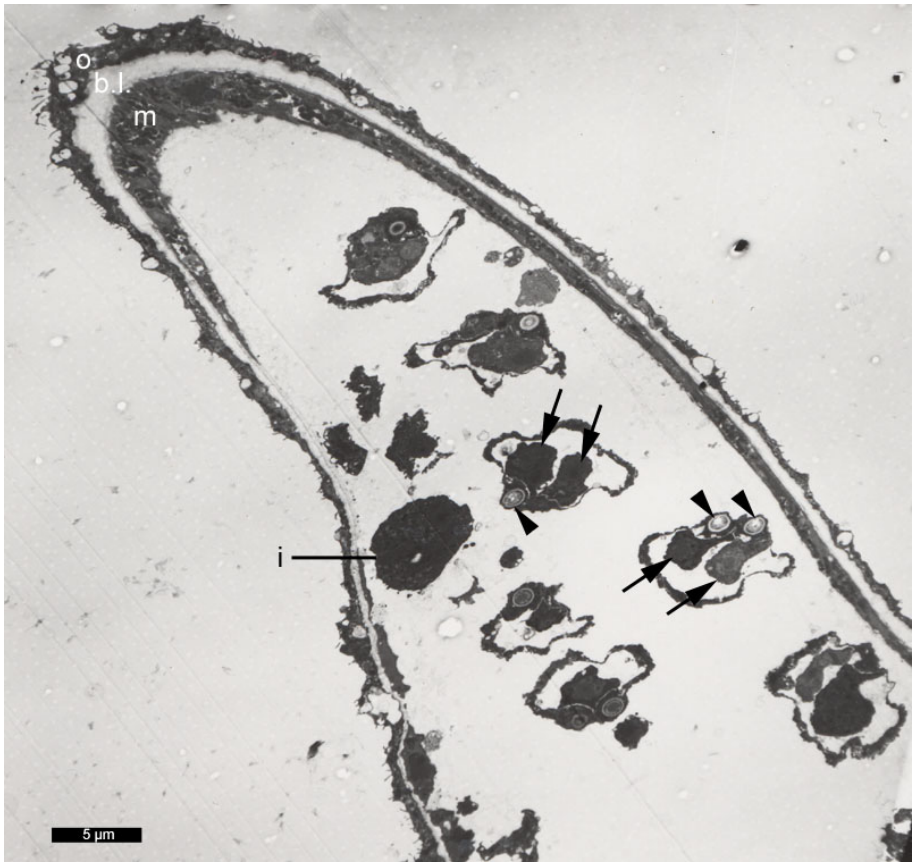

Figure 3.37: Ultra-thin section of *B. plumatellae* 'worm'

"o" = outer mural layer; "b.l." = basal lamina; "m" = muscle block; "i" = immature spore; arrowheads = polar capsules of maturing spores; arrows = sporoplasms. (uranyl acetate & lead citrate)

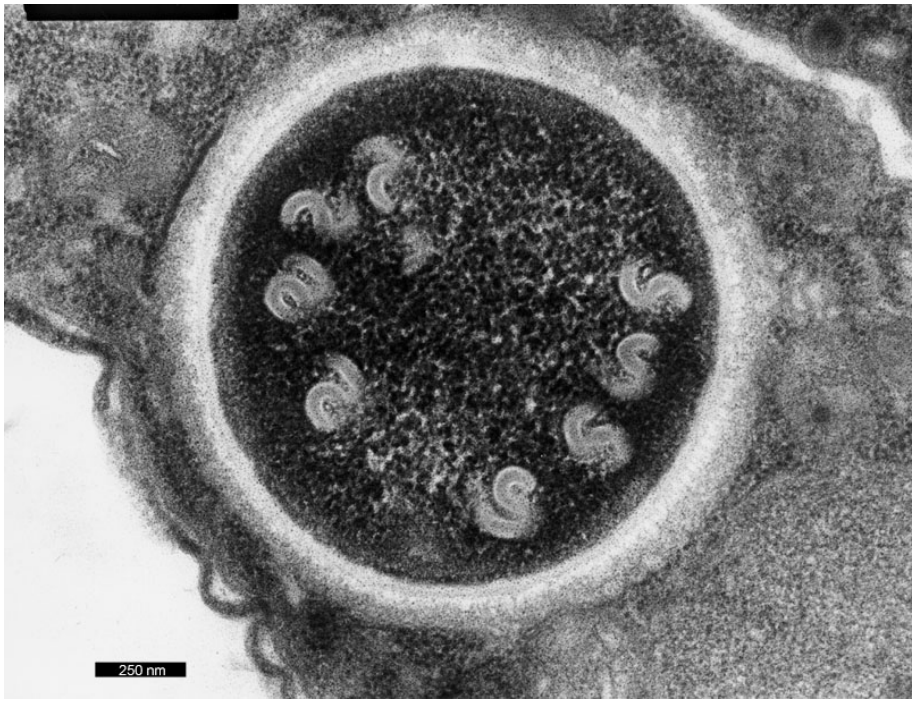

Figure 3.38: Polar capsule surrounded by an electron-lucent halo

Four pairs of sigmoid turns of the polar filament can be seen within the granular electron-dense matrix of the capsule. (uranyl acetate & lead citrate)

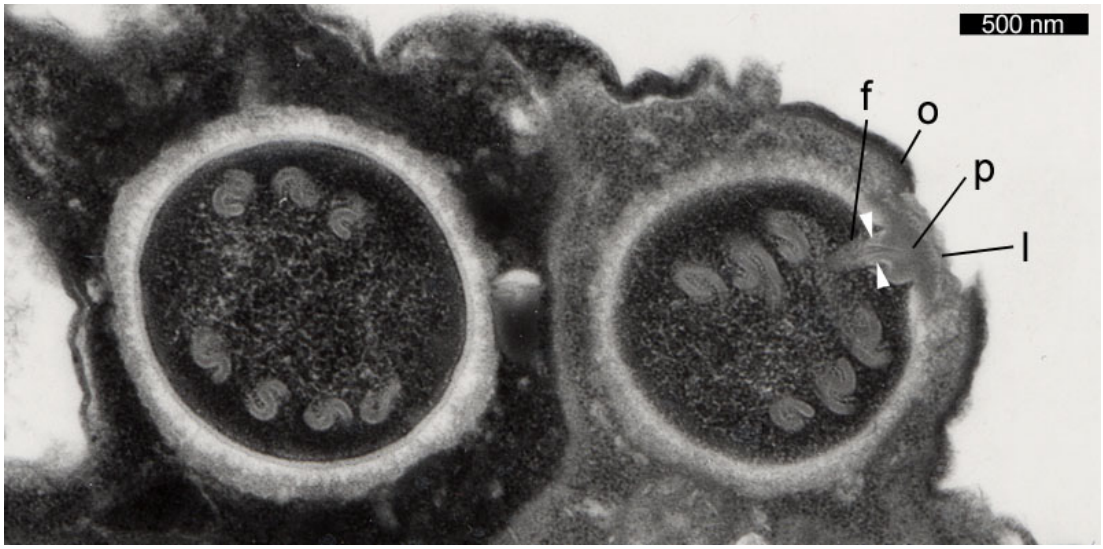

Figure 3.39: Polar capsules of *B. plumatellae* (detail of Figure 3.41)

Distal portion of polar filament ("f") can be seen contacting a mushroom-shaped plug ("p") overlain by a fine layer of material ("l"), but not by the outer layer of the spore wall ("o"); two proximal prongs of the plug can be seen (arrowheads). (uranyl acetate & lead citrate)

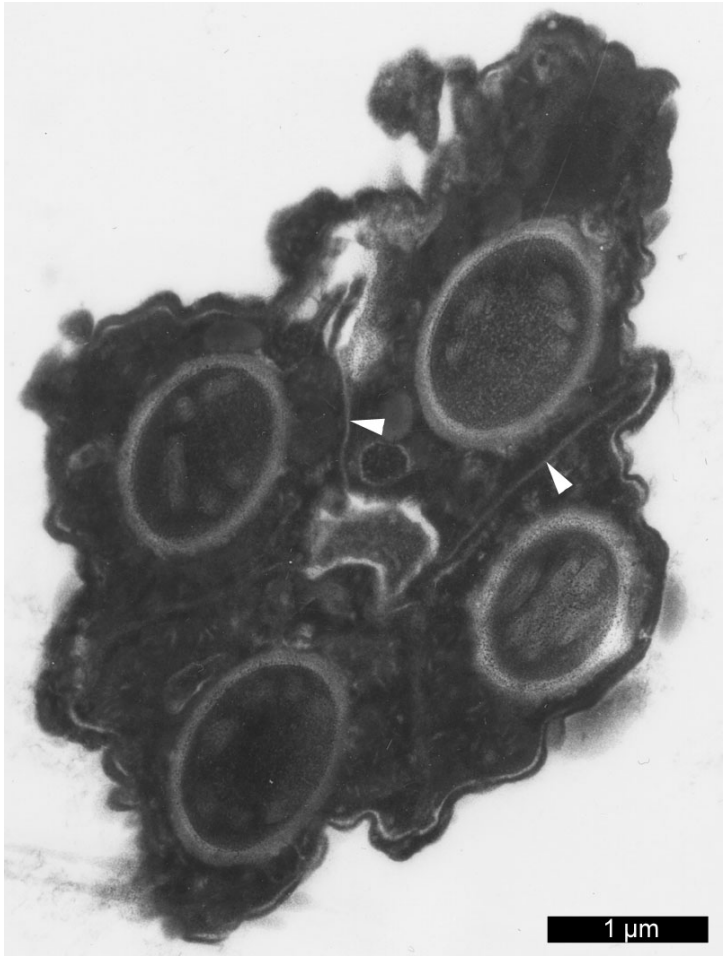

Figure 3.40: Spore containing four evenly spaced symmetrical polar capsules

Gap junctions (arrowheads) between adjacent capsulogenic cells appear regular in direction. (uranyl acetate & lead citrate)

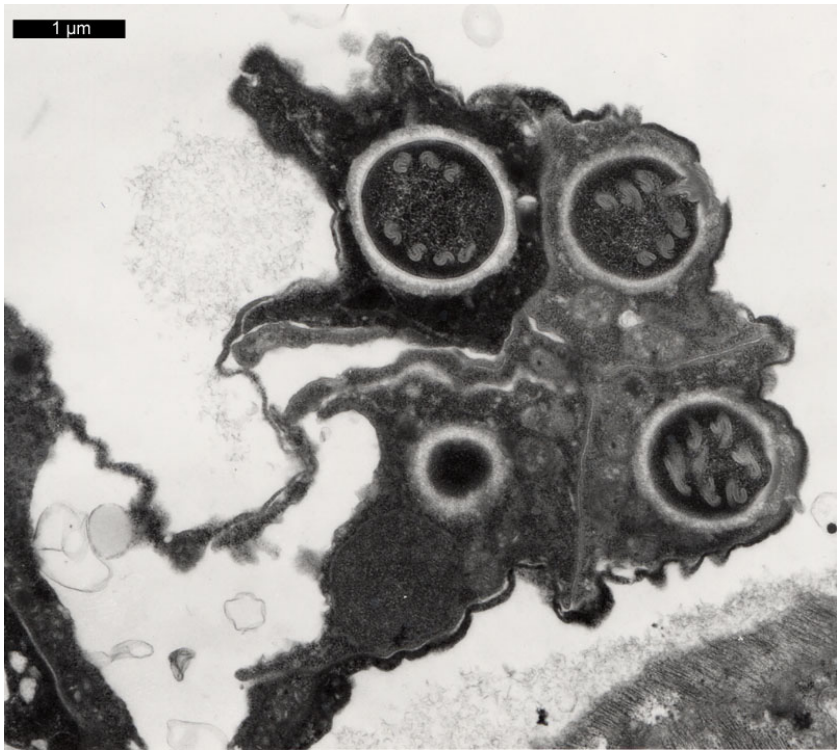

Figure 3.41: Spore of *B. plumatellae*

Three polar capsules can be seen in the plane of section, with the fourth capsule slightly offset. Regular gap junctions can be seen between the capsulogenic cells. (uranyl acetate & lead citrate)

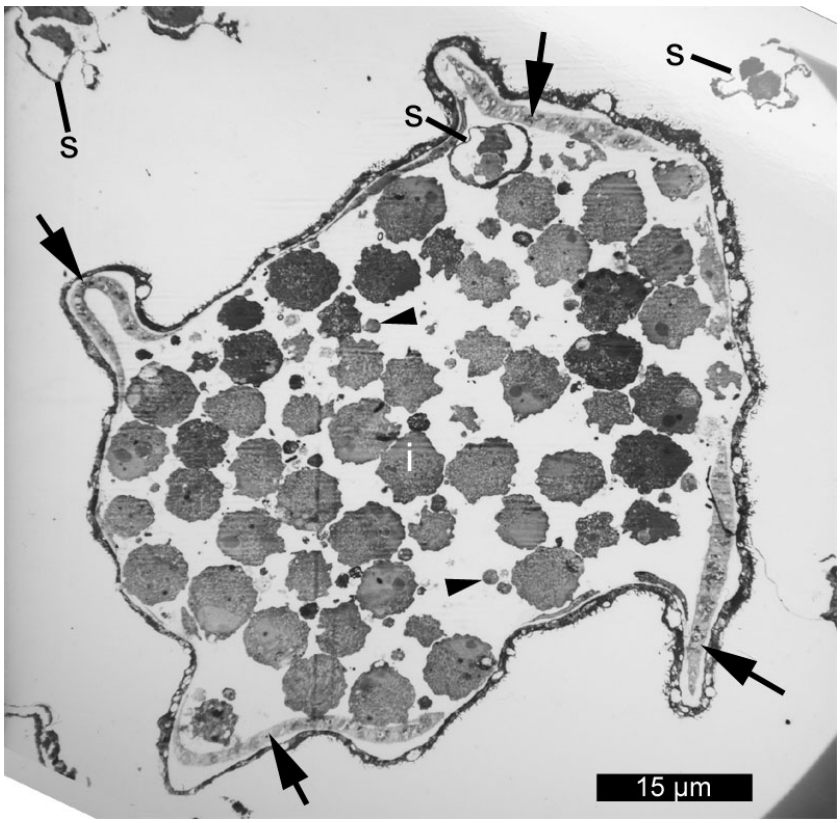

Figure 3.42: Cross section of immature 'worm'

Multiple immature spores ("i") & small cells (arrowheads) within 'worm' featuring four longitudinal muscle blocks (arrows); maturing spores ("s") within 'worm' & in metacoel of host. (uranyl acetate & lead citrate)

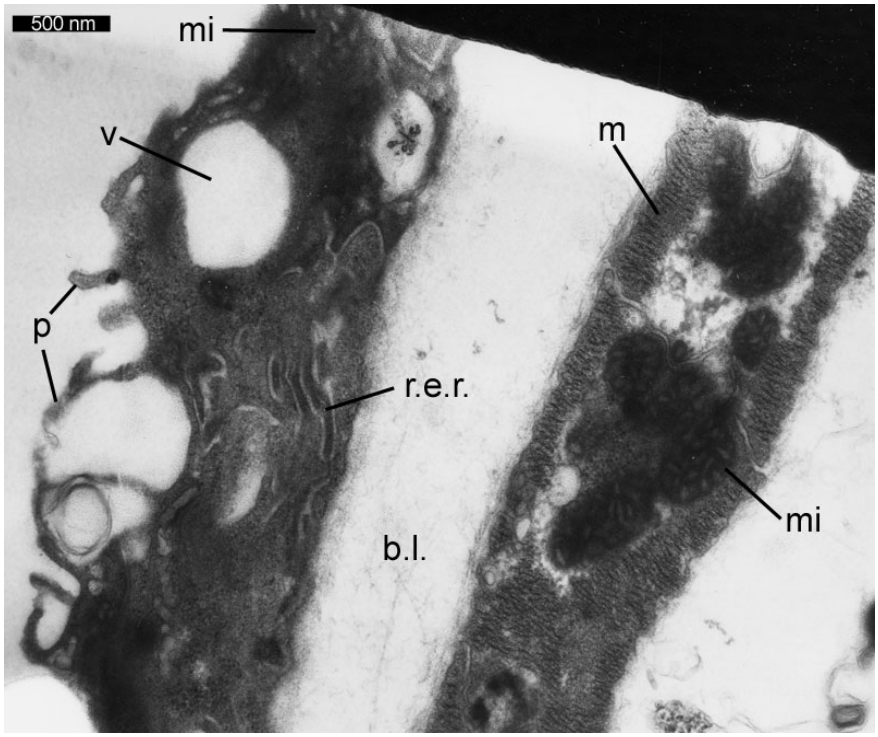

Figure 3.43: Wall of mature spore sac of *B. plumatellae*

Outer mural layer with finger-like projections (“p”): some of which coalesce to form vacuoles (“v”), rough endoplasmic reticula (“r.e.r.”) and mitochondria (“mi”); basal lamina (“b.l.”) and an inner layer including muscle fibres (“m”) and mitochondria. (uranyl acetate & lead citrate)

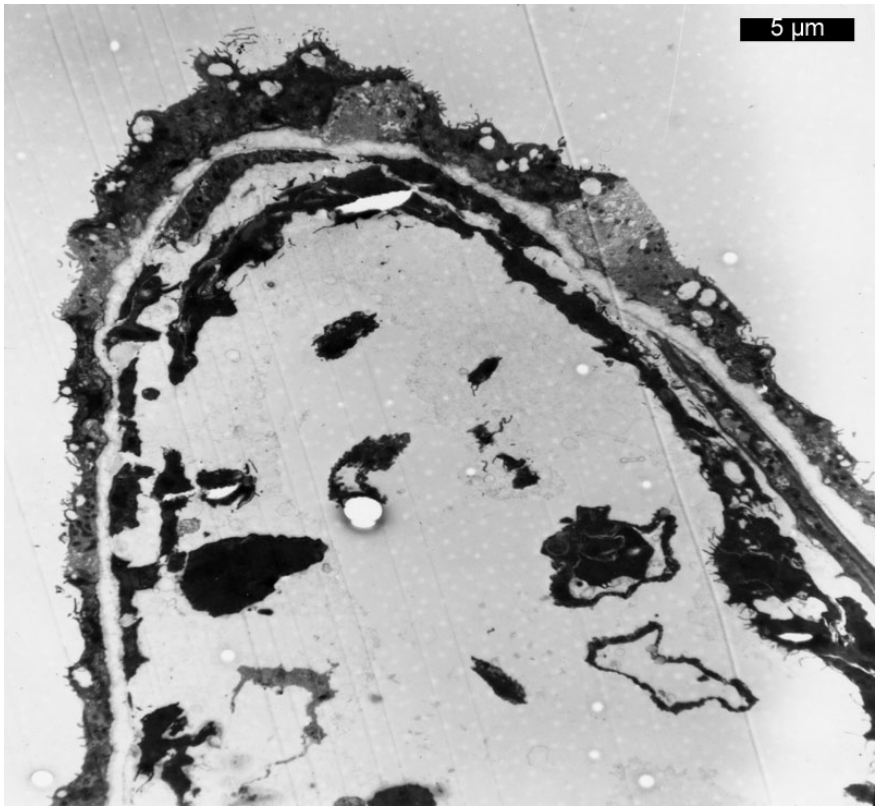

Figure 3.44: Wall of ‘worm’ of *B. plumatellae*

Multiple dactylate projections can be seen from the outer wall; pale cells can be seen interspersed in the wall; developing spores are seen within the lumen. (uranyl acetate & lead citrate)

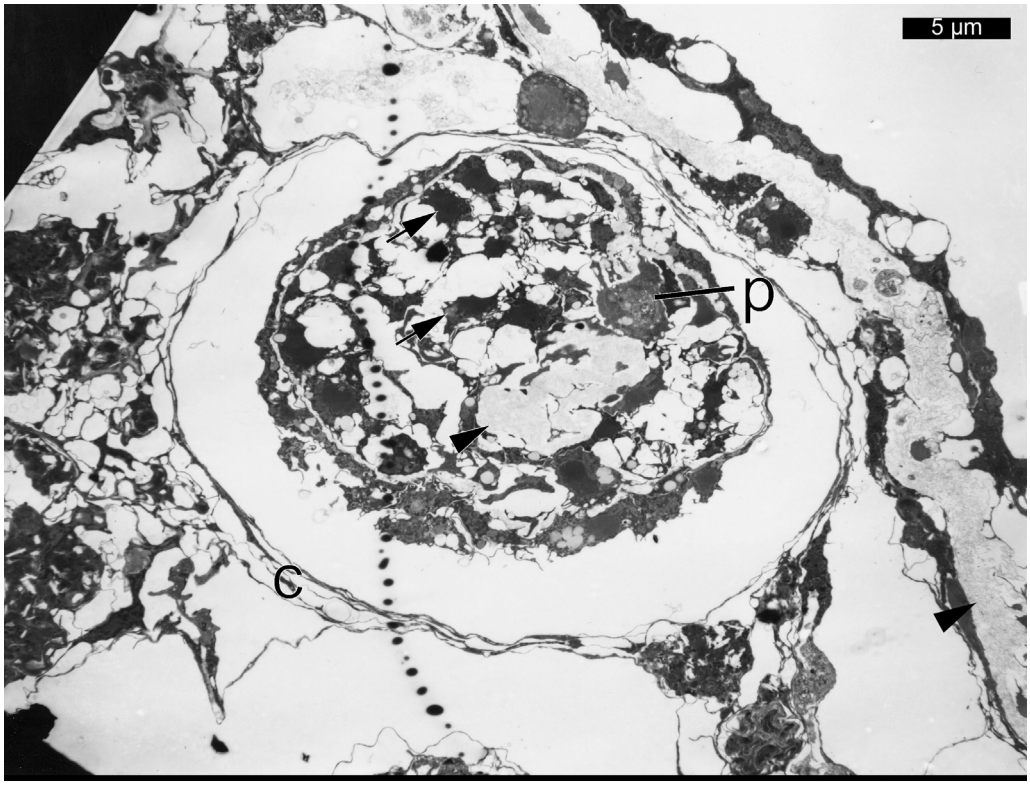

Figure 3.45: Encapsulated mass within *P. repens*

A mass of undifferentiated cells (arrows) can be seen, with a pale cell ("p") and basal lamina (arrowhead) surrounded by a pseudocapsule ("c"). Bryozoan wall can be seen on the right including vacuolated outer cells and basal lamina (arrowhead). (uranyl acetate & lead citrate)

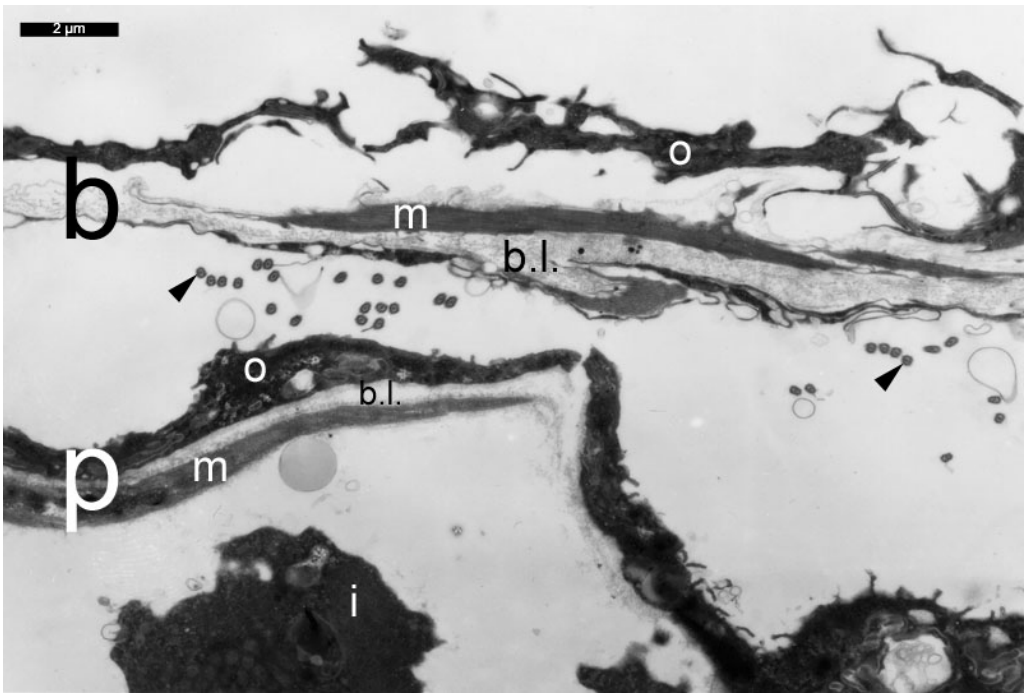

Figure 3.46: Bryozoan peritoneal wall and parasitic spore sac wall

The bryozoan peritoneum ("b") consists of an outer layer ("o"), muscle blocks ("m"), basal lamina ("b.l.") a thin inner layer and nearby cilia (arrowheads). Similar layers exist in the parasite wall ("p") below which is located an immature spore ("i") within the lumen of a 'worm'. (uranyl acetate & lead citrate)

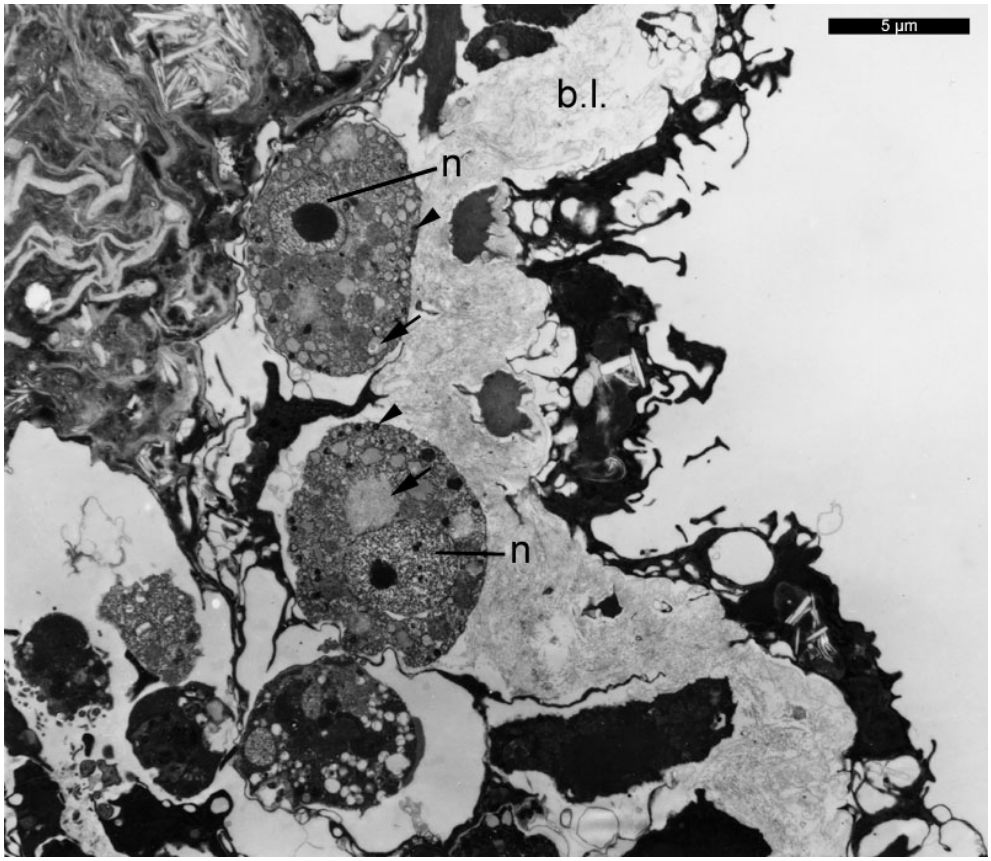

Figure 3.47: Distinctive cells within bryozoan material

Pale cells possessing nuclei ("n") with dense nucleoli, electron-dense bodies (arrowheads) and vacuoles (arrow). The cells are closely associated with host basal lamina ("b.l."). (uranyl acetate & lead citrate)

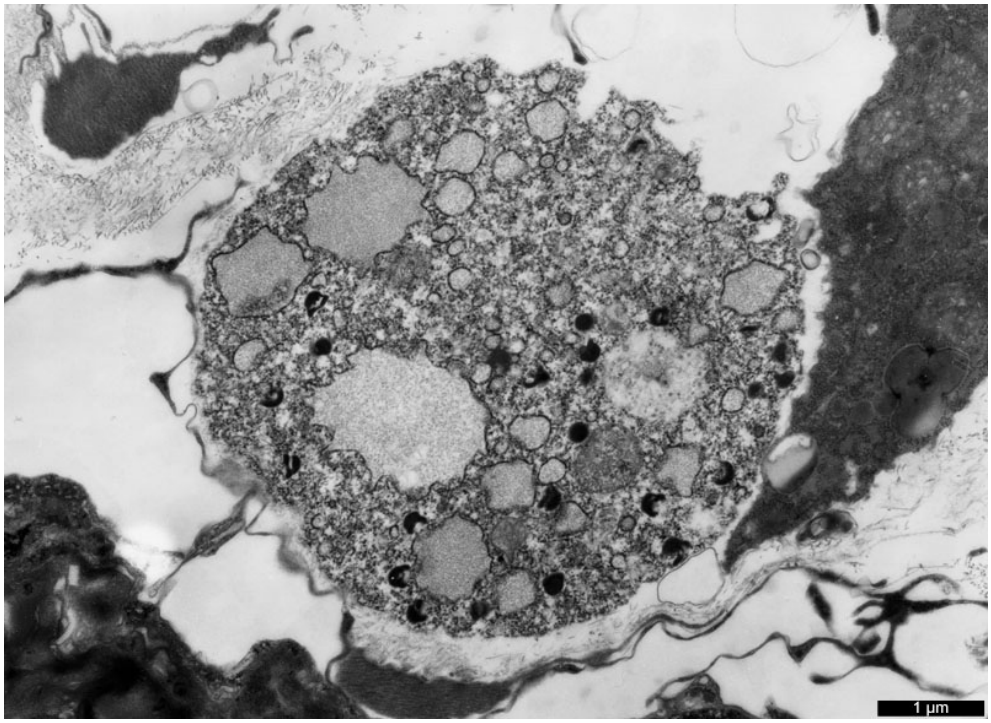

Figure 3.48: Pale cell in bryozoan containing multiple EDBs and vacuoles

Many of the electron-dense bodies are crescent-shaped. (uranyl acetate & lead citrate)

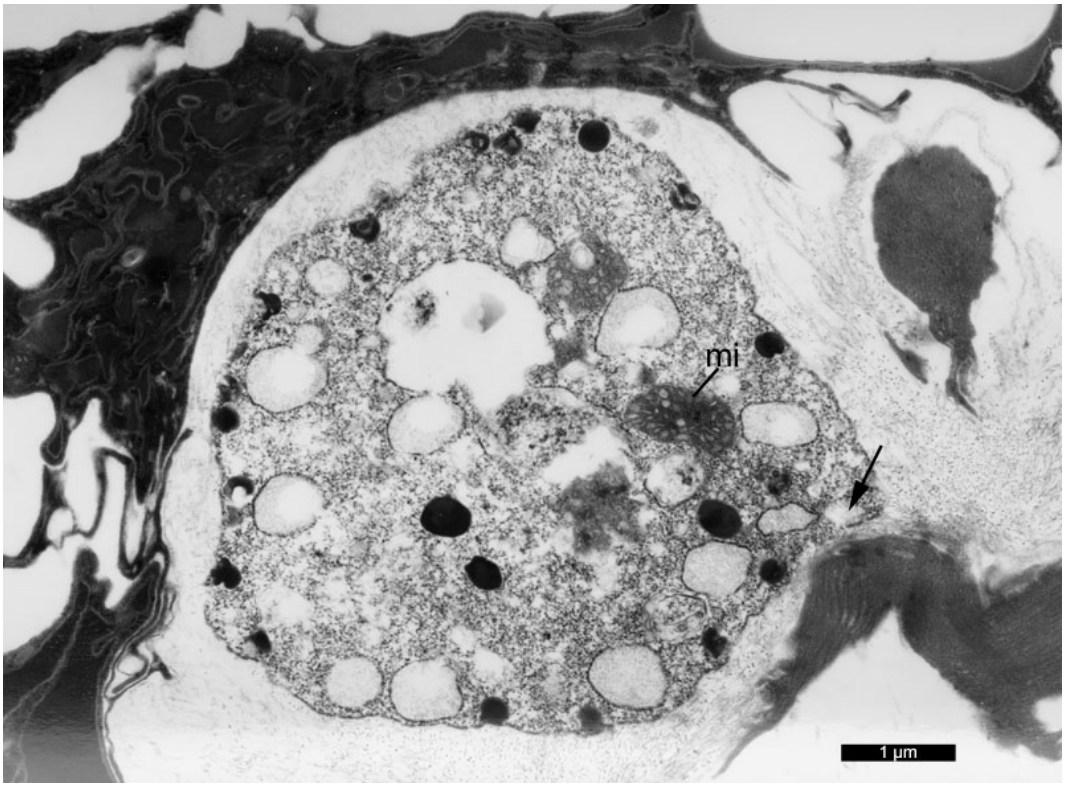

Figure 3.49: Cell containing vacuoles and EDBs within host basal lamina

Vacuoles contain material similar to surrounding basal lamina. An area where a vacuole appears to communicate with the basal lamina can be seen (arrow); one mitochondrion ("mi") is shown. (uranyl acetate & lead citrate)

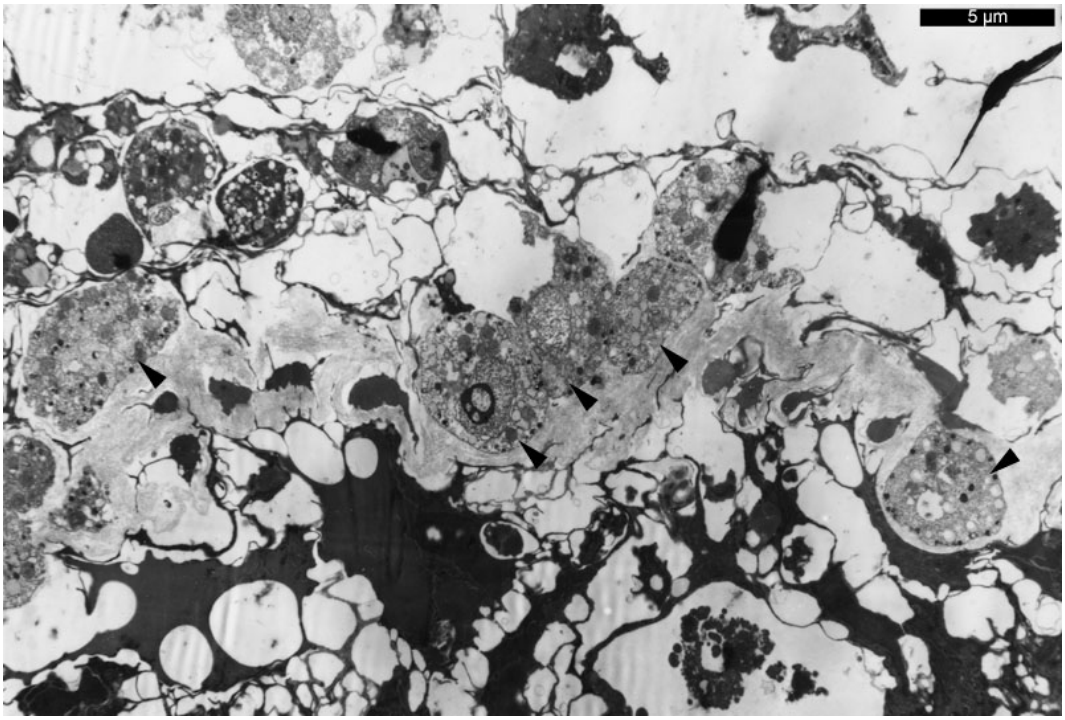

Figure 3.50: Multiple pale cells within bryozoan basal lamina

Pale cells of putative parasitic origin can be seen (arrowheads). (uranyl acetate & lead citrate)

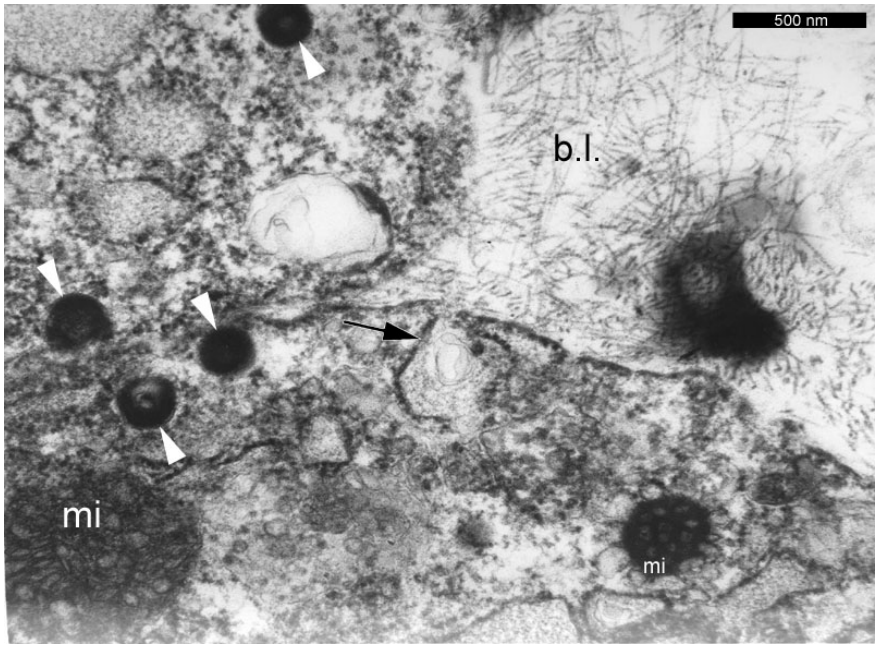

Figure 3.51: Vacuole apparently communicating with host basal lamina

Arrow = vacuole; arrowheads = electron-dense bodies; “mi” = distinctive mitochondria; “b.l.” = host basal lamina. (uranyl acetate & lead citrate)

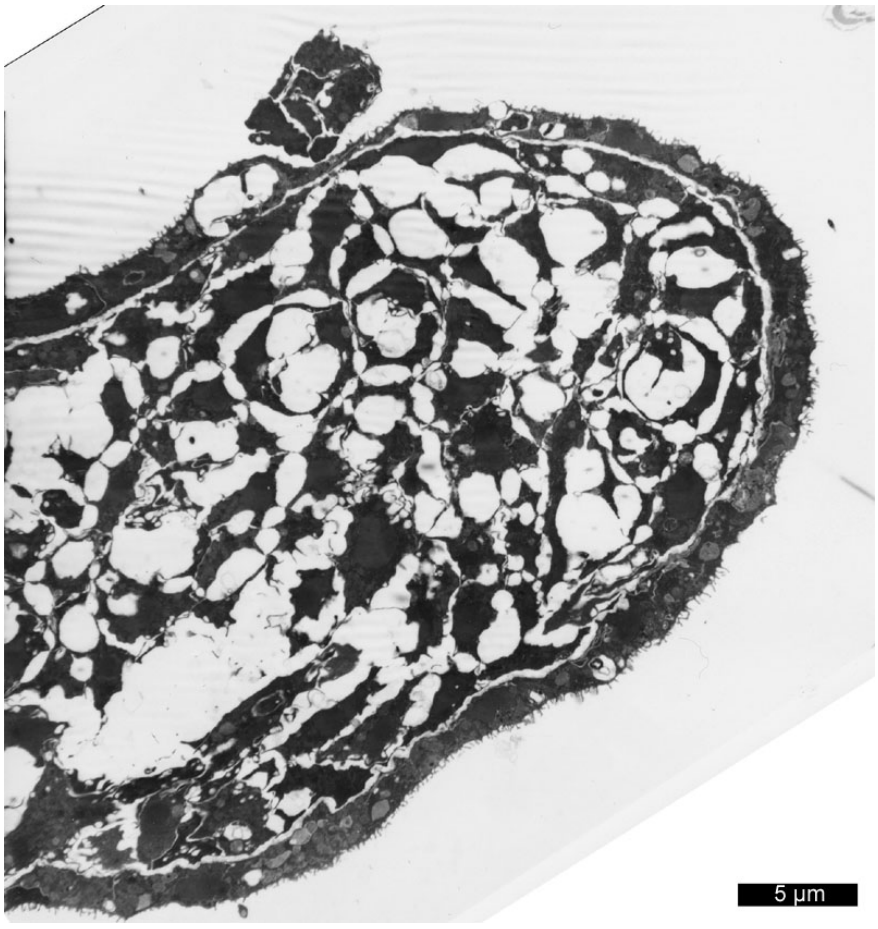

Figure 3.52: Mass of undifferentiated cells surrounded by outer layer

The outer layer contains distinctive vacuoles and dactylate projections. (uranyl acetate & lead citrate)

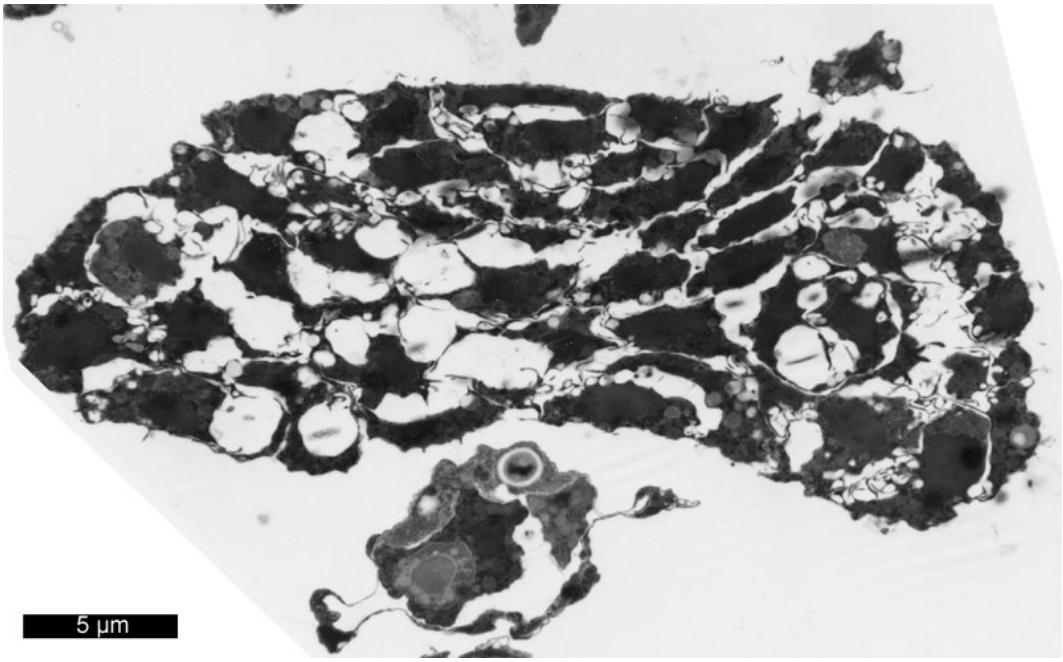

Figure 3.53: Mass of undifferentiated cells lacking a surrounding membrane

Below the mass an immature spore can be seen. (uranyl acetate & lead citrate)

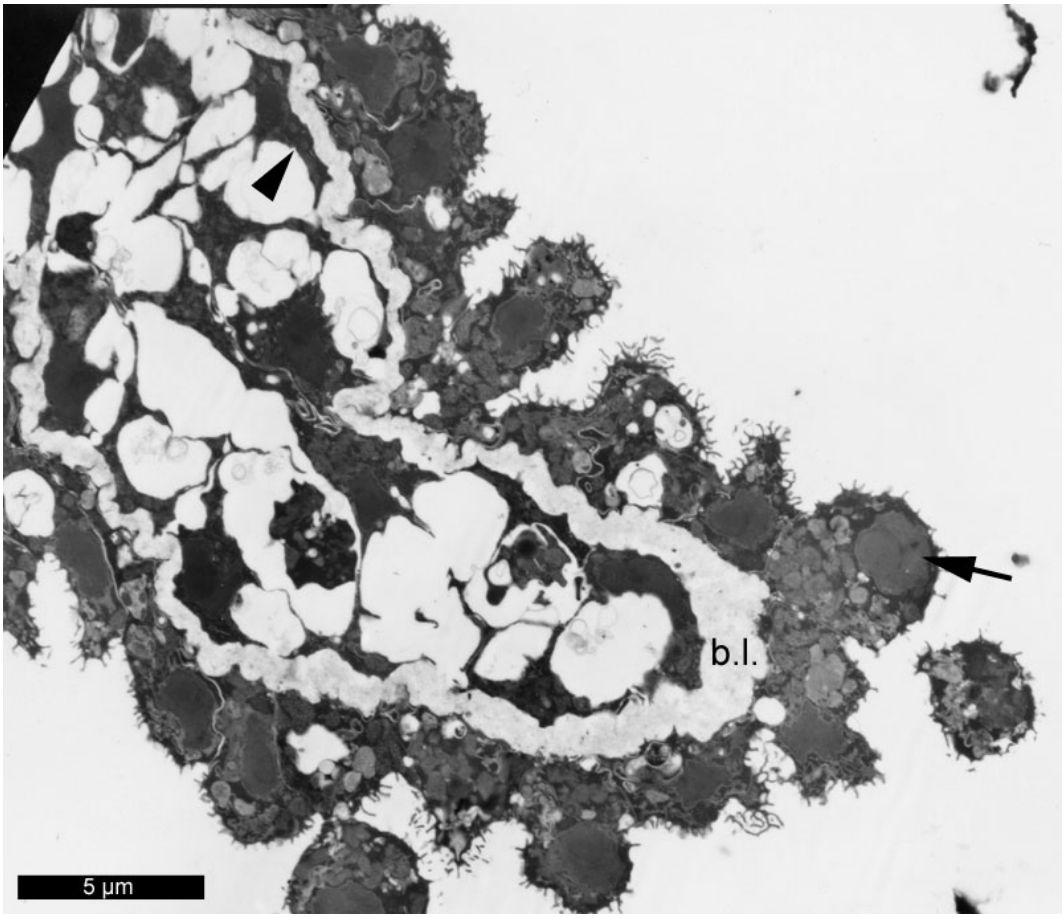

Figure 3.54: Mass of undifferentiated cells with surrounding membrane

Undifferentiated cells (arrowhead) are surrounded by basal lamina ("b.l.") with vacuolated outer cells (arrow). (uranyl acetate & lead citrate)

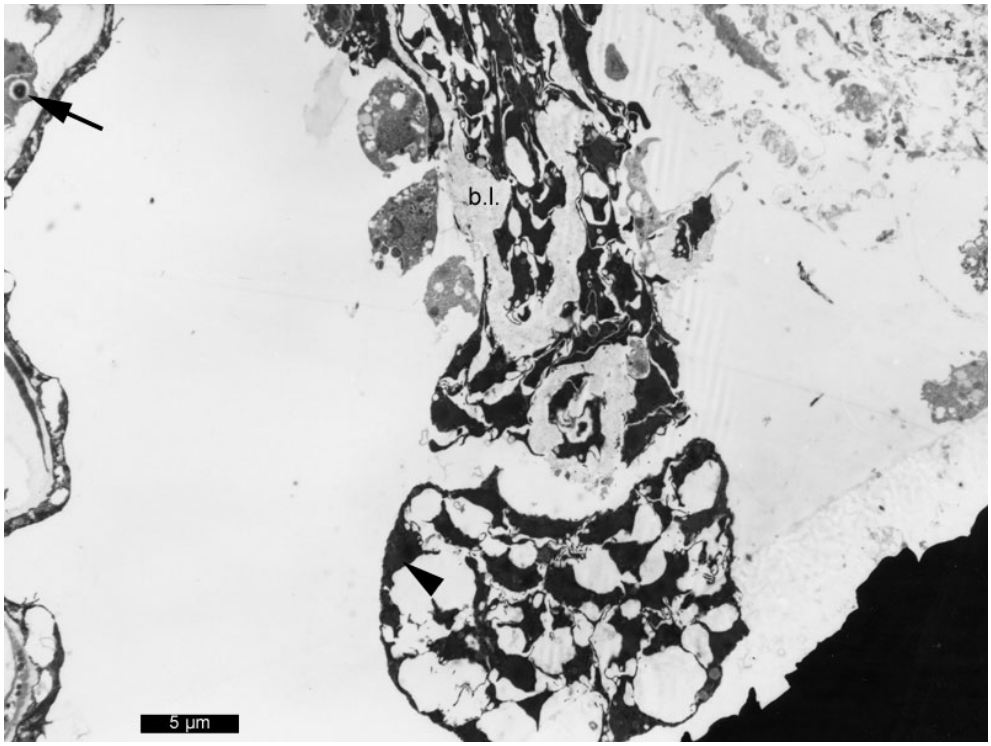

Figure 3.55: Mass of undifferentiated cells

Areas of basal lamina ("b.l.") can be seen associated with undifferentiated cells (arrowhead); in an adjacent sac the polar capsule (arrow) of an immature spore can be seen. (uranyl acetate & lead citrate)

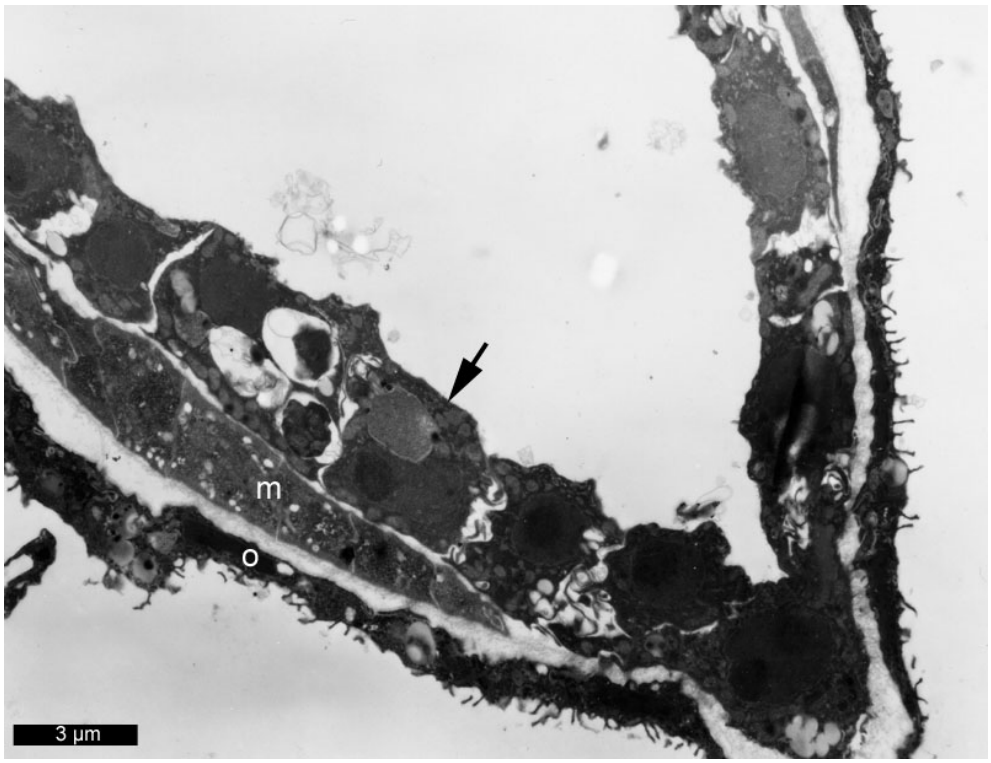

Figure 3.56: Cross section of immature spore sac showing mural layers

The inner layer of the wall of the sac comprises polygonal cells (arrow), with muscle blocks ("m") and outer vacuolated cells ("o") also seen. (uranyl acetate & lead citrate)

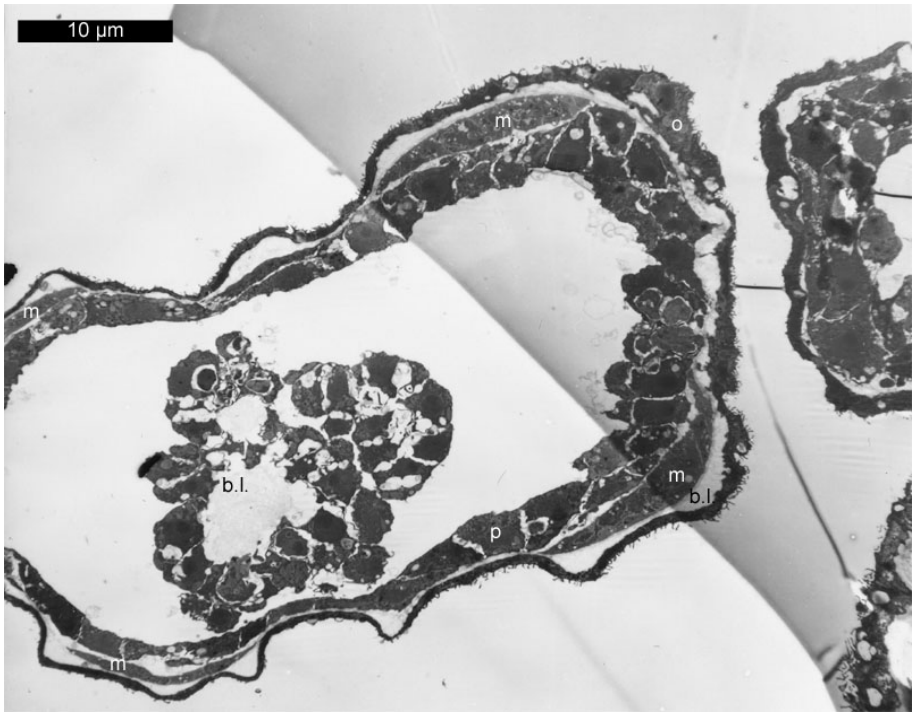

Figure 3.57: Cross section of immature 'worm' showing internalised mass

The mural layers comprises outer cells ("o"), muscle blocks ("m"), basal lamina ("b.l.") and polygonal cells ("p") which surrounded a lumen containing an internal mass also including basal lamina. (uranyl acetate & lead citrate)

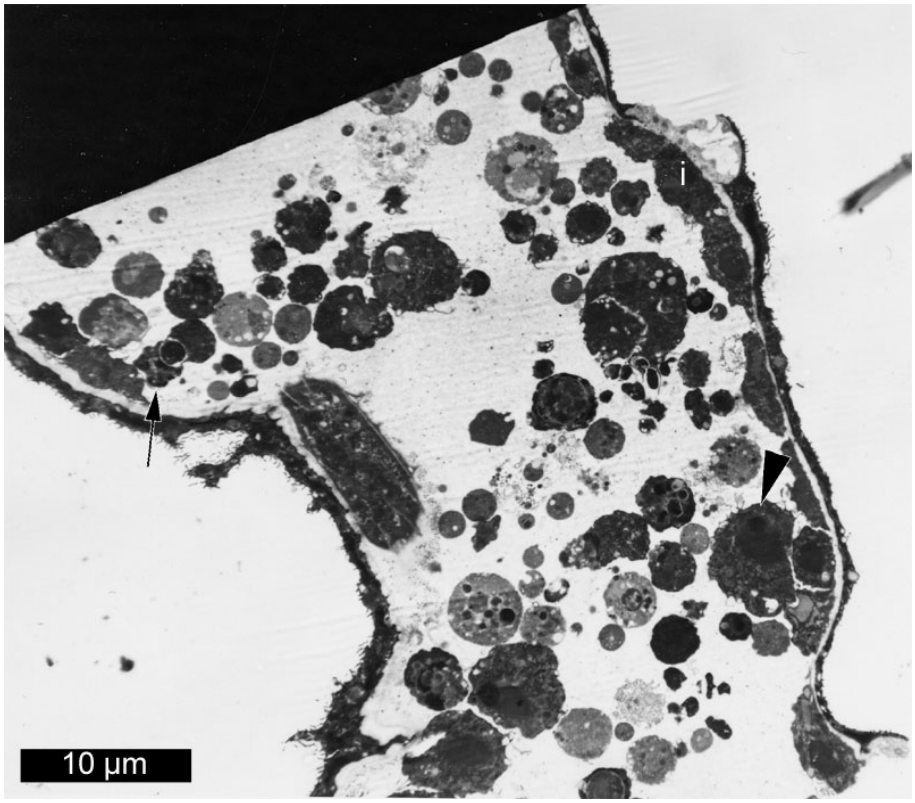

Figure 3.58: Immature sac with multiple intraluminal cells of various sizes

Some cells ("i") remain of the inner layer of the wall. A cell (arrowhead) can be seen apparently budding from the wall. Another (arrow) appears to be dividing subsequent to budding from the wall. (uranyl acetate & lead citrate)

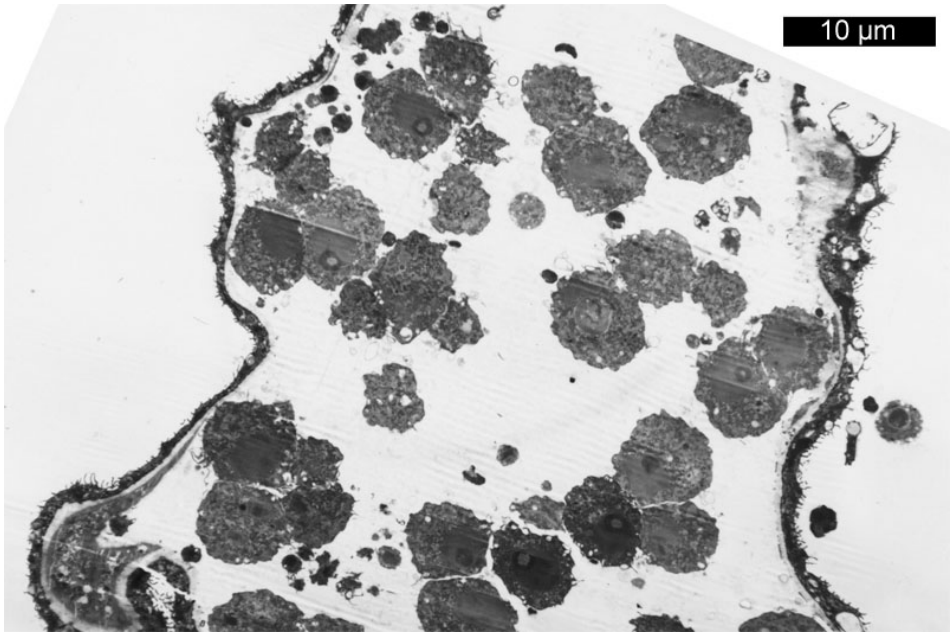

Figure 3.59: Dividing intraluminal cells

The inner mural layer is no longer apparent, although part of a muscle block can be seen in the bottom left corner. Multiple cells are closely apposed, presumably undergoing fission. (uranyl acetate & lead citrate)

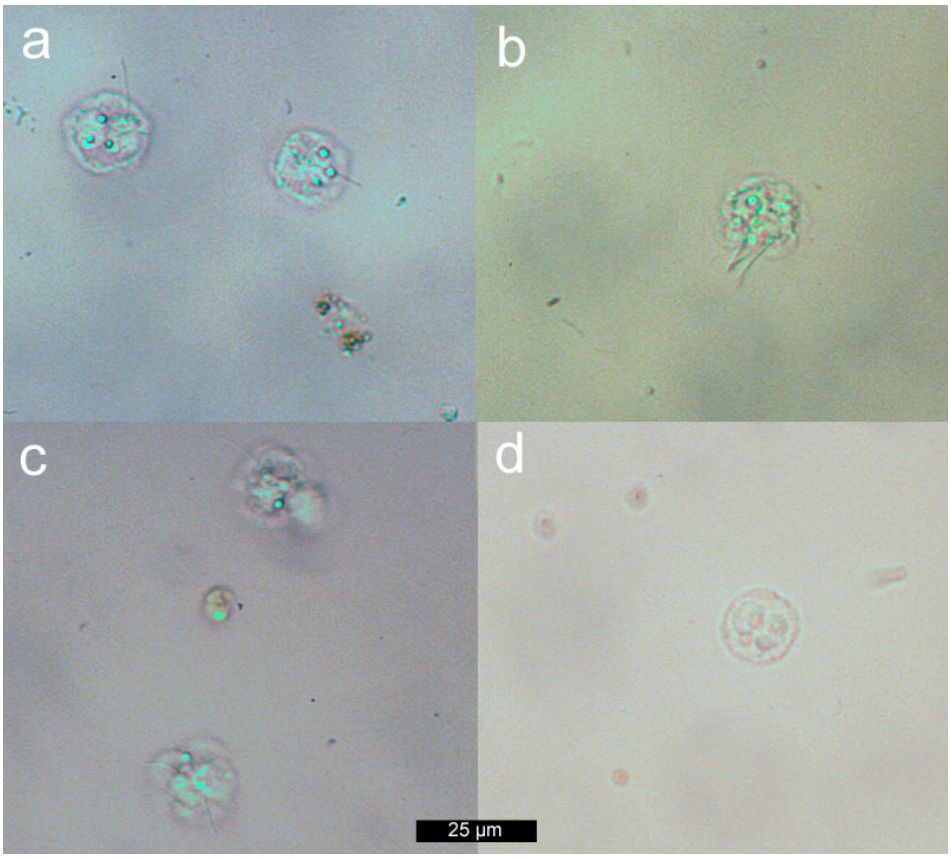

Figure 3.60: Spores of *B. plumatellae* following exposure to trout mucus

Spores were placed onto slides coated with: (a) PKD-affected rainbow trout mucus, (b) PKD-recovered rainbow trout mucus, (c) PKD-naïve rainbow trout mucus, (d) Chalkley's medium (control slide). Polar filaments can be seen extruded from polar capsules in (a–c). Scale bar applies to all images.

3.4. Discussion

The finding that bryozoans collected from field sites are generally coated with a matrix of opaque material has previously hindered the study of malacosporean parasites in their hosts (Canning *et al.* 2002). In his original incisive studies, Schröder (1910, 1912) resorted to utilising fixed bryozoan material for his observations. Morris *et al*. (2002a) described a methodology for extended laboratory maintenance of bryozoan populations, resulting in the formation of translucent colonies devoid of an opaque coating. This allowed these authors to observe the development of a malacosporean in living material. This protocol has been modified in the current study, allowing observation of developmental stages of *B. plumatellae* within the bryozoan host for a period of three months. The sessile nature of Bryozoa in conjunction with the development of translucent body walls allowed extensive monitoring of myxozoan development within the invertebrate host. This led to the possibility of chronological tracking of the cycle of myxozoan parasitism in individual bryozoan colonies over an extended time-scale.

Canning *et al*. (2002) reported the manifestation of apparent *B. plumatellae* infection in zooids of *P. fungosa* six days after having exhibited no signs of infection. In the current study, vermiform stages of the parasite were observed in laboratory cultured bryozoans 14 days following collection from the field site. However, neither of these findings provides much information on the relative time-scale of development of infection, as evidently each colony must have been infected at an undetermined time previous to collection, meaning that the baseline value could not be discerned. However, in the current study, the observation of primary signs of infection in a colony derived from a single zooid allowed the opportunity to follow the sequential stages of parasitic development.

Through observation of a growing bryozoan colony it was noted that demonstrable infection was invariably preceded by the presence of numerous small swirling particles within the metacoel. As these stages were not recognised on TEM examination, it was not possible to state their origin or function. The regularity with which they were observed as a prequel to overt infection suggested that they were correlated with a rudimentary stage of parasitism, but it could not be inferred whether they were either parasitic in origin or indicative of a host protective response. The infection in the colony described in Section 3.3.3.2 was presumed to have originated from an adjacent zooid, although it could not be stated whether transmission was by direct infiltration along the peritoneal wall, or via transfer of coelomic contents, *i.e.* the swirling particles.

Synchronous to the presence of swirling particles, regularly round stages were observed adhered to the bryozoan peritoneum. Schröder (1912) first described the presence of ellipsoid parasitic formations of diameter 20-30 µm in the body cavity wall of *Plumatella*. Canning *et al*. (2002) also suggested that *B. plumatellae* may undergo primary development within the host epithelium. In the current TEM and light microscopy studies, cells possessing myxozoan morphological features were observed throughout bryozoan epithelial tissue, with a particular affinity for the host basal lamina observed. Both the pale cells observed within bryozoan tissue and the mural cells of developing *B. plumatellae* spore sacs bore similarities to previously described malacosporean cells (Canning *et al.* 1996, 2000). Marked resemblances were noted between the walls of bryozoan peritoneum and of malacosporean spore sacs. Both were seen to possess outer vacuolated walls with numerous external dactylate projections and discrete muscle blocks surrounded by organised basal lamina. The identities of the walls could be ascertained by the presence of either cilia (which are not recognised in the Myxozoa (Lom and Dyková 1995)) adjacent to the bryozoan wall, or immature spores close to the myxozoan spore sac wall. The

distinctive structure of the outer wall of the spore sac was a consistent finding across the range of development stages in the current material, whereas Okamura *et al*. (2002) described limited presence of the finger-like projections in malacosporean material from *P. repens* and an apparent absence of the feature in sections of infected *Hyalinella punctata*.

If the encapsulated mass observed within bryozoan tissue represented a formative developmental stage of *B. plumatellae* then it would correlate with previous suggestions of malacosporean 'cryptic' stages (Canning *et al.* 2002) and with observations within the current light microscopy study of parasitic stages attached to the bryozoan peritoneum. The presence of organised zones of basal lamina within developing *B. plumatellae* tissue, morphologically similar to host tissue, leads to the proposition that the parasitic pale cells may be utilising host material in the production of parasitic architecture. Previously, the parasitic worm *Schistosoma mansoni* Sambon, 1907 has been described as acquiring an extensive range of host antigens which are subsequently expressed on the tegument of the parasite (Abath and Werkhauser 1996). Recently, studies of the development of the myxozoan parasite *Myxobolus pendula* Guilford, 1966 have shown that the growth of parasite-derived cysts was associated with degradation of host connective tissue and remodelling of fibrillar collagen (Martyn, Hong, Ringuette and Desser 2002). These authors compared and contrasted the morphology of host and parasitic collagen fibres, and suggested that host material was reorganised in the development of parasitic cysts. In the current study, it would appear that to facilitate parasitic development, initial stages of *B. plumatellae* would be likely to obtain products from the host. It could not be concluded whether these were in the form of nutritional substances aiding the parasite in producing its morphological components *de novo*, or if the parasite utilised and remodelled host tissue to form its architecture. However, the striking similarities in structure between bryozoan peritoneum and

malacosporean wall (Figure 3.46) would seem remarkably coincidental if the former was the accurate hypothesis.

Schröder (1912) described pyriform parasitic stages anchored to the bryozoan peritoneum. Comparable bodies within the present study appeared relatively homogenous by light microscopy (Figure 3.12), with no discernable lumina observed. Ultrastructural examination showed parasitic stages packed full of undifferentiated material, surrounded by an outer membrane of vacuolated cells with external projections (Figure 3.52). The undifferentiated cells were similar to those seen within the encapsulated mass, and also consistent with descriptions of developmental cells previously reported in *T. bryosalmonae* which were named 'stellate cells' (Canning *et al.* 2000), with the overall appearance markedly similar to previous observations (*cf.* Figure 24 in Canning and Okamura (2004)). It seems likely that these TEM-observed stages of *B. plumatellae* were consistent with described pyriform bodies, representing the intermediate stage between peritoneal and unattached coelomic *B. plumatellae*. The accumulations of undifferentiated material that were not surrounded by an identifiable outer layer (Figure 3.53 and Figure 3.55) may have represented a route of dissemination of infection throughout the bryozoan colony; alternatively, they may have been remnants of intraluminal masses of 'worms' separated from their outer membrane as artefacts during tissue processing.

Following detachment from the bryozoan peritoneum, multiple progressively elongated immature spore sacs were observed in the bryozoan metacoel. By both light and electron microscopy observations, a defined outer membrane was seen which was composed of polygonal cells surrounding an empty central lumen (Figure 3.13, Figure 3.32 and Figure 3.56). Okamura *et al*. (2002) classified the mural cells into separate populations known as types A and B, although such distinctions could not be made in the material studied herein. Some of the 'worms' appeared to have

bulbous protrusions at one tip (Figure 3.20), presumably being the point of previous attachment with the peritoneum, corroborating previous findings of a disparity between the opposing ends of individual 'worms' (Schröder 1910).

Evidently, the polygonal cells of the 'worm' wall were proliferative cells, developing in the wall before being released into the cavity. Intraluminal unattached masses were observed within the 'worms' by both light microscopy and TEM. The inner mural layer of the sacs was seen to become progressively hyperplastic, with aggregation of increasing quantities of intraluminal material (Figure 3.18), eventually occluding the lumen resulting in a distinctive 'corrugated' appearance of the 'worm' (Figure 3.17). Ultrastructurally, it was previously noted that the inner layer of proliferative mural cells was progressively diminished as spore sacs matured (Canning *et al.* 1996, 2002). Thus, in the current TEM study it was possible to ascertain the sequence of development of various sections of maturing spore sacs by the relative appearance of this inner layer of the spore sac wall. Sacs with continuous inner mural layers, containing defined intraluminal clumps of cells (Figure 3.57) were superseded by many circular intraluminal cells – some of which could be seen budding directly from the then sparser inner wall (Figure 3.58). Subsequently, large numbers of round cells undergoing fission were present in conjunction with an absence of the inner layer (Figure 3.59), leading to the appearance of immature spores (Figure 3.42). Within three days of the 'corrugated' appearance by light microscopy, the 'worms' were seen to be packed full of individual round bodies of approximately 18 µm, representing spore development (Figure 3.18).

The mean spore diameter of 17.67 µm was less than that previously described of ornamented spores dissected from a malacosporean 'worm' in *P. repens* (19.0 µm) (Morris *et al.* 2002a), and spherical spores of *T. bryozoides* (*i.e.* the alternative manifestation of *B. plumatellae*) from *C. mucedo* (19.2 µm) (Canning *et al.* 1996). In

the current study, each of the spores possessed characteristic malacosporean features, including four spherical polar capsules and two sporoplasms (Figure 3.37). In their ultrastructural study, Okamura *et al*. (2002) observed polar capsules in the mural cells of immature *B. plumatellae* spore sacs, however, no such findings were made in the current material: polar capsules only being seen in developing spores. The number of coils of polar filaments within the capsules of spores of vermiform *B. plumatellae* had not previously been reported, although five turns were noted in its alternative developmental form in *C. mucedo* (Canning *et al.* 1996). In the current study, up to four turns of the polar filaments were seen within capsules (Figure 3.38).

Upon exposure of *B. plumatellae* spores to microscope slides coated with rainbow trout mucus, polar filaments were seen to be extruded (Figure 3.60). Eversion occurred regardless of the PKD-status of the fish used, suggesting that endogenous specific immunological products in trout mucus were not significant in the process. Indeed, it has previously been shown that rainbow trout mucus contains glycosylated proteins which induce the formation of ion channels, resulting in changes in membrane permeability (Ebran, Julien, Orange, Auperin and Molle 2000). Previously, upon examination of exposure of six different types of actinosporean spores to fish mucus, polar filament extrusion and sporoplasmic ejection was witnessed within 1-2 min, although strict mucus specificity was not noted for any of the species examined (Xiao and Desser 2000). In the current study, it took over 10 minutes for any firing to be seen in the spores, and even after such a period, the development was not ubiquitous, as several spores did not so react. In many of the spores, less than four filaments fired, suggesting that each polar capsule was stimulated for release independently, reinforcing the hypothesis that the trigger in this case was unlikely to be specific to trout mucus. Presumably, exposure of the spores to the mucus led to alterations in the physiological structure of the mushroom-shaped plug and the overlying thin layer of tissue, which together sealed

the coiled filament within the capsule (Figure 3.39). The polar filament could be seen articulating with the plug with two apparent prongs at this location and straddled the electron-lucent layer which encircled the remainder of the polar capsule. As previously described, the outer membrane of the capsulogenic cell was discontinuous over the plug, allowing an exit point for the filament (Canning *et al.* 2002).

A previous report of *B. plumatellae* has described the ejection of intact mature spore sacs from *Plumatella fungosa* (Canning *et al.* 2002). This event was witnessed following 'gentle external prodding' of zooids, but was also described as occurring spontaneously, resulting in the slow ejection of mature 'worms' lasting 15 minutes. The authors reported that subsequent to such exits, the lophophore of the infected zooid was traumatised, resulting in malfunction of the lophophore retraction mechanism. Despite extensive observations (including many hours of video footage) of infected colonies of *P. repens* in the current study, no mature spore sacs were ever witnessed spontaneously exiting infected zooids. In addition, no zooids were recognised suffering from lophophore 'dislocation' as described in *P. fungosa*. Free spores of *B. plumatellae* were witnessed being released from zooids in the region of the base of the lophophore during its retraction, possibly exiting via the vestibular pore, the release route of mature statoblasts from bryozoans (Morris *et al.* 2002a). Individual zooids were seen to lose their complement of released spores within 12 hours of appearance. Thus, it can be ascertained that upon spore maturation of *B. plumatellae* in *P. repens*, vermiform sacs rupture within the metacoel, releasing spores which are then disseminated throughout the bryozoan before being voided into the surrounding water.

In the infected colony which was studied sequentially, the completion of developmental cycles of *B. plumatellae* was appreciated by the release of spores

into the metacoel after 20 days at 21°C. Following the production of spores, immature stages of *B. plumatellae* development in the form of adhered peritoneal buds and small swirling cells were evident within the same colony. These stages matured, resulting in a new wave of maturation, a sequence which was ongoing, resulting in different life cycle stages of *B. plumatellae* being present within a single bryozoan colony. Although infection was maintained, the colonies continued to steadily increase in their number of zooid units. However, deleterious effects were witnessed in the bryozoans. Pinching of the peritoneum was noted between contiguous zooids (Figure 3.24). It had been suggested that the resultant stenosis of the connecting channel would result in the annexing of infected colonial zones, allowing newly developed zooids to remain uninfected and to continue development of a clonal bryozoan line (Canning *et al.* 2002). However, in the current study, putative *B. plumatellae* material in the form of small swirling particles and mature spores was seen to pass readily through the narrow channels before division could occur. Moreover, although the colonies became fragmented as a result of the occlusion of the metacoel, newly developed zooids invariably showed subsequent signs of *B. plumatellae* infection, leading to dissemination of infection to the most marginal zones of growth. Thus, it appeared that if the bryozoan response was a protective mechanism as previously suggested, then it was seriously flawed. In light of these findings, it seemed more likely that the stenosis was a pathological sequel, triggered by traumatic effects of the parasite on bryozoan tissue. The resultant fragmentation of infected colonies was marked, with conspicuous differences in the apparent vitality between uninfected and infected colonies evident (Figure 3.28).

Whereas Schröder (1910) illustrated the development of statoblasts in *Buddenbrockia*-infected *P. repens*, subsequent reports noted a distinct absence of fresh statoblast production in malacosporean-infected bryozoan colonies (Canning *et al.* 2002; Morris *et al.* 2002a). In the current study, statoblasts were seen in zooids

which had shown signs of parasitism since their earliest developmental stages, thus the statoblast formation could not have preceded *B. plumatellae* infection. Although statoblasts continued to be formed throughout infected colonies, markedly fewer were seen when compared to uninfected colonies adhered to the same Petri dish (Figure 3.28). The development of statoblasts fuels the hypothesis that malacosporean infection could be harboured in these asexual stages over the winter period; alternative possibilities being the survival of the parasites in the sole phylactolaemate genus (*Fredericella*) which overwinters as live zooids or the existence of reservoirs of infection in alternative hosts. Unfortunately, attempts at germinating statoblasts from infected colonies were unsuccessful, negating the possibility of witnessing true vertical transmission of infection in Bryozoa.

Following comprehensive field studies involving the collection of numerous bryozoans from multiple sites, Canning *et al*. (2002) concluded that the natural prevalence of *Buddenbrockia* in wild bryozoans was low and that it seemed unlikely that direct horizontal transmission occurred between bryozoan colonies. In the current study, no direct horizontal bryozoan transmission was observed despite repeated attempts involving the direct injection of *B. plumatellae* material between zooids and separate prolonged cohabitation trials of uninfected and infected colonies. These findings reinforce the assertion that additional hosts may be involved in the life cycle of *Buddenbrockia*. Whether these potential hosts would be in the form of other invertebrates or perhaps teleosts can only be hypothesised.

Morris *et al*. (2002a) described malacosporean development in *P. repens* following exposure to homogenised PKD-infected rainbow trout kidney. However, due to morphological and developmental findings, it was judged by the authors that the malacosporean present was neither *B. plumatellae* nor *T. bryosalmonae*. Indeed, several marked differences exist between the previously described malacosporean

and *B. plumatellae* examined in the current study. Morris *et al*. (2002a) reported an absence of stages attached to the peritoneum, limited deleterious effects on the bryozoans, an absence of statoblast production, isolation of infection by zooid degeneration and formation of non-spherical ornamented spores. The contrast of these observations to those of the current study supports their suggestion of the existence of a malacosporean species distinct but related to *B. plumatellae*. Despite continued efforts, Morris *et al*. (2002a) were successful neither in infecting rainbow trout with the malacosporean nor in repeating the incidence of infection in bryozoans following exposure to PKD-infected kidney. However, the possibility that the malacosporean parasite that they studied was a vermiform alternative manifestation of *T. bryosalmonae* is highly intriguing. In the current study, no sign of malacosporean infection was seen in fish exposed to *B. plumatellae* spores. In light of the fact that the fish farm from which the infected bryozoans were collected also stocked common carp – a species in which an unidentified myxozoan parasite possessing electron-dense bodies similar to those seen in the Malacosporea has been described (Voronin and Chernysheva 1993) – it had been hoped to conduct an experimental transmission trial of *B. plumatellae* to carp. Unfortunately, all of the cultures of infected *P. repens* material died before this experiment could be undertaken.

Canning *et al*. (2002) suggested that *B. plumatellae* could appear rapidly within bryozoans, and vanish equally swiftly. While the former assertion has been supported by the current observations, the latter has been challenged by the maintenance of infection in *P. repens* for three months and the resultant development of waves of malacosporean infection. The previous authors stated that sub-optimal laboratory culture conditions may have contributed to the premature demise of infected colonies. It would appear that this may also have led to the previous inability of colonies to develop statoblasts. In the current study, greater

growth of infected bryozoans was witnessed following increased concentration of algal and protozoal components in the culture medium by centrifugation. This resulted in the production of a highly turbid culture medium, consistent with the water conditions described by Canning *et al*. (2002) in an American lake harbouring infected *Hyalinella* sp. Jullien colonies. In the current study, all of the cultured bryozoans – both uninfected and infected – died after three months of laboratory culture. As the uninfected colonies had been actively thriving until this point, it seems likely that the culture demise was due to a collapse in the culture system rather than a natural termination of infection.

Thus, it could be concluded that under ideal culture conditions, bryozoans harbouring *B. plumatellae* could be reared for extended periods, with consecutive waves of infection maintaining the presence of parasites for as long as the bryozoans were adequately provided with culture requirements. The observation of sequential developmental stages studied by light- and electron-microscopy has revealed further details of malacosporean parasitism, while transmission trials have suggested that another (possibly teleost) host is involved in the life cycle. The close relationship between *B. plumatellae* and *T. bryosalmonae* should mean that research into the former species could reveal potential insights into the biology of the latter. Further elucidation of the life cycles of the Malacosporea would be an aim in future research in trying to understand the complex dynamics of these intriguing parasites.

Chapter 4: Developmental stages of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) in phylactolaemate bryozoans (Bryozoa: Phylactolaemata)

4.1. Introduction

Although they were originally described almost 150 years ago (Allman 1856), relatively little is known of the developmental stages of malacosporean parasites within their bryozoan hosts. Canning *et al*. (1996) renewed the current interest in these parasites with their description of the development of *Tetracapsula bryozoides* (now known to be a junior synonym of *Buddenbrockia plumatellae*) within *Cristatella mucedo*. This first ultrastructural study of malacosporean development allowed the authors to propose a rudimentary structure for a malacosporean spore. The subsequent discovery of *Tetracapsuloides bryosalmonae* within bryozoans further intensified the momentum to elucidate the morphological characteristics of these now economically pertinent parasites (Anderson *et al.* 1999a).

Further ultrastructural studies have demonstrated the presence of various developmental stages of *T. bryosalmonae* within bryozoans, including the formation of mature spores (Canning *et al.* 1999, 2000; Canning and Okamura 2004). In addition, light microscopical studies of malacosporean development have revealed details of the morphological configuration of the parasites (Gay *et al.* 2001; Morris *et al.* 2002a; Canning and Okamura 2004), allowing schematic diagrams of the spores to be devised. However, artefacts inherent from the processing of material for transmission electron microscopy (Hayat 1981), the paucity of available spores of *T. bryosalmonae* (de Kinkelin *et al.* 2002) and the limitations of the resolution of conventional light microscopical techniques have curtailed the comprehensive study of the spore, an intrinsically significant parasitic developmental stage with regard to PKD. Resultantly, spores of *T. bryosalmonae* have been described as being of approximate diameter 20 µm, roughly spherical in shape and relatively soft in structure (Canning *et al.* 1999). Although the constituent cells of the spore have been described (Canning *et al.* 2000), their three-dimensional relationship has not been

previously proposed, meaning that the complex morphology of the spore could not be interpreted.

Following collection of the bryozoan *Fredericella sultana* from waterways known to be endemic for PKD, rainbow trout have been successfully infected with *T. bryosalmonae* following experimental exposure trials (Feist *et al.* 2001). While exposure of disrupted bryozoan material to trout in static water (putatively resulting in a high ratio of spores to fish) resulted in infection, it was also found that cohabitation of infected bryozoans with trout in a flow-through system (resulting in a lower challenge ratio) resulted in PKD development. This suggested that exposure to a relatively low number of spores was capable of infecting trout; however, no quantification of the infectious dose could be deduced at that time. Additionally, Feist *et al*. (2001) demonstrated that intraperitoneal injection of *T. bryosalmonae* spores did not result in development of PKD in rainbow trout, suggesting that the spores required initial exposure to the teleost integument in order to undergo the subsequent changes necessary to elicit infection. Further studies have demonstrated the presence of early stages of *T. bryosalmonae* within the gills and skin tissue of rainbow trout following experimental exposure to spores or PKD-endemic water (Morris *et al.* 2000b; Longshaw *et al.* 2002). The latter authors asserted that spores of *T. bryosalmonae* were highly effective at locating and infecting trout, with exposure to very low numbers of spores being capable of causing PKD. Within the Myxozoa, investigations into quantifying an infective dose of spores to fish have been documented for *Myxobolus cerebralis* (Markiw 1991, 1992), but no such information has previously been gathered for *T. bryosalmonae*.

The aim of this study was to collect bryozoan colonies infected with *T. bryosalmonae* and subsequently culture them for prolonged periods. This would supply material to allow:

- Examination of the developmental stages of *T. bryosalmonae* within its bryozoan hosts by light microscopy.

- Confocal laser scanning microscopy (CLSM) examination of spores allowing three-dimensional (3D) models of the morphological structure to be produced.

- Optimisation of experimental challenge methods, potentially being intrinsic in future efficacy testing of prospective PKD vaccine candidates.

- Corroboration of previous assertions of a low infectious dose of spores of *T. bryosalmonae* to trout by quantifying the number of spores capable of eliciting infection in individual rainbow trout.

Success with such studies would allow further insight into the parasitic interaction between bryozoan and teleost hosts of this intriguing myxozoan.

4.2. Materials and Methods

4.2.1. Collection of bryozoan colonies

Bryozoan colonies were collected from fish farms in southern England known to be endemic for PKD as described in sections 2.2.1 and 3.2.1. Between June 2002 and June 2004, eight field trips were made in the months between February and October. On each visit, multiple colonies (confirmed to be *Fredericella sultana* by colony and statoblast morphological analysis as described in Section 3.3.2) were collected by incising portions of a willow tree root system submerged within the River Cerne. Colonies of *Plumatella* sp. (identified by colony morphology) adhered to black plastic "bubble-wrap" were also removed from the River Avon.

4.2.2. Maintenance and study of the bryozoans in the laboratory

The resulting colonies were maintained in 5 L tupperware tanks as described in Section 2.2.4, with the culture vessels kept at 18°C in an incubator. Stages of malacosporean development were monitored by light microscopy as described in Section 3.2.3 for between 48 and 84 days depending on the viability of the colonies.

4.2.3. Experimental challenge of fish with culture media from colonies of *F. sultana* infected with *T. bryosalmonae*

Twenty four rainbow trout of mean weight 85 g, from a source known to be free of PKD were segregated evenly and randomly allocated into two 100 L flow-through tanks supplied with dechlorinated tap water at a temperature of 18°C in the ARF. After acclimatisation, the water flow was stopped to both tanks and the level reduced to 21 L with continuous vigorous aeration applied. To one of the tanks, one litre of culture media from a tank containing colonies of *F. sultana* observed to contain suspected spores of *T. bryosalmonae* was added. To the other tank – acting as a

negative control, freshly made CM was added. After 60 min, the water supplies to both tanks were restarted. The exposure procedure was repeated after four days.

After 50 days maintenance at a water temperature of 18°C, the fish were overdosed in 10 mg L^{-1} benzocaine and euthanased by severing the spinal cord. Samples of kidney, spleen and liver were fixed in 10% neutral buffered formalin for 24 hours. The samples were then processed for histology as outlined in Section 3.2.8.1 and subsequently examined by immunohistochemistry as described in Section 3.2.8.2. Samples of kidney were also taken from each fish and frozen for subsequent DNA analysis.

4.2.4. DNA amplification and detection

4.2.4.1. DNA extraction

Samples of kidney were stored in sterile 0.5 ml Eppendorf tubes at -20°C until DNA extraction was carried out. The samples were then defrosted and transferred to sterile 1.5 ml Eppendorfs. A DNA extraction kit (Genisol Maxi-Prep Kit, ABgene) based on the protocol of Miller, Dykes and Polesky (1988) was used, resulting in the salting-out of protein contaminants.

Firstly, 0.5 ml of Digestion Buffer was added to the sample which was ground with a sterile pestle and incubated at 60°C for up to one hour. Next, 160 µl of Precipitation Buffer was added and incubated for two minutes at room temperature, followed by centrifugation in a microcentrifuge (IEC Micromax, Thermoquest, UK) for five minutes at 1500 × *g*. The supernatant containing DNA was decanted to a fresh sterile 1.5 ml Eppendorf containing 660 µl of 80% (v/v) isopropanol and gently mixed by inversion. Precipitated DNA was pelleted by centrifugation at 1000 × *g* for five minutes. The supernatant was discarded and the pellet flushed well with 600 µl of 80% (v/v) isopropanol. The alcohol was discarded, taking care to conserve the pellet,

and any excess alcohol was allowed to evaporate from the tube. The resultant DNA was dissolved in 100 µl of ultra-pure water for 15 minutes at 60°C. The DNA samples were either analysed immediately or stored at -20°C for subsequent use.

4.2.4.2. Primer preparation

The *Tetracapsuloides bryosalmonae* specific primers 5F (CCTATTCAATTG-AGTAGGAGA) and 6R (GGACCTTACTCGTTTCCGACC) designed by Kent *et al*. (1998) were used for DNA amplification. The primers were dissolved at 55°C for 10 minutes in sufficient ultra-pure water to result in a concentration of 100 µM and then briefly vortexed (WhirliMixer, Fisher, UK). Aliquots of 10 µl were stored at -20°C. For use, 390 µl of ultra-pure water was added to an aliquot resulting in a working concentration of 2.5 µM.

4.2.4.3. Polymerase chain reaction (PCR)

DNA was amplified using the method of Kent *et al*. (1998) with modifications. To a sterile 200 µl PCR tube, 2.5 µl DNA template, 1 µl of each primer, and 8 µl of ultra-pure water were added to 12.5 µl of PCR master mix solution (2 × Reddymix, ABgene, UK: 1.25 units Thermoprime Plus DNA Polymerase, 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween 20, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, precipitant and red dye for electrophoresis). For each reaction, positive control samples including DNA extracted from a PKD-affected rainbow trout kidney and negative control samples were included alongside the samples being analysed.

The tubes were placed in a thermocycler ('T Gradient', Biometra, Goettingen) and heated to 94°C for 3 minutes, followed by 35 cycles of the following sequence: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by a final incubation of 5 min at 72°C. The samples were then stored at 4°C, awaiting analysis by electrophoresis.

4.2.4.4. Gel electrophoresis analysis

A 1.5% agarose gel was created by dissolving 0.9 g agarose in 60 ml TBE buffer (0.09 M Tris, 0.09 M boric acid, 2 mM EDTA Na_2 (pH 8.0)) in a round-bottomed flask and heated in a microwave oven for 90 sec at 45 Watts. Six µl of 5 mg ml^{-1} ethidium bromide was then added to the dissolved gel and mixed by gentle swirling. The solution was then poured into a horizontal gel caster (Flowgen) and one or two 20 tooth combs were inserted depending on the number of samples to be analysed. The gel was allowed to set for approximately 30 min before being placed in electrophoresis running apparatus and covered with 340 ml of TBE buffer.

DNA molecular weight marker (pHC624 TAQ I/PMJ3 NC11, ABgene) was mixed 50:50 (v/v) with gel loading dye (ReddyRun Gel Loading Buffer, ABgene) and 10 µl was instilled into the first well. Into the second well, 10 µl of PCR product from positive control PKD kidney was added, while to the third well, negative control PCR product was added. To subsequent wells, PCR products from samples were added. The gel was subjected to a potential difference of 80 volts for 60 min, after which DNA bands were visualised under ultra violet (UV) illumination (UVS White / UV Transilluminator). Using this protocol, positive bands would be seen at 435 base pairs. The image files (.bmp) were saved on a personal computer.

4.2.5. Quantification of the minimum infective dose of *T. bryosalmonae* spores to rainbow trout

4.2.5.1. Trial 1

4.2.5.1.1. Collection of *T. bryosalmonae* spores

Colonies of *F. sultana* collected and maintained as described in sections 4.2.1 and 4.2.2 were examined using an inverted microscope until spores of *T. bryosalmonae* became apparent within the metacoel. The bryozoans were then teased apart on microscope slides viewed with a dissecting microscope resulting in release of spores

into the surrounding media. The slides were then placed on the stage of an inverted microscope, with attached microinjection and manipulation accessories as described in Section 3.2.6.1. Spores were carefully counted as they were slowly drawn into the micropipette. Known numbers of spores from each withdrawal were then expelled into individual bijoux containers. To each of six replicate containers, one spore was added, while six replicate containers were also made with five and ten spores each. To each of six negative control containers, freshly made CM was added.

4.2.5.1.2. Experimental exposure of *T. bryosalmonae* spores to fish

Twenty four rainbow trout (mean weight 16 g) originating from a source known to be free of PKD were each individually allocated to one of 24, ten litre flow-through tanks maintained at a water temperature of 18°C in the ARF. On the day of challenge, the water supply to each tank was suspended and the volume of each tank reduced to 2.5 L. To each tank, one of the containers described in Section 4.2.5.1.1 was randomly assigned. The content of each container was added to its respective tank, with thorough rinsing of the bijoux with the tank water to allow maximum expulsion of the spores from bijoux to tank. The tanks were maintained with vigorous aeration at 2.5 L for one hour, after which the water supply to each tank was restarted.

The fish were maintained at 18°C for 56 days after which they were overdosed in 10 mg L^{-1} benzocaine and euthanased by severing the spinal cord. Samples of kidney, spleen and liver were fixed in 10% neutral buffered formalin for 24 hours. The samples were then processed for histology as outlined in Section 3.2.8.1 and subsequently examined by immunohistochemistry as described in Section 3.2.8.2. Samples of kidney were also taken from each fish and frozen for subsequent DNA analysis as described in Section 4.2.4.

4.2.5.2. Trial 2

A second experimental trial was undertaken including further modifications to the methodology in attempting to achieve more robust results than in the first trial.

4.2.5.2.1. Collection of *T. bryosalmonae* spores

Spores were dissected from *F. sultana* as described in Section 4.2.5.1.1, and drawn into micropipettes using the apparatus described therein. On this occasion, however, the collected spores were not counted as they were drawn into the micropipette but rather as they were slowly expelled onto individual cavity microscope slides viewed using an inverted microscope. Ice packs were placed on the microscope stage in an attempt to maintain the temperature of the specimens. One spore was added to each of six microscope slides, two spores being added to a further two slides, three spores to two slides, five spores to two slides, six spores to one slide, 14 spores to two slides, 15 spores to one slide and 16 spores to two slides. These slides were kept cool on the surface of a container filled with ice until being transported within 10 minutes to the ARF.

4.2.5.2.2. Experimental exposure of *T. bryosalmonae* spores to fish

Twenty four rainbow trout (mean weight 25 g) from a PKD-free source were allocated to the ARF tanks described in Section 4.2.5.1.2. Again, on the day of exposure, the water level was reduced to 2.5 L, and to each tank one slide, as described in Section 4.2.5.2.1, was randomly assigned, the slide being well rinsed in the tank water and then attached to metal clips (Cytoclip, Shandon) and suspended by string in the tank for one hour. To each of six control tanks, a slide including uninfected CM was added. The fish were then maintained for 63 days at 18°C before being euthanased, sampled and examined as described in Section 4.2.5.1.2.

4.2.6. Examination of spores of *T. bryosalmonae* using confocal microscopy

4.2.6.1. Collection of material

Spores of *T. bryosalmonae* were identified circulating within the metacoel of colonies of *F. sultana* following collection from the River Cerne in September 2003 and February 2004 (Table 4.3) as described in Section 2.2.1. Infected colonies were suspended in CM in nine cm Petri dishes and carefully dissected while viewed with a dissecting microscope. Released spores were pipetted on to microscope slides, in preparation for fluorescent staining.

4.2.6.2. Fluorescent staining of *T. bryosalmonae* spores

The fluorophores, Blankophor (4,4'-bis[(4-anilino-6-substituted 1,3,5-triazine-2-yl)amino]stilbene-2,2'-disulfonic acid), BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) and DAPI (4,6-diamidino-2-phenyl-indole, dilactate) were diluted in dechlorinated tap water to appropriate working dilutions (Table 4.1). These fluorescent stains were then added to the medium containing spores. Cover slips were then placed over the samples, and sealed with nail varnish to prevent evaporation of the medium. The slides were incubated in the dark for between 30 min and two hours to allow fluorescent staining of the spores and they were then examined by confocal laser scanning microscopy.

Table 4.1: Fluorescent stains used for confocal microscopy of *T. bryosalmonae*

Fluorophore	Manufacturer	Working concentration	Staining specificities
Blankophor	ICN Biomedicals	0.1%	Beta-glycosidically linked polysaccharide stain
BODIPY 505/515	Molecular Probes	80 µM	Neutral lipid stain
DAPI	Molecular Probes	300 nM	Nucleic acid stain

4.2.6.3. Scanning of *T. bryosalmonae* spores by confocal microscopy

A TCS SP2 AOBS confocal laser scanning microscope (Leica) coupled to a DM IRE2 inverted microscope (Leica) employing ×63 oil- / glycerol-immersion objective lenses (HCX PL APO CS 63X NA 1.3) was used to examine the microscope slides of spores of *T. bryosalmonae*. Nine scans of spores were carried out using the settings described in Table 4.2. In addition to laser excitation of fluorochromes, transmitted light was also used to visualise some of the samples. Sequences of images were taken along the optical Z axis using the confocal optical sectioning facility. The images were acquired on a PC running Windows XP using Leica Confocal Software (version 2.5), with three-dimensional (3D) reconstructions generated using the same application.

4.2.7. 3D modelling of the spore of *T. bryosalmonae*

Using the data of spore morphology obtained from the confocal laser scanning microscopy examinations, schematic 3D models of the spore structure were developed using a Windows XP PC running 3D Canvas software (version 6.5.0.2, Amabilis Software). Three-dimensional models of the spore were produced using spheres of various sizes to represent the components of the structure. Still and video images were captured of the spore in various positions, including dynamic rotational views. In addition, Dr. J.E. Bron (Institute of Aquaculture, University of Stirling, UK) programmed a PC running the imaging software 3DS Max 7 (Discreet Software) to develop static schematic 3D models of the spore including internal constituents of the spore.

Table 4.2: Analyses performed on *T. bryosalmonae* spores using confocal laser scanning microscopy

Scan number	Fluorophore(s) used	Number of sections captured	Thickness of each section (nm)	Wavelength(s) of excitation (nm)	Wavelengths of detection (nm)
1	Blankophor	50	488	405; 514; 633	402-480; 509-523
2	Blankophor	100	244	405	458-601
3	Blankophor	100	238	405; 594	416-462; 543-593
4	Blankophor	200	146	405; 594	419-482; 551-591
5	Blankophor	200	169	405; 488	412-464; 475-547
6	DAPI	20	1154	405; 594	412-517; 544-621
7	BODIPY; DAPI	195	107	405; 488	510-520
8	BODIPY	150	214	488	510-520
9	BODIPY	150	176	405; 488	500-535

4.2.8. Immunohistochemistry of sections of *F. sultana* infected with *T. bryosalmonae*

Colonies of *F. sultana* seen to include developing stages of *T. bryosalmonae* were dissected and fixed in 10% neutral buffered formalin for 24 hours. The samples were then processed as described in Section 3.2.8.1. Immunohistochemistry was conducted as described in Section 3.2.8.2, however in addition to using monoclonal antibody (MAb) P01, neat supernatants of the MAbs B4 and D4 originally produced by Morris (1996) were also used for incubation. As a negative control, slides were also incubated with fresh hybridoma tissue culture medium (Dulbecco's modified essential medium, DMEM, Sigma).

4.3. Results

4.3.1. Study of the development of *T. bryosalmonae* within phylactolaemate bryozoans using light microscopy

4.3.1.1. Recognition of *T. bryosalmonae* infection

On each of the eight field trips to the River Cerne, colonies of *F. sultana* were found attached to the willow root and collected. Following maintenance of the colonies in the laboratory at 18°C, signs of malacosporean development were invariably seen. The time interval between collection and identification of malacosporean parasite development varied from 2-38 days with a mean period of 22 days (Table 4.3). Following initial recognition of infection, characteristic spores were always observed within five days. More rapid appearance of infection was witnessed in colonies collected during the earlier part of the season (from February to April) than later in the year. On a single occasion, *T. bryosalmonae* development was observed in two colonies of *Plumatella* sp. collected adhered to "bubble-wrap" plastic from the River Avon. The identity of the observed parasites as *T. bryosalmonae* was confirmed by the development of PKD in fish exposed to the spores as described below in Section 4.3.2.

Table 4.3: Collection of *F. sultana* from the River Cerne and the timing of recognition of *T. bryosalmonae* infection

Date of collection	Time after collection when infection was first recognised (days)
27/06/2002	21
01/10/2002	38
29/04/2003	2
18/06/2003	37
05/09/2003	26
17/02/2004	13
01/06/2004	17
29/06/2004	20

4.3.1.2. Developmental stages of *T. bryosalmonae* within bryozoans

It was often observed that initial recognition of malacosporean infection followed noticeable growth of the bryozoans. This period of proliferation consistently included sexual development, resulting in the production of spermatozoa (Figure 4.1) from the testes. The initial indicator of *T. bryosalmonae* infection involved the presence of numerous small bodies of approximate diameter 7 µm (Figure 4.2) swirling within the bryozoan metacoel. These particles moved freely within the coelomic currents, with no attachments observed between parasite and host. Within a day, round stages of diameter 8-15 µm could be seen attached to the bryozoan peritoneum (Figure 4.3), with many evident near the retractor muscles of the lophophore.

After a period of one to three days, irregularly-shaped bodies of 40-100 µm diameter were seen circulating within the metacoel (Figure 4.4). These varied in basic shape from spherical to elongated (Figure 4.5), with irregular protrusions evident on their external surfaces. Within three days, distinctly spherical bodies of approximate diameter 50-100 µm (Figure 4.6) became evident within the metacoel. In some colonies, abundant numbers of these spherical sacs of *T. bryosalmonae* were seen within single zooids (Figure 4.7). The range in size of the sacs presumably resulted from varying levels of maturity of the stages. Initially, the sacs appeared translucent (Figure 4.8), but subsequently, refractive bodies could be seen internally. At first, the refractive bodies appeared to be localised in peripheral zones within the sacs, but later these zones expanded, eventually coalescing. This gave the appearance of each sac containing a central lucent area surrounded by a shell of refractive bodies (Figure 4.9). Eventually, the coalesced zones filled the lumina of the sacs. Upon observation of some sacs that were moving less rapidly in the coelomic currents, protrusions could be seen from the external walls of the otherwise regularly spherical sacs (Figure 4.10). The protrusions varied in size from 15-50 µm in diameter, some

being relatively innocuous, while others took on more elaborate botryoid formations (Figure 4.11).

Upon maturity, spore sacs of up to 350 μm in diameter appeared to be packed full with refractive bodies (Figure 4.12). Within one or two days, many malacosporean spores could be seen swirling alongside other intact spore sacs in the metacoel (Figure 4.13). Their fast movement within the bryozoan host made examination difficult, although closer details could be discerned where spores became located in slow moving coelomic currents (Figure 4.14). In some bryozoan colonies, innumerable quantities of malacosporean spores could be seen circulating within the coelomic currents. It was estimated that many thousands of spores were contained within one colony comprising five zooids. Following the observation of spores within individual colonies, their prevalence became markedly diminished within 12 hours, with few spores seen after 24 hours.

Bryozoan colonies containing mature spore sacs and spores were dissected. Examination using an inverted microscope confirmed the general structure of the spore sacs (Figure 4.15), with recognisable spores seen exiting mature spore sacs following dissection from the bryozoan host (Figure 4.16). Each spore of approximate diameter 20 μm was seen to include four spherical polar capsules and two sporoplasms (Figure 4.17). Nuclei could also be seen in the outer limiting valve cells of the spores, up to four being evident in a single plane of focus (Figure 4.18). Although some infected bryozoan colonies died following infection, this occurrence was not universal. Some colonies seemed to apparently recover following the release and dissipation of spores, with no infection evident immediately thereafter. However, if the colonies were successfully maintained, signs of infection would return within 10-20 days. This meant that consecutive waves of infection were observed within bryozoan colonies that were cultured for extended periods.

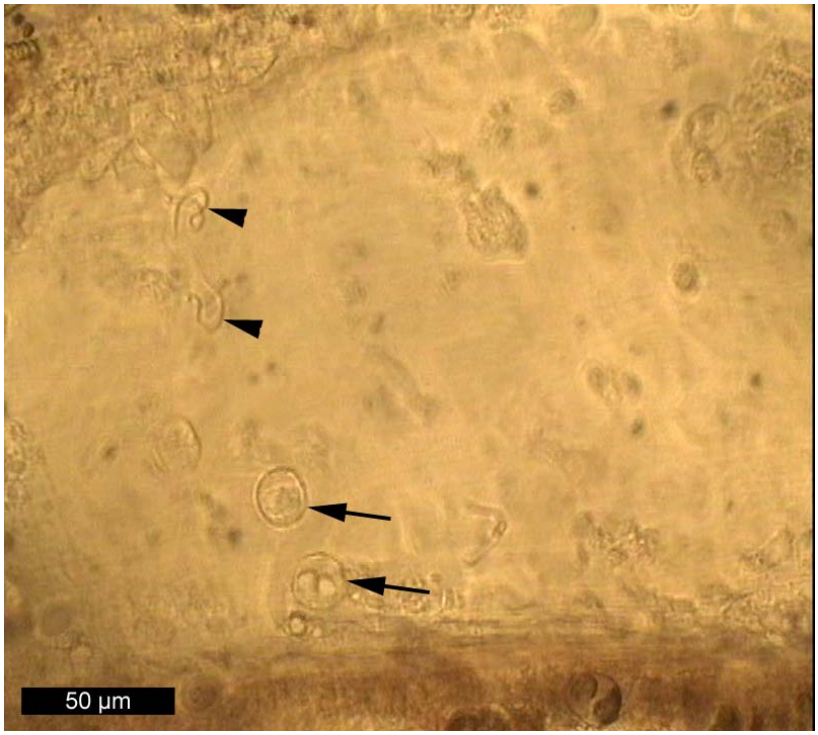

Figure 4.1: Metacoel of *F. sultana* containing both bryozoan sperm and spores of *T. bryosalmonae*

Arrowheads = bryozoan sperm; arrows = spores.

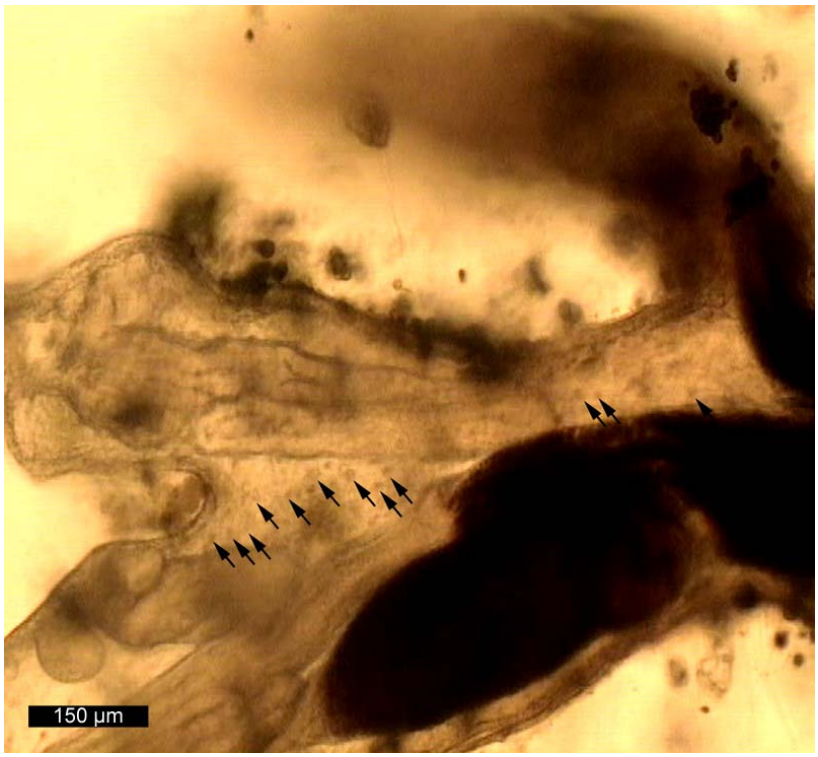

Figure 4.2: Colony of *F. sultana* containing numerous immature stages of *T. bryosalmonae*

Numerous small swirling cells (arrows) are present within the bryozoan metacoel.

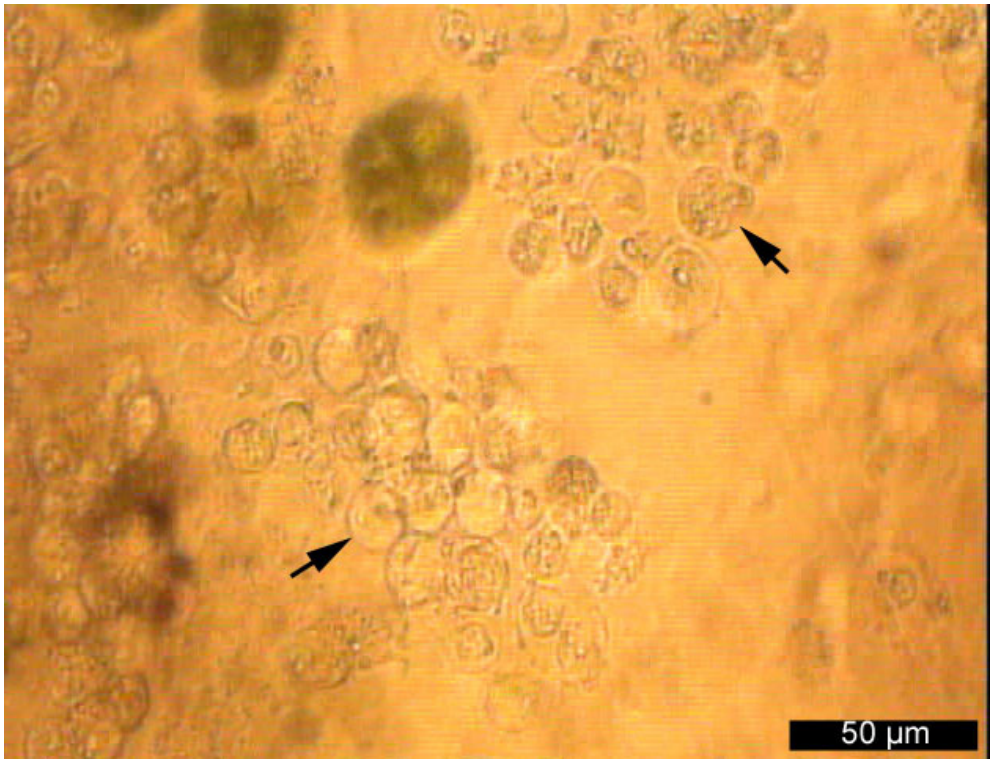

Figure 4.3: Peritoneal stages of *T. bryosalmonae* within *F. sultana*

Arrows mark parasitic stages.

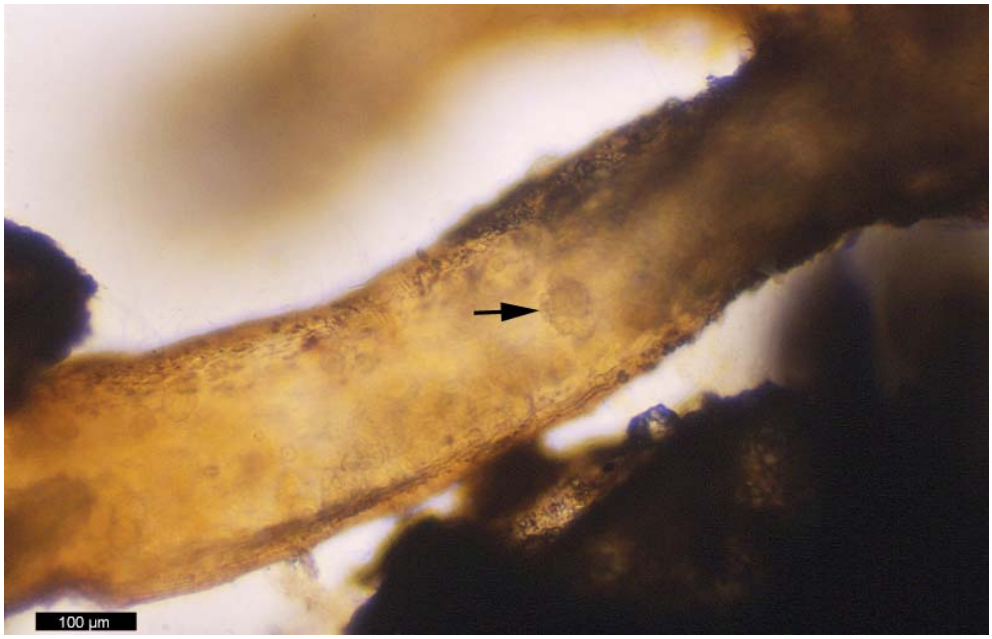

Figure 4.4: *F. sultana* containing immature stages of *T. bryosalmonae*

An irregularly-shaped stage of *T. bryosalmonae* could be seen swirling in the metacoel.

Figure 4.5: An irregularly-shaped stage of *T. bryosalmonae* within *F. sultana*

An elongated irregular malacosporean stage could be seen swirling in the coelomic current.

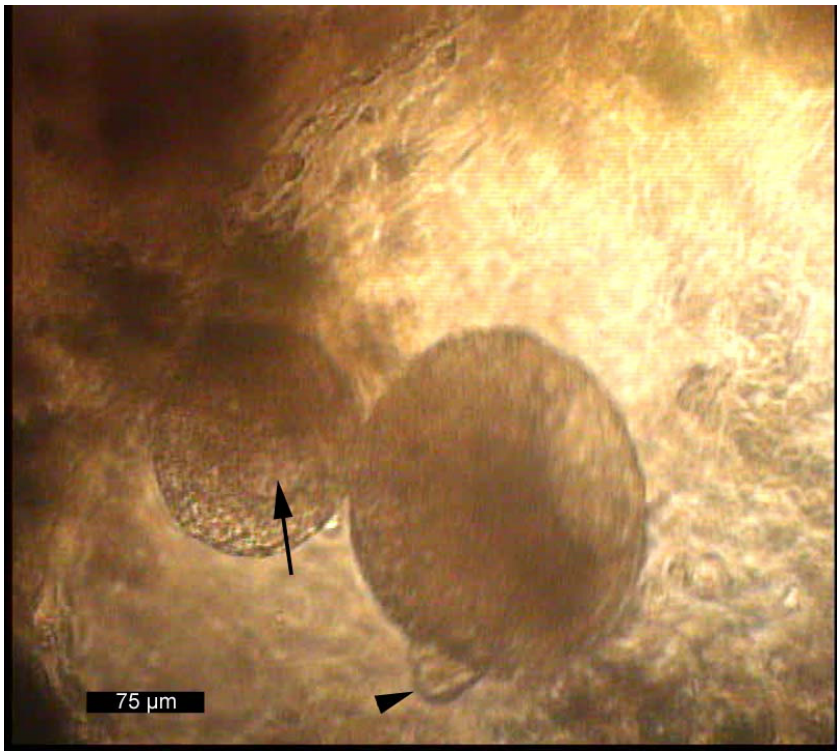

Figure 4.6: Maturing spore sacs of *T. bryosalmonae* within *Plumatella* sp.

Immature spore sacs including lucent zones (arrow) could be seen in conjunction with more mature stages featuring external projections (arrowhead).

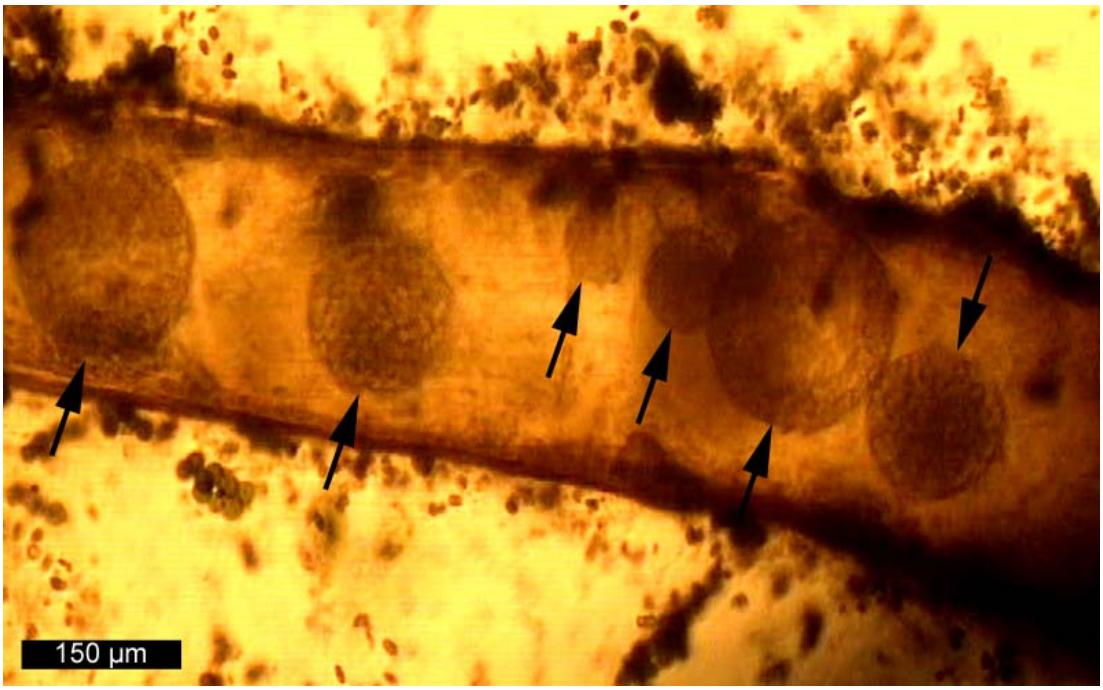

Figure 4.7: Multiple spore sacs of *T. bryosalmonae* within *F. sultana*

Multiple spore sacs (arrows) of varying size and maturity could be seen within bryozoan colonies.

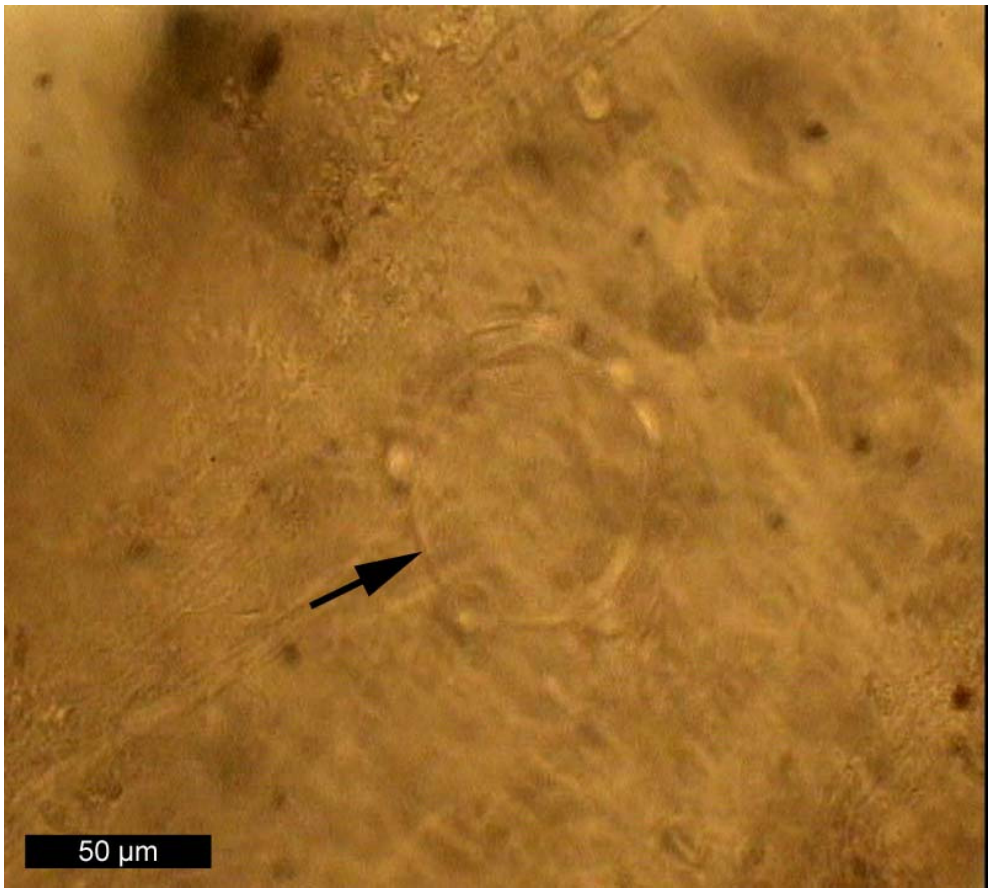

Figure 4.8: An immature spore sac of *T. bryosalmonae* within *F. sultana*

No internal contents could be visualised within the spore sac (arrow) which exhibited external projections.

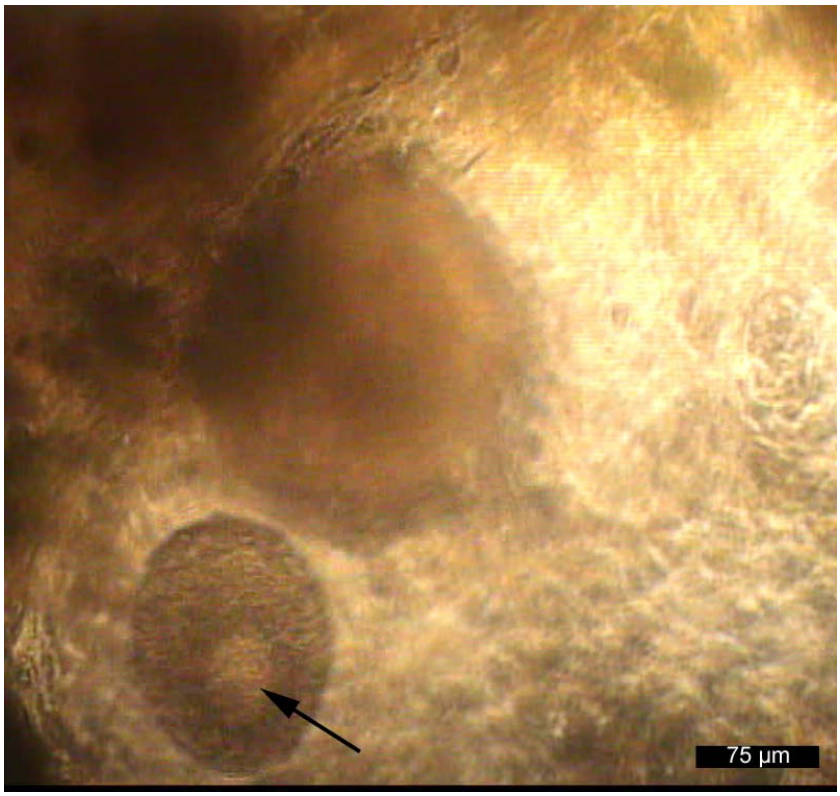

Figure 4.9: Developing spore sacs of *T. bryosalmonae* within *Plumatella* sp.

Central lucent zones (arrow) could be observed in some developing spore sacs.

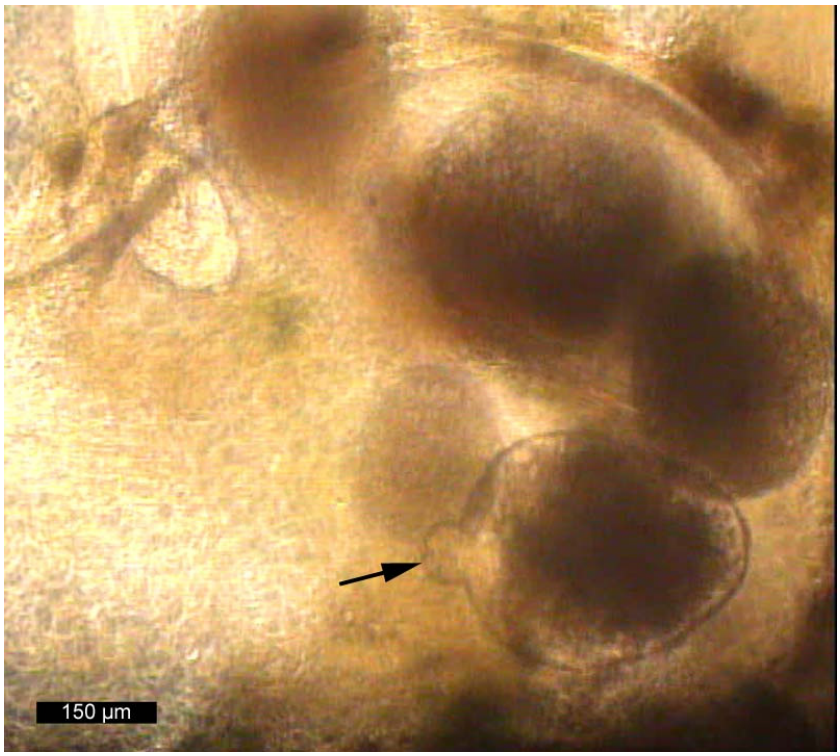

Figure 4.10: Spore sacs of *T. bryosalmonae* within *Plumatella* sp.

Regular bulges could be seen projecting from the surface of some spore sacs.

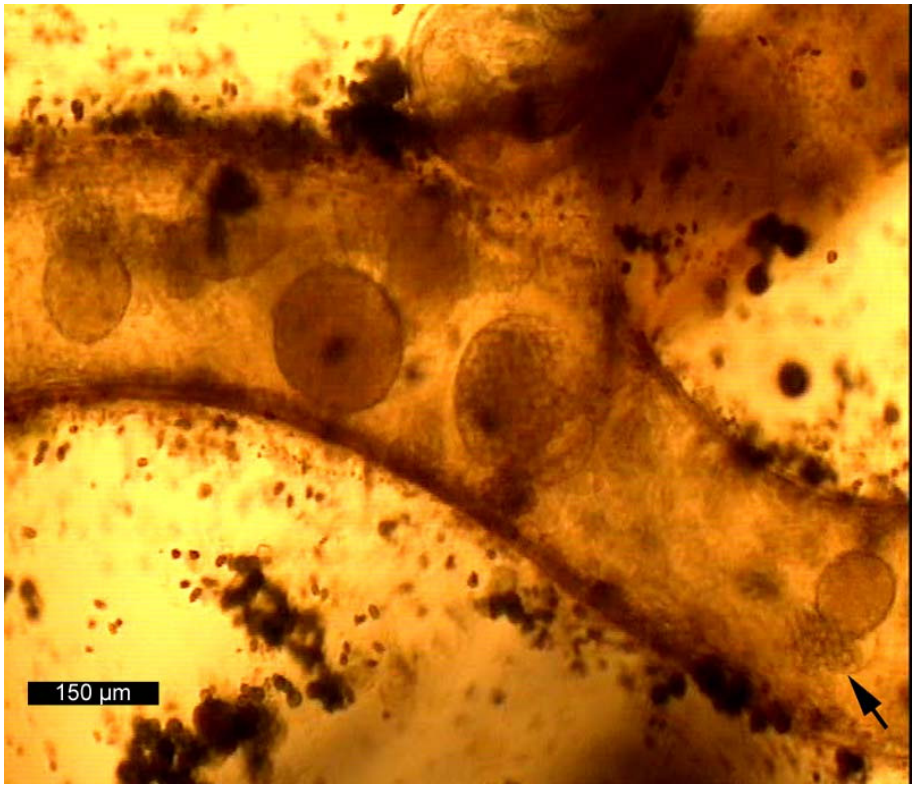

Figure 4.11: Spore sacs of *T. bryosalmonae* within *F. sultana*

Some spore sacs were seen to be irregularly shaped with prominent botryoid projections (arrow).

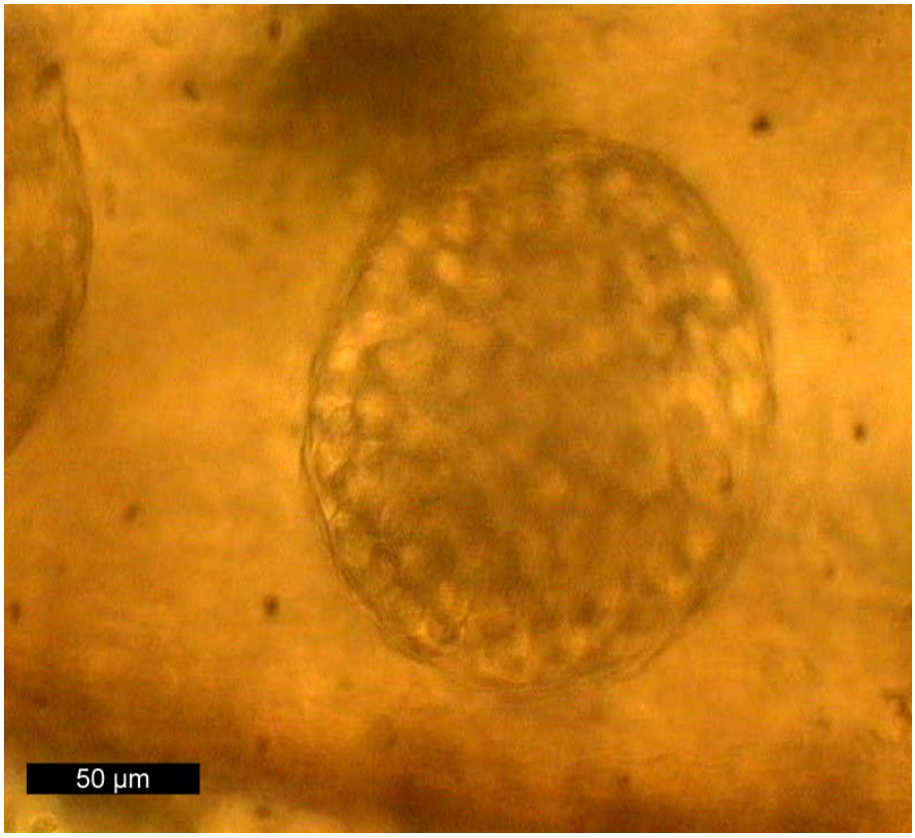

Figure 4.12: Mature spore sac of *T. bryosalmonae* within *F. sultana*

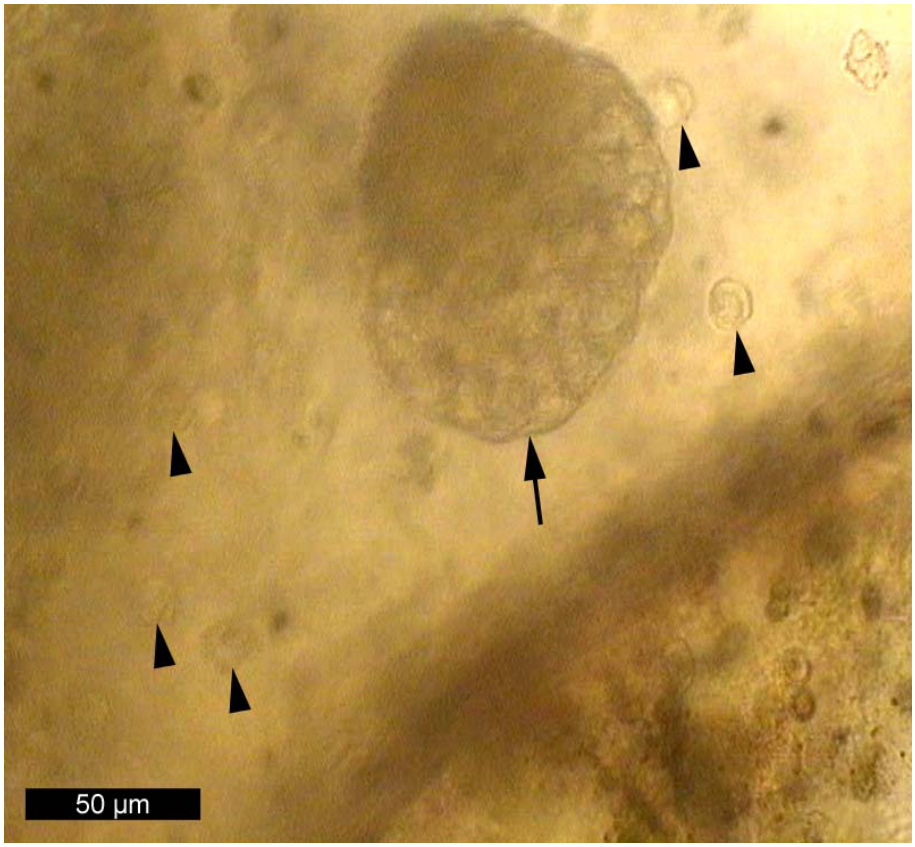

Figure 4.13: Spore sac and spores of *T. bryosalmonae* within *Plumatella* sp.

Spore sac (arrow) carried within the coelomic current amid multiple spores (arrowheads).

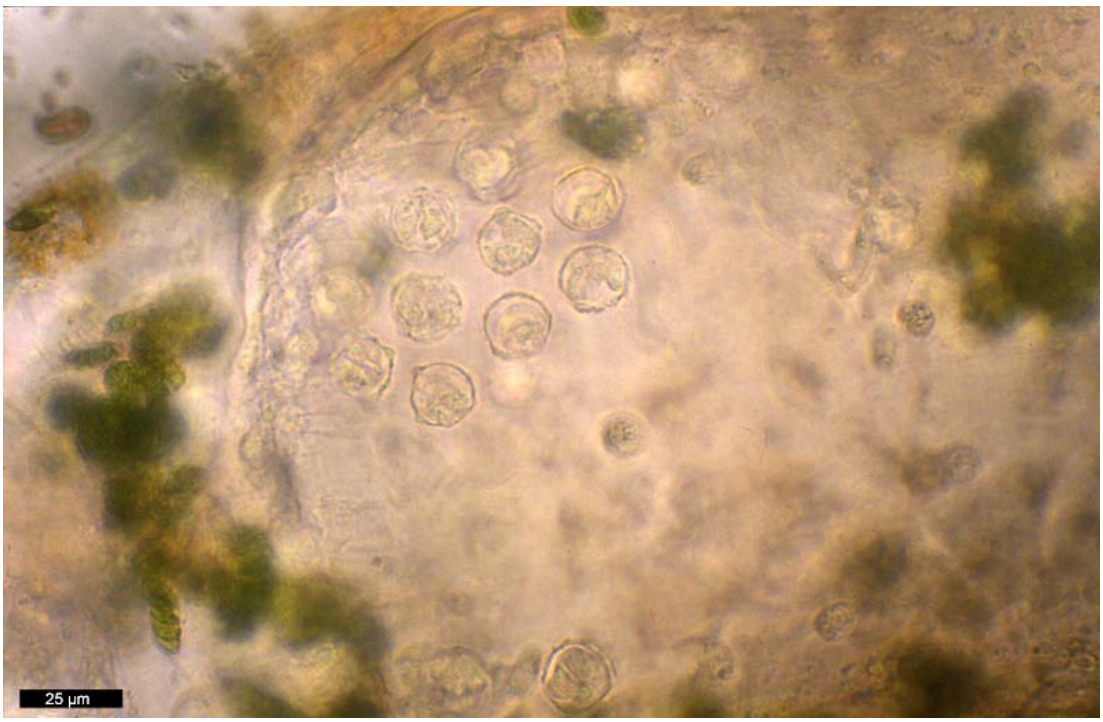

Figure 4.14: Released spores of *T. bryosalmonae* within *F. sultana*

Some spores were visualised within areas of slow-moving coelomic currents.

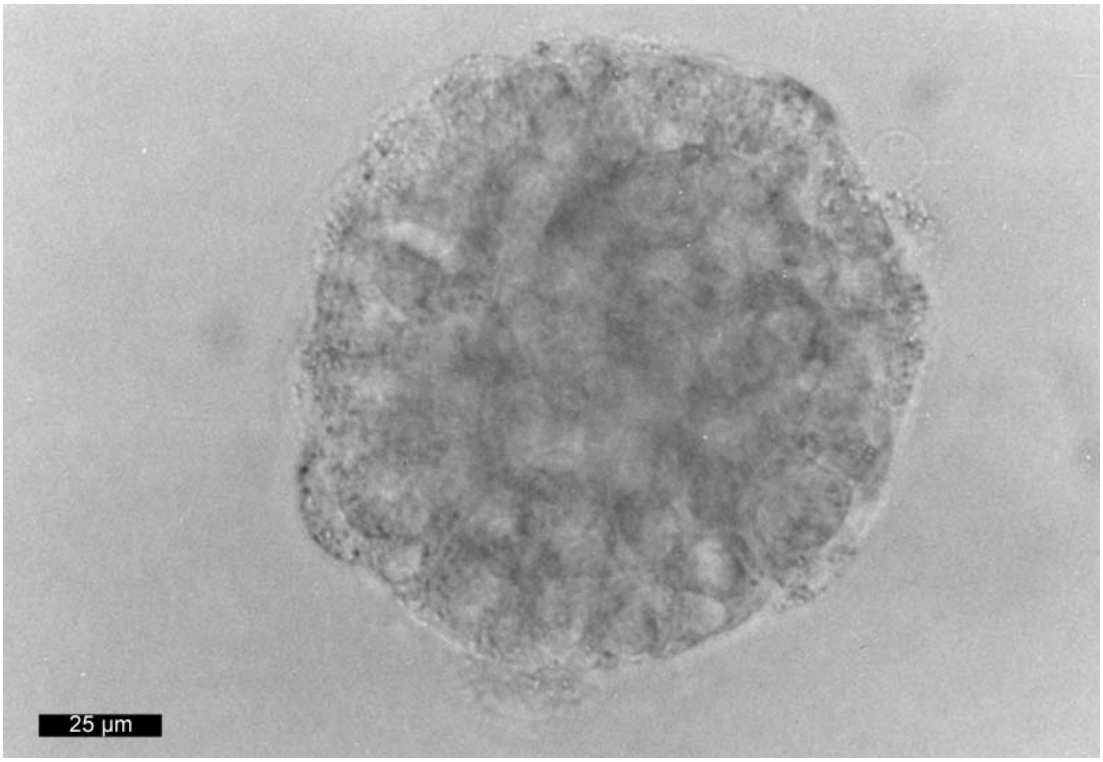

Figure 4.15: Mature spore sac of *T. bryosalmonae* dissected from *F. sultana*

The sac is composed of multiple round bodies, some of which could be seen protruding from the outer surface.

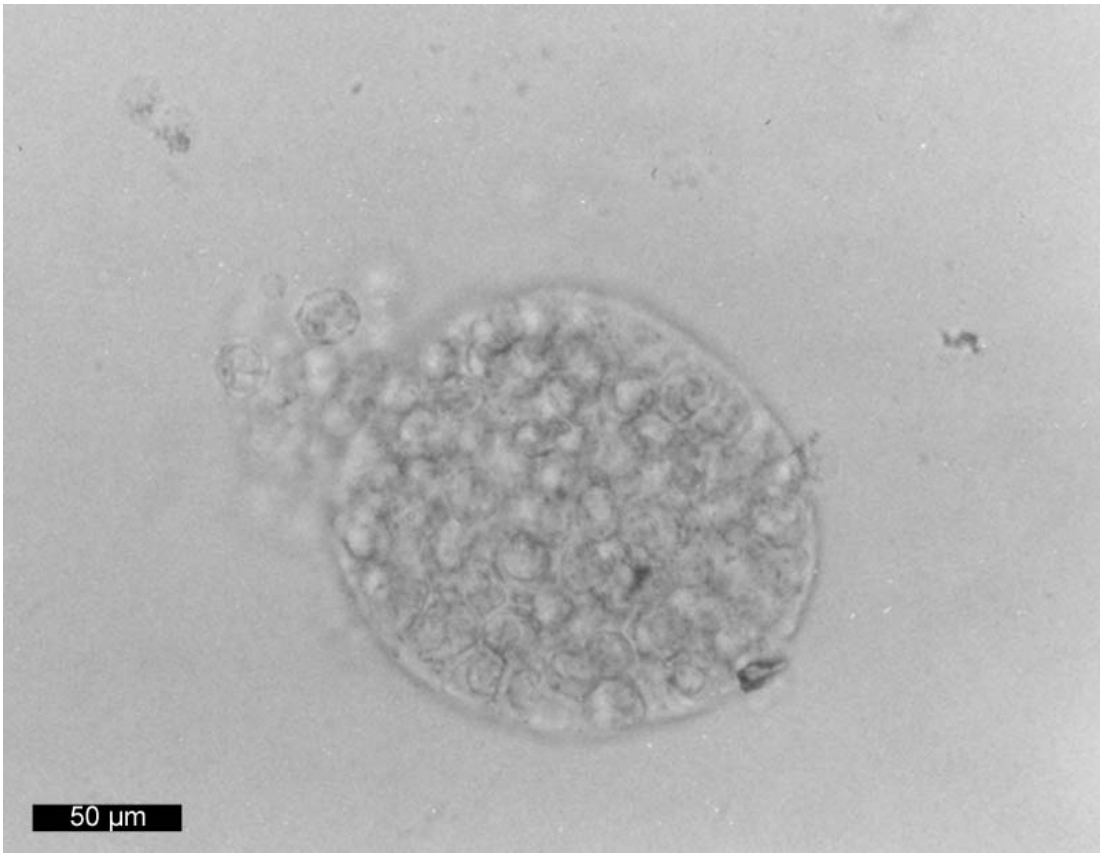

Figure 4.16: Spore sac of *T. bryosalmonae* dissected from *F. sultana*

Spores could be seen exiting at the top left periphery of the sac.

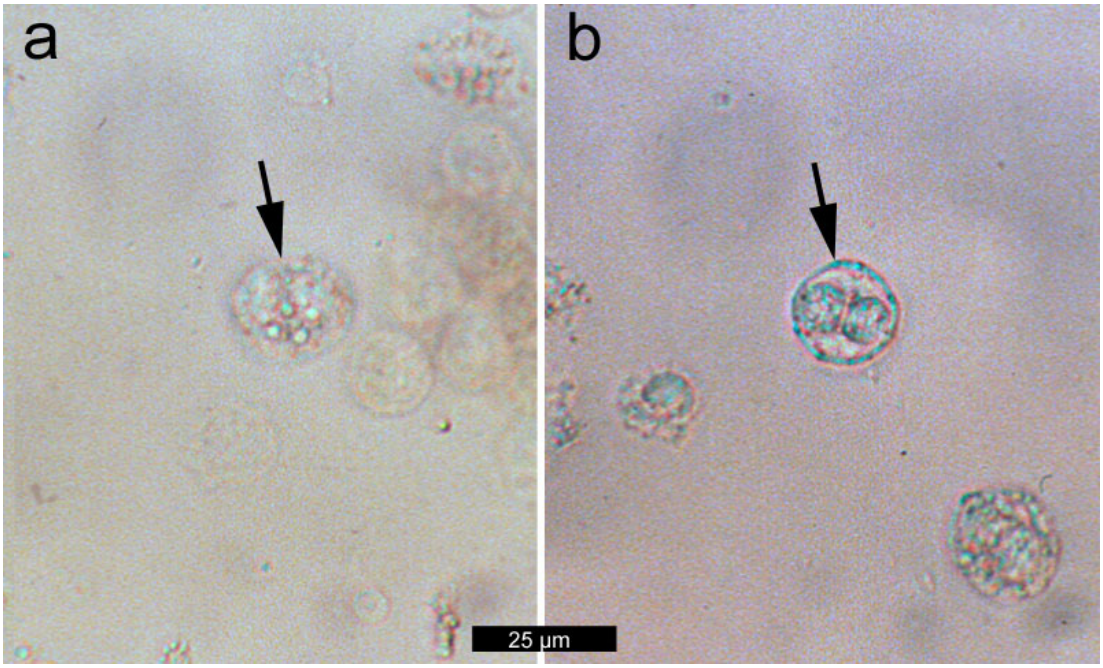

Figure 4.17: Spores of *T. bryosalmonae* dissected from *F. sultana*

(a) Plane of focus showing a spore (arrow) with four refractive spherical polar capsules.
(b) Plane of focus showing a spore (arrow) containing two sporoplasms.

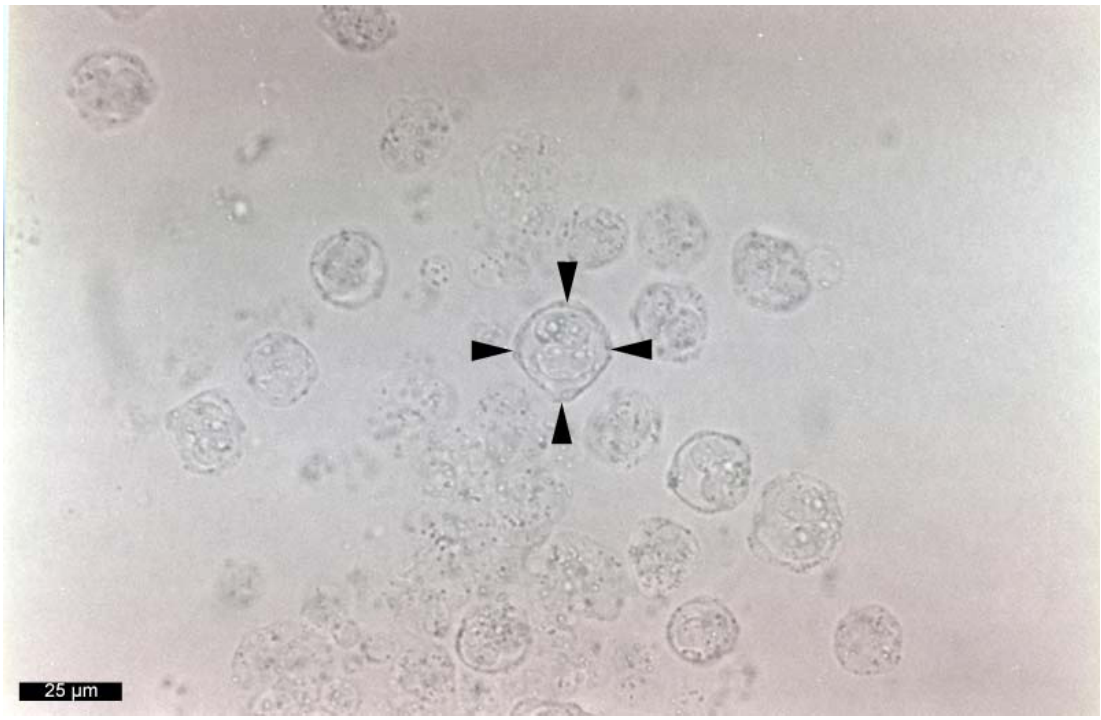

Figure 4.18: Spores of *T. bryosalmonae* dissected from *F. sultana*

Four nuclei (arrowheads) could be visualised in the outer membrane of a spore.

4.3.2. Challenge of fish with culture media from infected *F. sultana*

4.3.2.1. Examination of kidney sections using immunohistochemistry

At the end of the trial, the mean weight of the fish was 83.5 g (s.d. = 52.4). All of the 12 fish exposed to media from infected bryozoans showed nephromegaly upon necropsy. Eleven showed signs of PKD by immunohistochemistry, fish number two giving the only negative result. None of the sections from the negative control fish showed signs of PKD (Table 4.4).

4.3.2.2. PCR analysis of kidney samples

Eleven out of 12 fish exposed to infected media showed positive results by PCR for the presence of *T. bryosalmonae*, although the result of fish number 2 was only weakly positive (Figure 4.19). Fish number six gave a negative result. All 12 of the negative control fish tested negative for *T. bryosalmonae* by PCR (Table 4.4).

4.3.3. Quantification of the infective dose of *T. bryosalmonae* to rainbow trout

4.3.3.1. Examination of kidney sections using immunohistochemistry

In the first trial, the only fish yielding a positive result for *T. bryosalmonae* by immunohistochemistry was fish number 10 (Figure 4.20a), which had been exposed to only one spore (Table 4.5). In the second trial, four fish as noted in Table 4.6 exhibited characteristic signs of PKD (Figure 4.20b-e). Kidney sections of fish 22 (exposed to 2 spores) appeared equivocal by immunohistochemistry, with a few small cells observed which were stained by the DAB substrate that were suspected to represent immature extrasporogonic stages of *T. bryosalmonae* (Figure 4.20f). There was no trend detected between challenge level and resultant renal parasite burden. None of the negative control fish yielded positive results by immunohistochemistry.

Table 4.4: Analysis of kidney samples from rainbow trout exposed to culture media from *F. sultana* infected with *T. bryosalmonae*

"fish no." = fish designation number: "1-12" = exposed to infected media, "13-24" = exposed to uninfected media (negative control); "IHC" = *T. bryosalmonae* immunohistochemistry result; "PCR" = *T. bryosalmonae* polymerase chain reaction result.

fish no.	1	2	3	4	5	6	7	8	9	10	11	12
IHC	+	-	+	+	+	+	+	+	+	+	+	+
PCR	+	+	+	+	+	-	+	+	+	+	+	+

fish no.	13	14	15	16	17	18	19	20	21	22	23	24
IHC	-	-	-	-	-	-	-	-	-	-	-	-
PCR	-	-	-	-	-	-	-	-	-	-	-	-

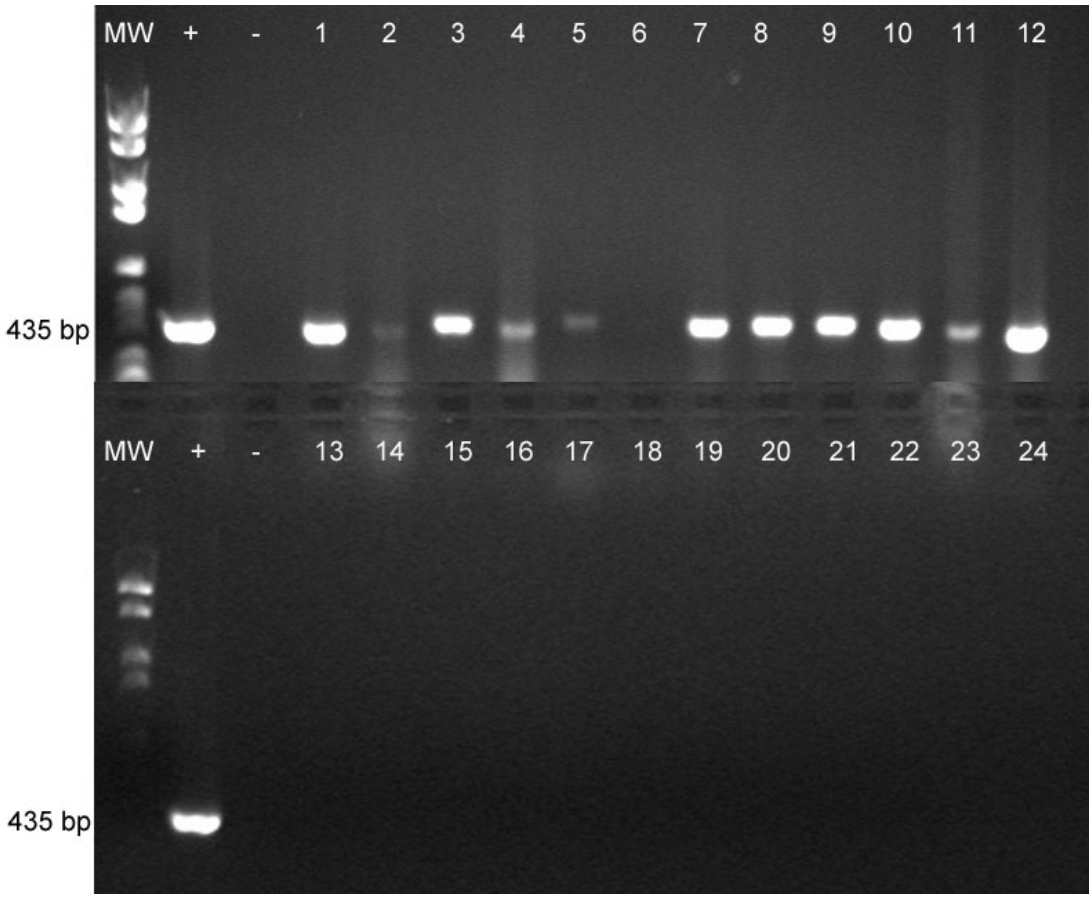

Figure 4.19: PCR analysis of kidney samples from rainbow trout exposed to culture media from *F. sultana* infected with *T. bryosalmonae*

Lanes: "MW" = molecular weight markers; "+" = positive control samples; "-" = negative control samples; "1-12" = fish exposed to infected media; "13-24" = fish exposed to uninfected media. (UV illumination of 1.5% agarose gel containing ethidium bromide)

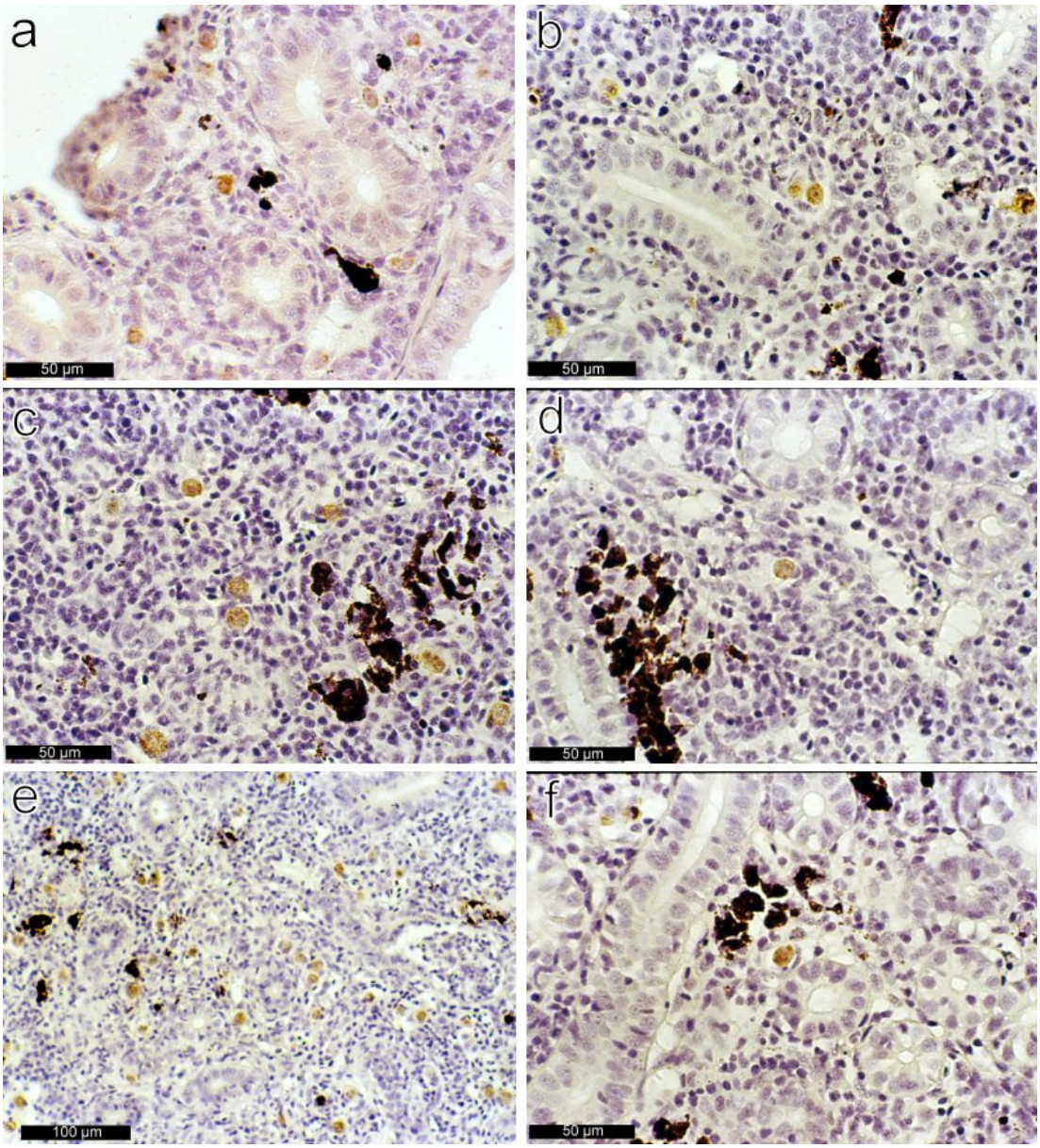

Figure 4.20: Immunohistochemistry of kidney sections of rainbow trout exposed to known numbers of *T. bryosalmonae* spores

(a) fish 10 from trial 1: exposed to 1 spore
(b) fish 4 from trial 2: exposed to 14 spores
(c) fish 11 from trial 2: exposed to 5 spores
(d) fish 12 from trial 2: exposed to 16 spores
(e) fish 20 from trial 2: exposed to 6 spores
(f) fish 22 from trial 2: exposed to 2 spores

(haematoxylin & DAB substrate)

Table 4.5: Analysis of kidney samples from rainbow trout exposed to known numbers of *T. bryosalmonae* spores from 1st trial

"fish no." = fish designation number; "spores" = the number of spores added to the tank; "IHC" = *T. bryosalmonae* immunohistochemistry result; "PCR" = *T. bryosalmonae* polymerase chain reaction result.

fish no.	1	2	3	4	5	6	7	8	9	10	11	12
spores	0	10	10	5	5	5	0	5	10	1	0	1
IHC	-	-	-	-	-	-	-	-	-	+	-	-
PCR	-	-	-	-	-	-	-	-	-	+	-	-

fish no.	13	14	15	16	17	18	19	20	21	22	23	24
spores	1	10	1	0	0	0	1	10	1	10	5	5
IHC	-	-	-	-	-	-	-	-	-	-	-	-
PCR	-	-	-	-	-	-	-	-	-	-	-	-

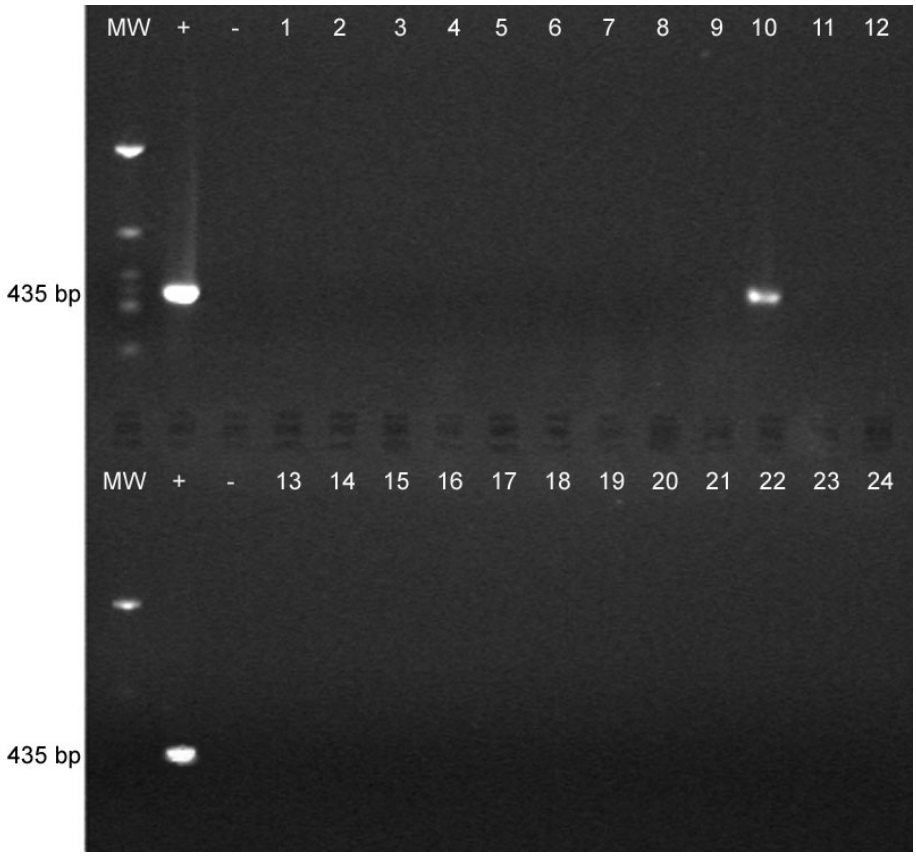

Figure 4.21: PCR analysis of kidney samples from rainbow trout exposed to known numbers of *T. bryosalmonae* spores from 1st trial

Lanes: "MW" = molecular weight markers; "+" = positive control samples; "-" = negative control samples; "1-24" = fish exposed to known numbers of spores as described in Table 4.5. (UV illumination of 1.5% agarose gel containing ethidium bromide)

Table 4.6: Analysis of kidney samples from rainbow trout exposed to known numbers of *T. bryosalmonae* spores from 2nd trial

"fish no." = fish designation number; "spores" = the number of spores added to the tank; "IHC" = *T. bryosalmonae* immunohistochemistry result; "PCR" = *T. bryosalmonae* polymerase chain reaction result.

fish no.	1	2	3	4	5	6	7	8	9	10	11	12
spores	0	1	2	14	5	15	1	16	0	3	5	16
IHC	-	-	-	+	-	-	-	-	-	-	+	+
PCR	-	-	-	+	-	-	-	-	-	-	+	+

fish no.	13	14	15	16	17	18	19	20	21	22	23	24
spores	1	1	0	1	1	3	0	6	0	2	14	0
IHC	-	-	-	-	-	-	-	+	-	?	-	-
PCR	-	-	-	-	-	-	-	+	-	+	-	-

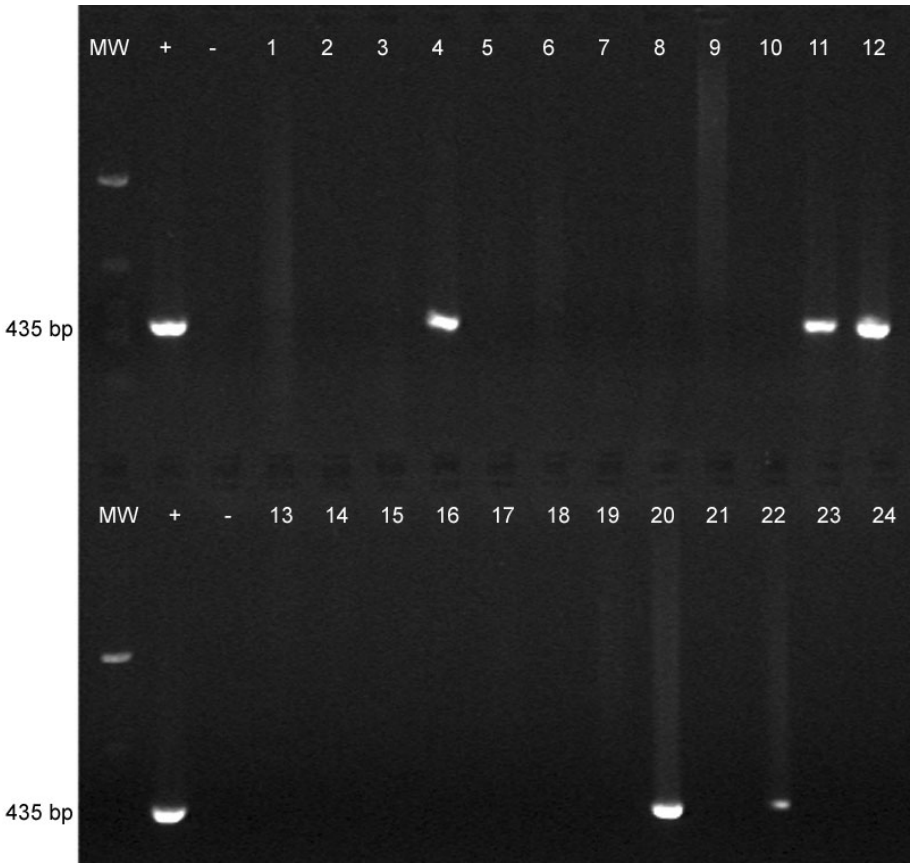

Figure 4.22: PCR analysis of kidney samples from rainbow trout exposed to known numbers of *T. bryosalmonae* spores from 2nd trial

Lanes: "MW" = molecular weight markers; "+" = positive control samples; "-" = negative control samples; "1-24" = fish exposed to known numbers of spores as described in Table 4.6. (UV illumination of 1.5% agarose gel containing ethidium bromide)

4.3.3.2. PCR analysis of kidney samples

From both trials, all of the fish which tested positive by immunohistochemistry also tested positive by PCR for *T. bryosalmonae* (Table 4.5, Figure 4.21, Table 4.6 and Figure 4.22). In addition, from the second trial, fish number 22 tested positive by PCR. None of the negative control fish yielded positive results by PCR.

4.3.4. Study of spores of *T. bryosalmonae* using CLSM

Eighteen spores of *T. bryosalmonae* were examined during the course of nine confocal microscopy scans (as noted in Table 4.2). No morphological differences were noted between the spores resulting from the two collection times. The mean spore diameter was 19.16 µm (s.d. = 1.15), measured using the inbuilt quantification facility in the confocal software. Two additional scans were carried out without addition of fluorophores to the samples; however, the lack of fluorescence detected in these preparations excluded the likelihood of auto-fluorescence of the spores, thus necessitating the use of fluorescent dyes for visualisation.

4.3.4.1. Confocal scan 1

The two ranges of detection wavelength (Table 4.2) utilised during this scan allowed capture of images due to both fluorescence of Blankophor and white light transmission. Four measurements were taken of the diameter at different angles across the spore, the mean value being 20.44 µm (s.d. = 0.37). Basic morphological features of the spore could be discerned (Figure 4.23), including the identification of at least two spherical bodies (presumed to represent polar capsules) and a fine strand of length 12.41 µm and thickness 1.09 µm (presumed to be an extruded polar filament) communicating at one of its ends with the spore.

4.3.4.2. Confocal scan 2

This was a more detailed examination of the same spore as described in Section 4.3.4.1. The 100 sections captured during this scan allowed further morphological details to be discerned both in the raw sections and in 3D representations of the structure (Figure 4.24). Forty eight of the consecutive sections captured that best display details of spore morphology are shown in Figure 4.25 and Figure 4.26. A polar filament can be seen in images 1-5, observed in contact with a capsulogenic cell of the spore in image 6 and in Figure 4.24. Four capsulogenic cells could be seen forming a cruciate arrangement in images 8-14 of Figure 4.25, with the cell from which the polar filament had extruded appearing more fluorescent than its three cohorts, presumably due to increased entry of the fluorophore following extrusion of the filament. Four structural valve cells could be seen beneath the capsulogenic cells in image 14, nuclei being visible within these cells in images 9-22. The structure and arrangement of the valve cells and the positions of nuclei could be better appreciated in 3D reconstructions (Figure 4.24). From image 21 onwards of Figure 4.25, a central mass could be recognised, presumably representing sporoplasmic material. In images 28-31 of Figure 4.26, nuclei could be seen in two more valve cells. These nuclei were seen to be eccentrically positioned within the cells, this being more apparent in Figure 4.24b. An apparent artefact resulting from a deformation of the wall of the spore could be seen in the bottom left corner of the spore from image 30 onwards of Figure 4.26 and also in Figure 4.24.

4.3.4.3. Confocal scan 3

From four different measurements, the spore diameter was found to be 20.39 μm (s.d. = 0.79). Four well-defined spherical polar capsules were observed, from each of which a polar filament had extruded (Figure 4.27). In addition, four masses of fluorescent material could be seen, presumably representing primary and secondary cells of a pair of sporoplasms. Such bodies were not seen to be stained by

Blankophor in previous scans of intact spores which had not fired their polar filaments. Following extrusion of all four polar filaments, the overall form of the spore appeared different from previously examined specimens, being less regular in outline. Only one nucleus from a structural valve cell was evident in this specimen. One of the polar filaments was measured (the others were not positioned appropriately) and was found to be of length 12.46 μm and thickness 800 nm. The mean diameter of the polar capsules was 1.73 μm (n = 4, s.d. = 0.03).

4.3.4.4. Confocal scan 4

From four measurements, the spore diameter was calculated as 16.15 μm (s.d = 1.04). Four well-defined capsulogenic cells could be observed in a cruciate pattern (Figure 4.28), within one of which a spherical polar capsule of diameter 2.28 μm was observed. Another spherical object (diameter 2.52 μm) of unknown origin was positioned outwith but proximate to the spore. Upon 3D reconstruction, the front aspect of the spore possessed four valve cells surrounding the capsulogenic cells, whilst in side view three additional valve cells could be detected.

4.3.4.5. Confocal scan 5

From four measurements, the spore diameter was found to be 19.94 μm (s.d. = 0.88). In section no. 64 of 200 captured (Figure 4.29a), four capsulogenic cells could be observed, while at a deeper plane at section 90 (Figure 4.29b), the front row of four valve cells could be seen. Towards the rear aspect of the spore, four additional valve cells could be seen in section 155 (Figure 4.29c) making a total of eight. Movement of the spore during the scan due to currents formed by living protozoa introduced into the media from the dissected bryozoans precluded 3D reconstruction of the sections.

4.3.4.6. Confocal scan 6

From four measurements, the spore diameter was found to be 19.23 μm (s.d = 0.41). Various nuclei were detected by the DAPI stain. In section no. 6 of 20, representing a depth of 6.9 μm within the spore (Figure 4.30a), four peripheral valve cell nuclei and four capsulogenic cell nuclei could be detected. At a deeper plane, in section 10 at 11.5 μm depth (Figure 4.30b), four different valve cell nuclei could be observed, making a total of eight valve cells comprising the outer layer of the spore. In each section where valve cell nuclei were imaged, the four nuclei were seen to be positioned approximately equidistantly around the circumference. The orientation of the deeper layer of four valve cell nuclei (Figure 4.30b) was seen to roughly represent that of the shallower layer (Figure 4.30a) rotated through 25° anti-clockwise. The mean diameter of the valve cell nuclei was 2.46 μm (n = 8, s.d. = 0.09), while that of the capsulogenic cell nuclei was 2.37 μm (n = 4, s.d. = 0.18).

4.3.4.7. Confocal scan 7

The spore diameter from four measurements was 19.79 μm (s.d. = 1.31). The BODIPY lipophilic fluorophore penetrated the spore, staining two sporoplasms within the cytoplasm (Figure 4.31a) and multiple highly fluorescent round masses of approximate diameter 500 nm throughout the spore. Although the sample was stained with both BODIPY and DAPI, no fluorescence attributable to the latter dye could be detected.

4.3.4.8. Confocal scan 8

Nine spores were scanned, having a mean diameter of 19.14 μm (s.d. = 1.12). Many of the spores displayed twin sporoplasms, each of which included two non-fluorescent nuclei (Figure 4.31b). In several of the spores, one nucleus was consistently seen to be larger than its companion, the former presumably being in a primary cell and the latter in a secondary cell within the sporoplasm.

4.3.4.9. Confocal scan 9

Three spores were scanned, the mean diameter being 18.86 μm (s.d. = 0.34). Again, twin nuclei of different sizes were seen within individual sporoplasms of mean length 9.01 μm (n = 6, s.d. = 0.47) and mean width 6.39 μm (n = 6, s.d. = 0.43) (Figure 4.32). Additionally, small round non-fluorescent bodies of approximate diameter 500 nm, possibly representing vacuoles were seen within sporoplasms.

4.3.5. Three-dimensional modelling of the spore of *T. bryosalmonae*

The aim of the modelling was to configure the relative positioning of the known components of the spore, and to achieve this goal, spheres were used to represent the cells and polar capsules. As no single confocal scan showed all the components simultaneously, data gathered from multiple scans were combined to develop a proposed structure for the spore. Comparisons between 3D images from confocal scans and 3D models corroborated the postulated structure. The frontal view shown in Figure 4.33a correlated closely with Figure 4.28a; Figure 4.33b demonstrating a similar view to Figure 4.24a; the side view displayed in Figure 4.33c represented that shown in Figure 4.28b.

From the scans, it was found that the *T. bryosalmonae* spore was composed of four capsulogenic cells (containing polar capsules), eight valve cells and two sporoplasms (each including primary and secondary cells). As all of the spores examined were seen to be positioned in a similar orientation with the capsulogenic cells facing down towards the inverted objective lens, the aspect of the spore diametrically opposite the polar capsules could not be directly imaged due to poor fluorescent resolution at such a distance from the lens. Thus, an interpretation of the data gathered from multiple scans allowed representation of the relationship of the cells in this area to be proposed in the models. Due to limitations in displaying transparencies in the 3D Canvas software, only the external surface of the spore

could be represented by this method (Figure 4.33). By the use of transparency effects, the images produced in 3DS Max by Dr. J.E. Bron (Figure 4.34) allowed representation of all the known spore components, including the internal structures discerned using the nucleic acid and lipophilic stains (confocal scans 6-9).

4.3.6. Examination of sections of *F. sultana* infected with *T. bryosalmonae* by immunohistochemistry

All of the antibodies screened showed some reaction with the tissue, positive areas appearing brown due to the DAB substrate (Figure 4.35). The apparently non-specific staining with the P01 and B4 MAbs showed widespread immunostaining of the tissue of both *F. sultana* and *T. bryosalmonae*, whereas MAb D4 bound more selectively to the latter. The control slide with which culture medium was incubated gave negative results.

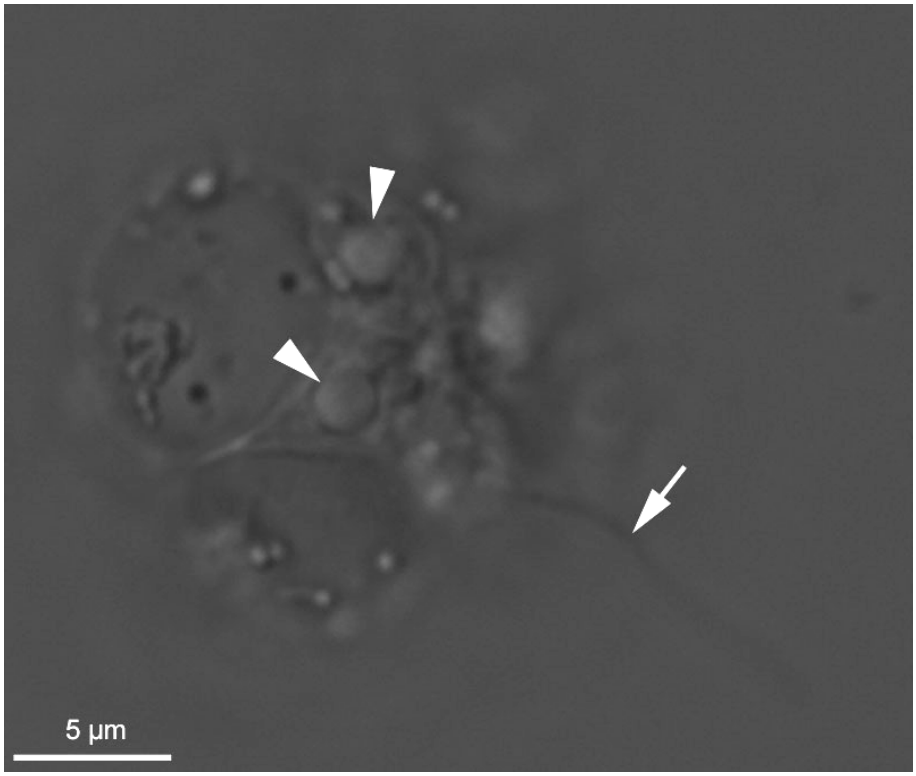

Figure 4.23: Section from 1st confocal scan of *T. bryosalmonae* spore

Transmitted light image showing two spherical polar capsules (arrowheads) and an extruded polar filament (arrow).

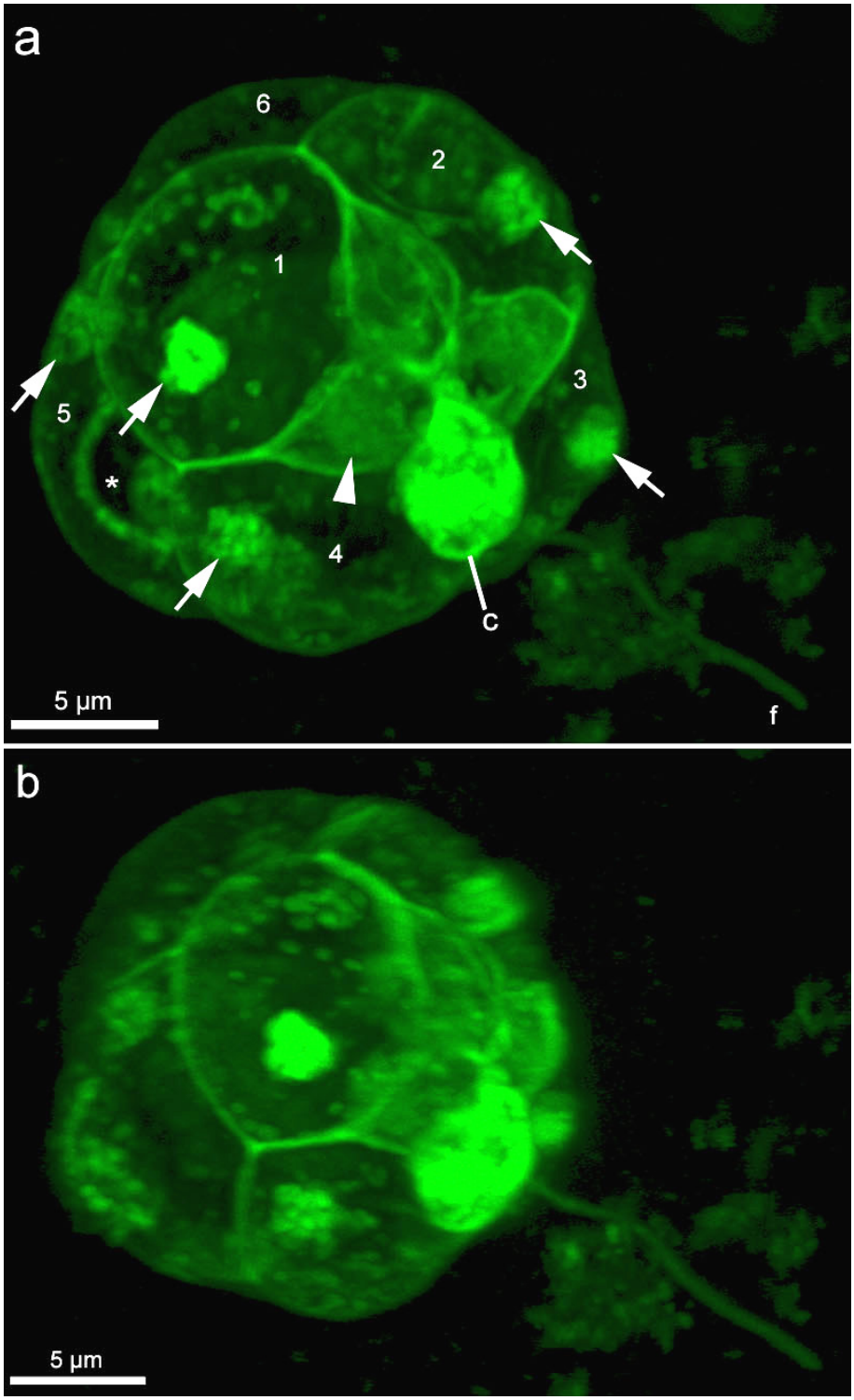

Figure 4.24: 3D views from *T. bryosalmonae* spore confocal scan 2

(a) A polar filament ("f") could be seen exiting from a capsulogenic cell ("c") which has absorbed fluorescent dye. A spherical polar capsule (arrowhead) can be seen in another one of the 4 capsulogenic cells. Six valve cells ("1-6") could be observed in this view, with nuclei (arrows) being visible in five of them. An artefact representing minor damage could also be seen ("★").

(b) A different angle of the spore showing the eccentric position of the nucleus of valve cell 5 from (a).

(stained with Blankophor)

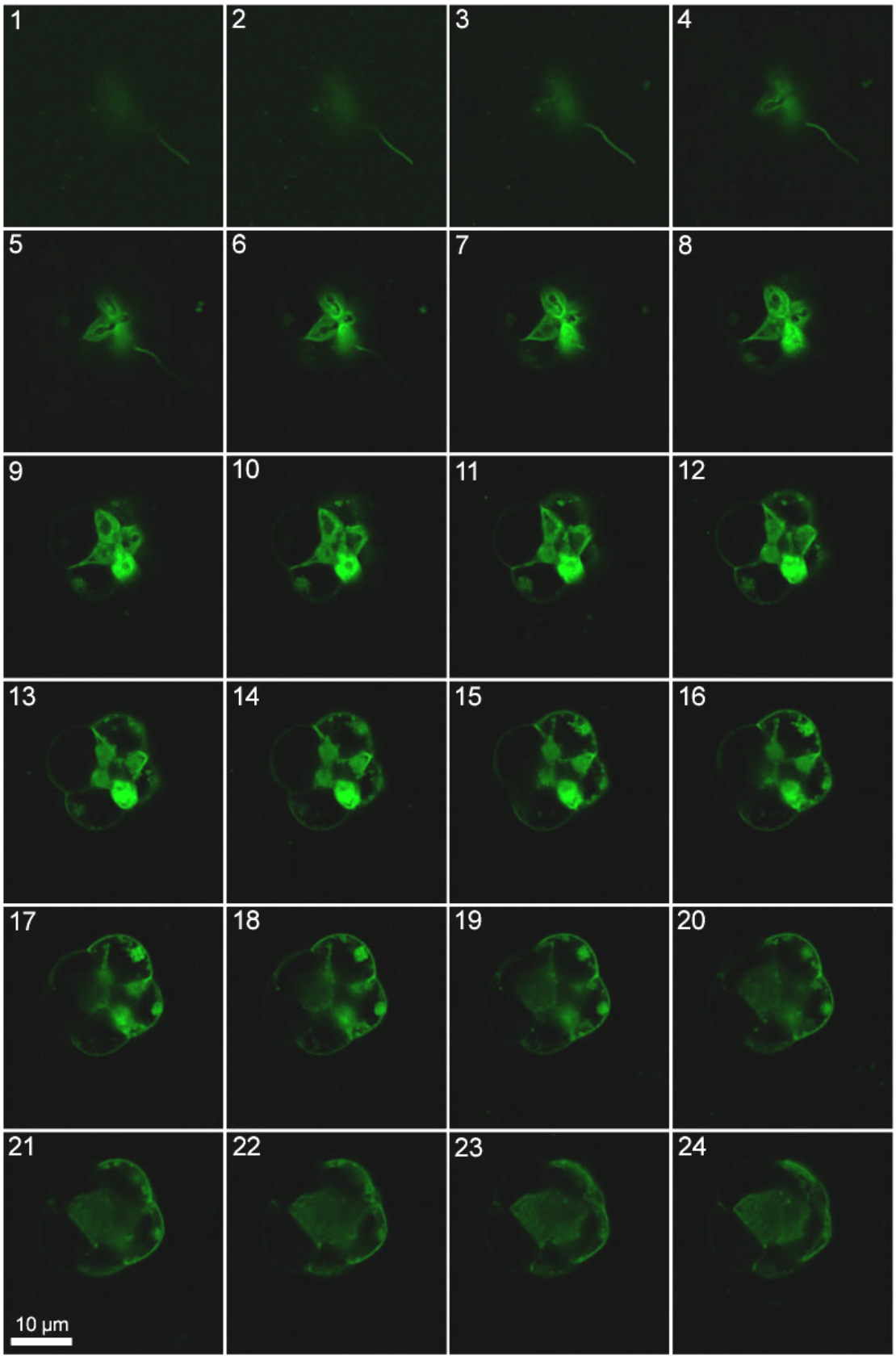

Figure 4.25: Series of sections from 2nd confocal scan of *T. bryosalmonae*

Images 1-24. (stained with Blankophor)

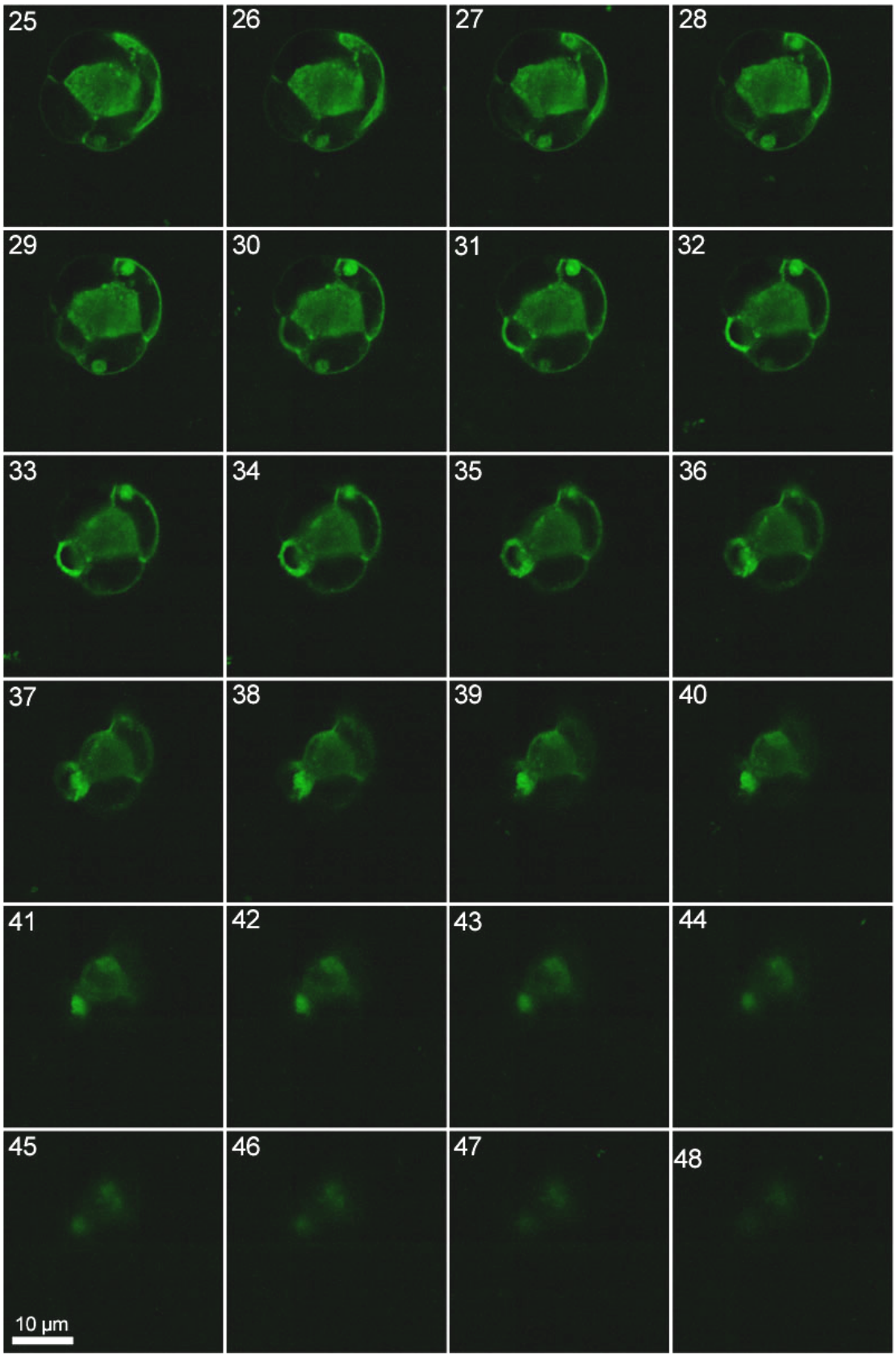

Figure 4.26: Series of sections from 2nd confocal scan of *T. bryosalmonae* (continued)

Images 25-48. (stained with Blankophor)

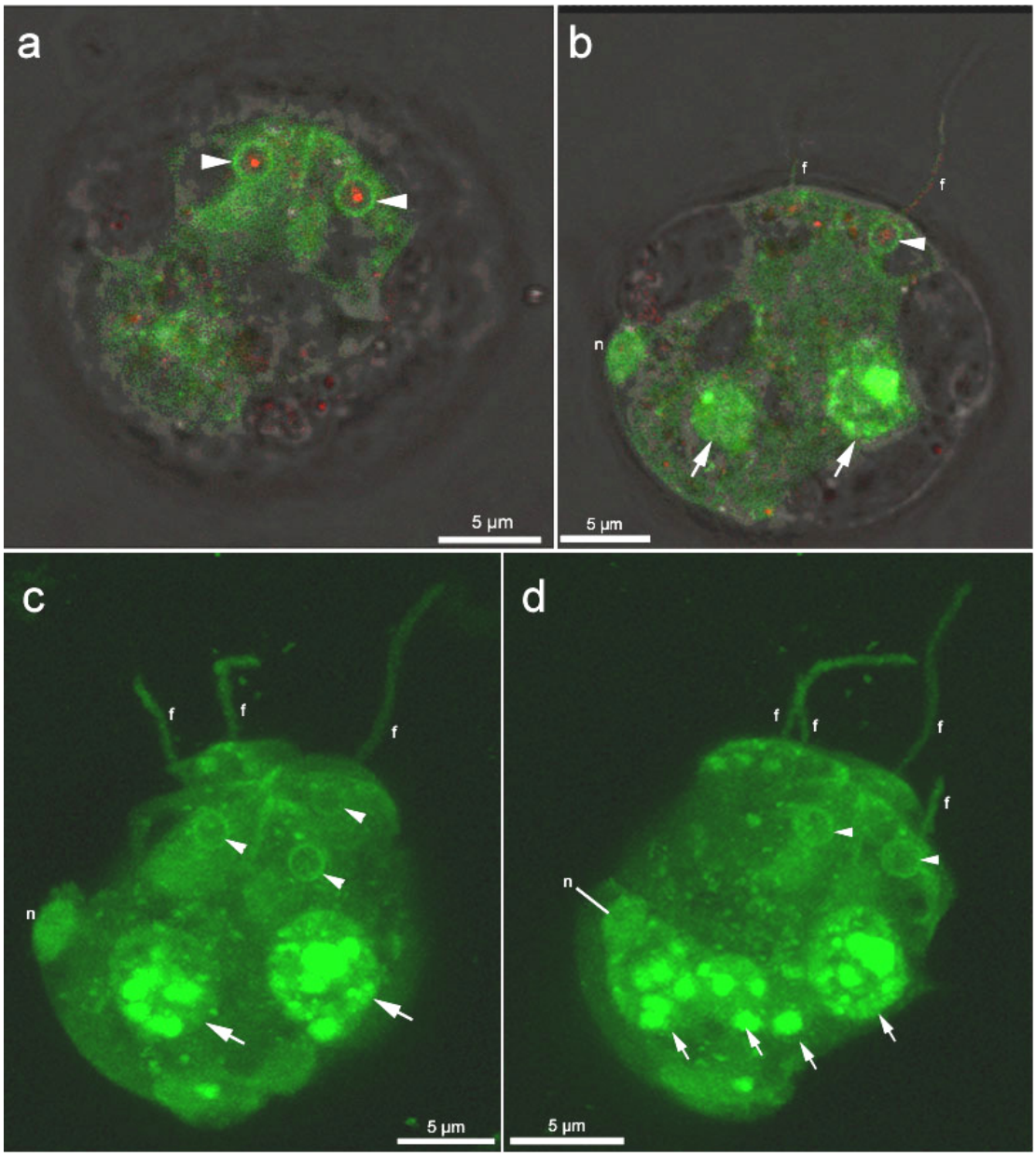

Figure 4.27: Images from *T. bryosalmonae* spore confocal scan 3

(a) and (b) show sections capturing fluorescence (green) and transmitted light (red).
(c) and (d) show 3D representations of the spore using fluorescent capture only.
Spherical polar capsules (arrowheads) can be seen, from each of which a polar filament ("f") exits. Four sporoplasmic masses (arrows) can be seen within the spore, with one nucleus ("n") of a structural valve cell also evident.

(stained with Blankophor)

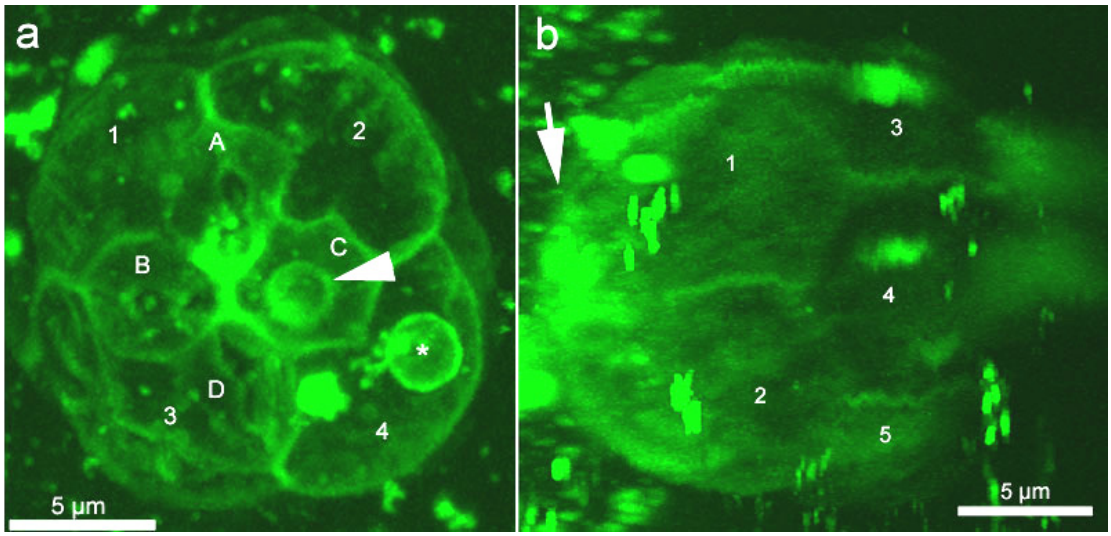

Figure 4.28: 3D representations from *T. bryosalmonae* spore confocal scan 4

(a) Four capsulogenic cells could be seen ("A-D") with one polar capsule (arrowhead) being apparent. Four structural valve cells ("1-4") could be seen on the front surface of the spore. A spherical mass ("*") was evident outwith the spore.
(b) Side view of the spore showing five valve cells ("1-5"), with the front surface (arrow) to the left.

Cells 2 & 4 in (a) correspond to cells 1 & 2 respectively in (b). (stained with Blankophor)

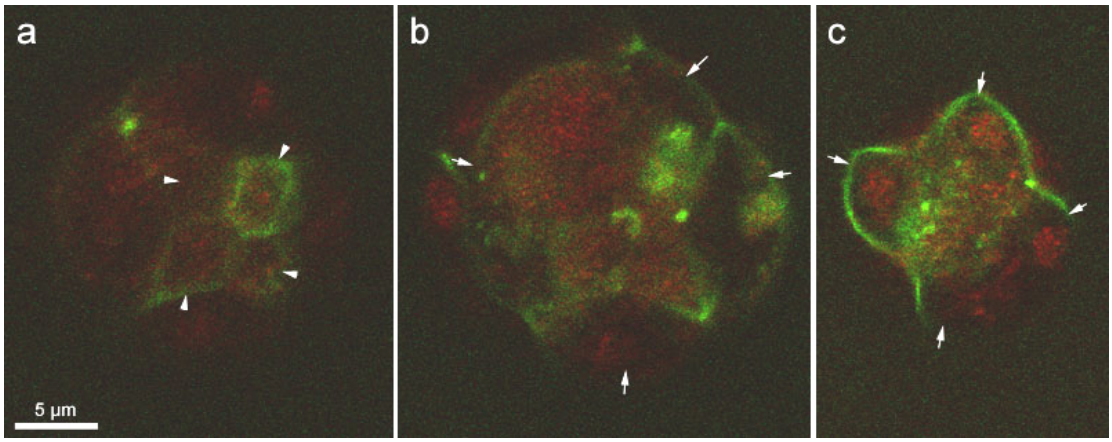

Figure 4.29: Sections from *T. bryosalmonae* spore confocal scan 5

(a) Four capsulogenic cells (arrowheads) could be seen on the front surface of the spore.
(b) Four structural valve cells (arrows) become evident further into the spore.
(c) At greater depth into the spore, four different valve cells (arrows) could be seen.
(bar applies to all images; stained with Blankophor)

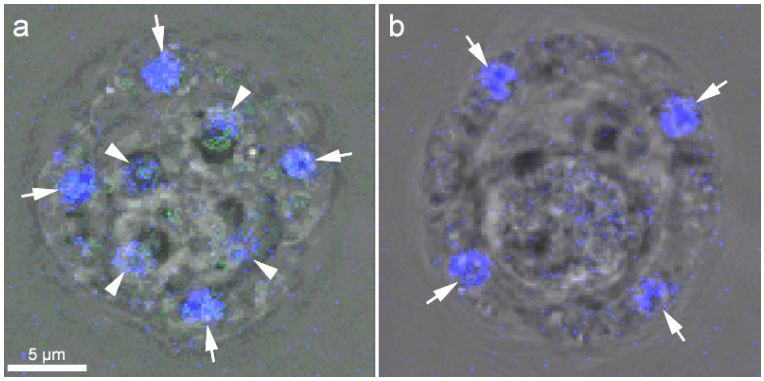

Figure 4.30: Sections from *T. bryosalmonae* spore confocal scan 6

(a) Showing four valve cell nuclei (arrows) and four nuclei of capsulogenic cells (arrowheads).
(b) At a deeper plane within the spore, four different valve cell nuclei (arrows) could be detected. (bar applies to both images; stained with DAPI)

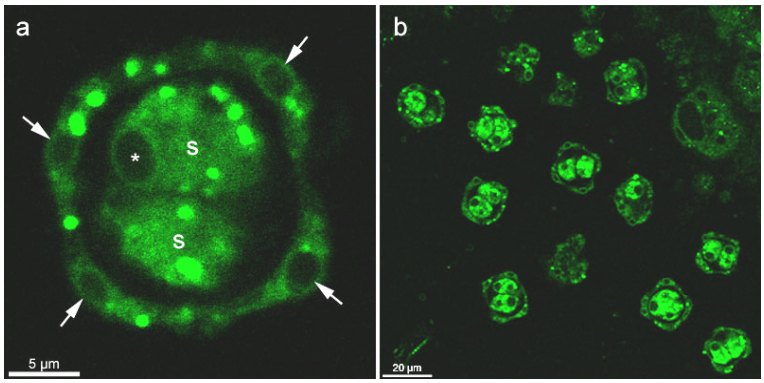

Figure 4.31: Sections from *T. bryosalmonae* spore confocal scans 7 and 8

(a) Scan 7: a single spore exhibiting two central sporoplasms ("s") within one of which a nucleus ("★") could be observed in this plane. Four valve cell nuclei (arrows) could be seen peripherally. Brightly-stained lipophilic deposits could be observed throughout the spore. (stained with BODIPY and DAPI)
(b) Scan 8: a group of spores, some of which demonstrate sporoplasms with twin nuclei. (stained with BODIPY)

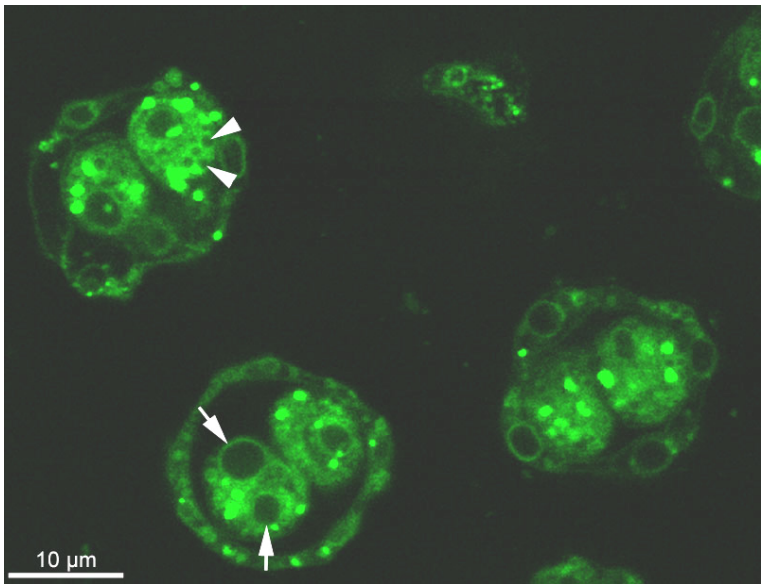

Figure 4.32: Section from *T. bryosalmonae* spore confocal scan 9

Nuclei (arrows) of both primary and secondary cells of a single sporoplasm could be observed. Some sporoplasms were seen to possess round non-fluorescent areas (arrowheads). (stained with BODIPY)

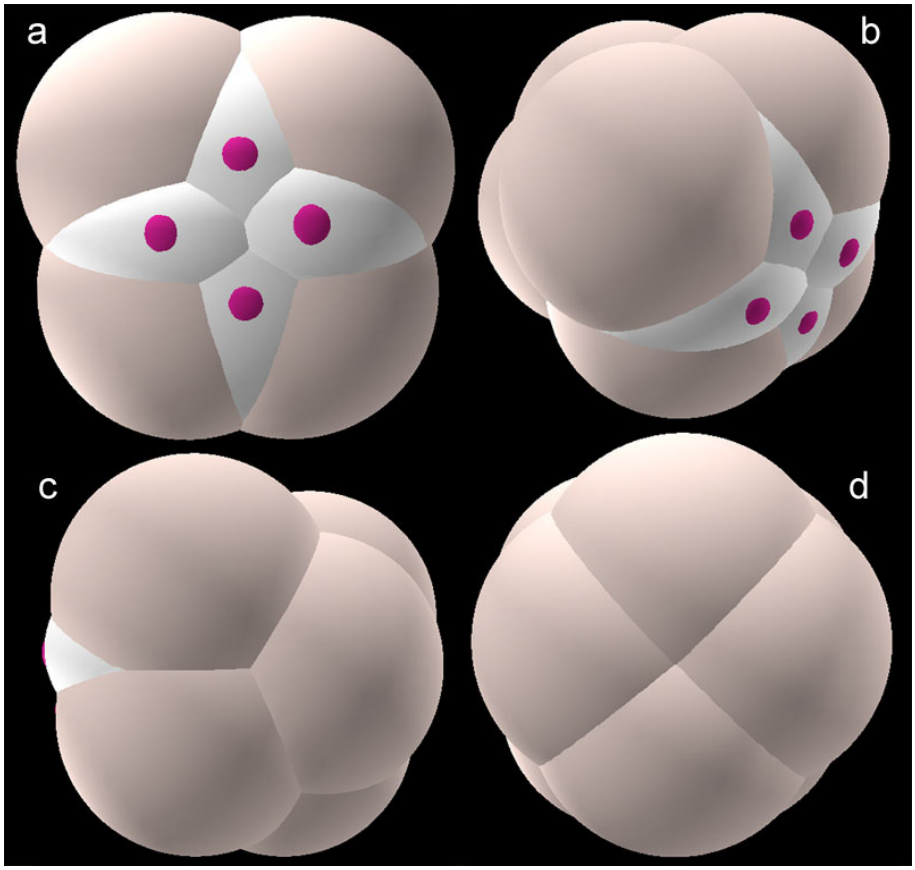

Figure 4.33: Images of a 3D model of a *T. bryosalmonae* spore

(a) Front view showing four red spherical polar capsules within four white capsulogenic cells surrounded by four beige valve cells; (b) Oblique view; (c) Side view; (d) Interpretation of rear view, showing four valve cells. (3D Canvas images)

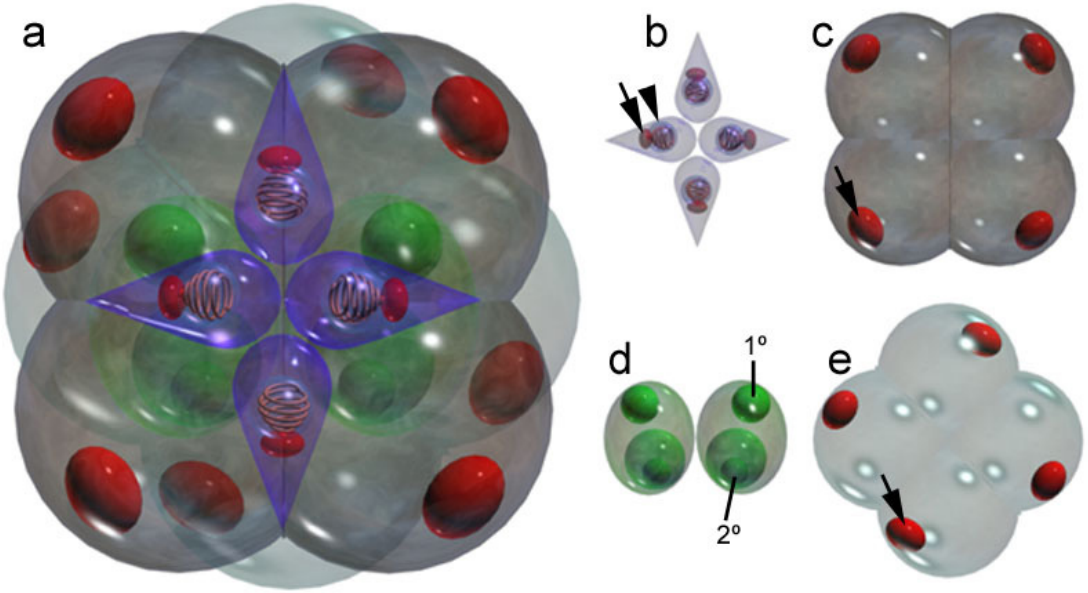

Figure 4.34: 3D computer model of a *T. bryosalmonae* spore

(a) Composite image showing all layers *in situ*. (b) Nearest layer from view, comprising 4 capsulogenic cells, each containing a spherical polar capsule (arrowhead) with internal coiled polar filament and a nucleus (arrow). (c) Deeper layer of four valve cells, each with one nucleus (arrow). (d) Middle layer of 2 sporoplasms, each comprising a primary cell with nucleus "1°" and a secondary cell with nucleus "2°". (e) Deepest layer from view, comprising 4 valve cells with nuclei (arrow). (3DS Max images)

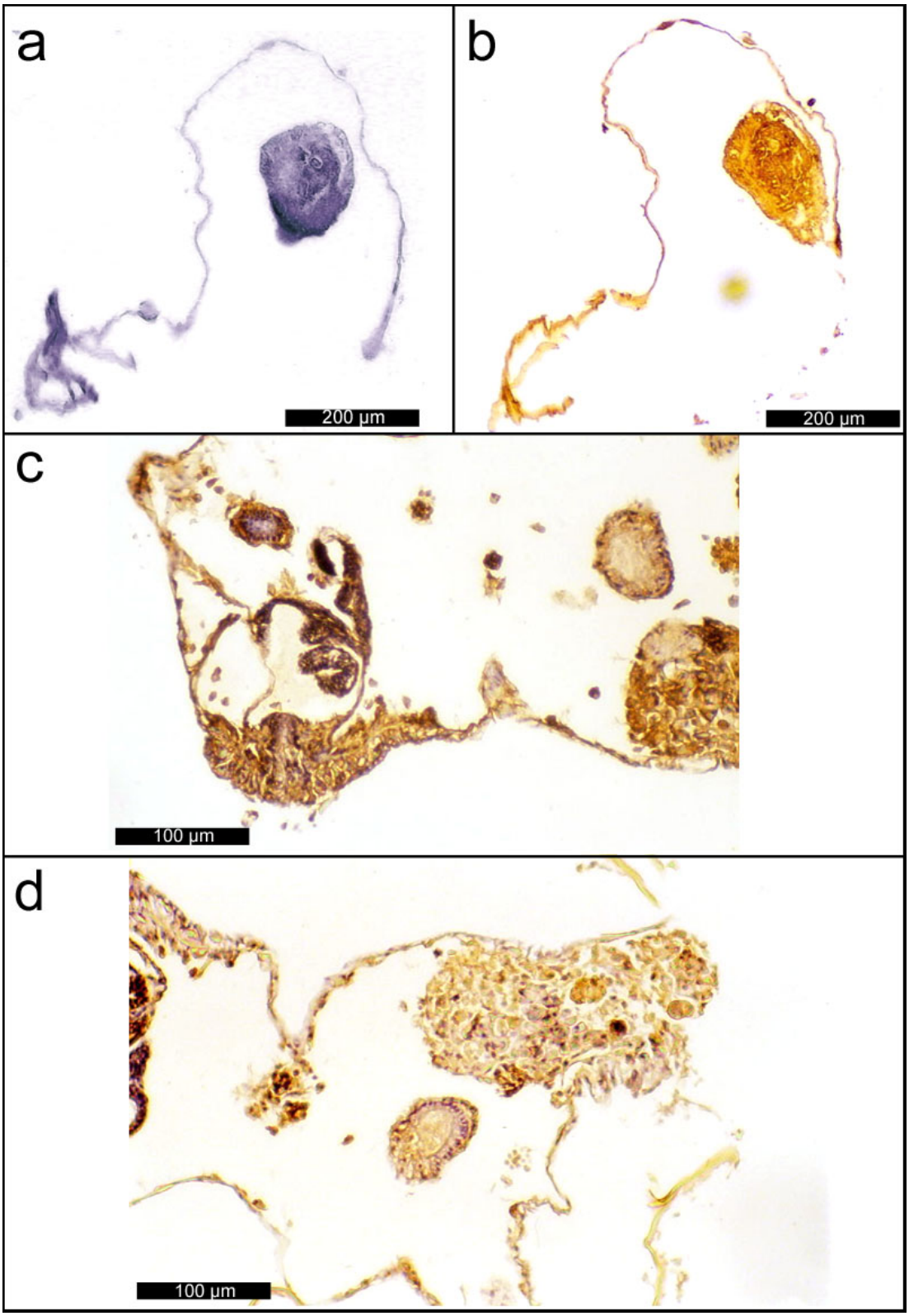

Figure 4.35: Immunohistochemistry of *T. bryosalmonae*-infected *F. sultana*

The sections were incubated with: (a) DMEM only (control slide); (b) MAb P01; (c) MAb B4; (d) MAb D4. (haematoxylin & DAB substrate)

4.4. Discussion

Gay *et al*. (2001) described a year-round presence of *T. bryosalmonae* in PKD-endemic waterways. These findings were confirmed in the current study, with development of *T. bryosalmonae* visualised within *F. sultana* regardless of the time of year when the material was collected from an infected river. In addition, a link seemed to occur between sexual proliferation of the bryozoan host (resulting in the development of spermatozoa) and subsequent recognition of *T. bryosalmonae* developmental stages. This correlation appeared more evident when there was a marked increase in the culture temperature, *i.e*. following spring or autumn collection of specimens from cooler water conditions. Gay *et al*. (2001) related that *F. sultana* collected from cold water conditions and subsequently maintained at 16°C exhibited observable signs of *T. bryosalmonae* development within 2-3 days. In the current study, the most rapid recognition of infection was made two days after the collection of material during early spring. Bryozoans collected during the warmer months did eventually exhibit infection, but the apparent prepatent phase was more protracted. It could not be stated from the findings whether the rate of development was directly linked to the magnitude of temperature increase, or if a number of extrinsic and intrinsic factors interacted, such as photoperiod, nutrition of the host or the time since first infection. Nevertheless, it was observed that parasitic proliferation was invariably noticed in rapidly growing bryozoans suggesting an interaction with host vitality.

Following extensive sampling of bryozoans from various sites it has been suggested that the general prevalence of *T. bryosalmonae* infection is relatively low (Canning and Okamura 2004). However, it was postulated that infection could have the potential of developing rapidly, with resultant brief periods throughout the year of high prevalence. Okamura *et al*. (2001) found a high prevalence of infection of *T. bryosalmonae* in *F. sultana* during a short summer period, and although infection

was also detected in the winter and spring, it was more difficult to diagnose. Although the current study did not specifically examine the incidence and prevalence of *T. bryosalmonae* infection in wild bryozoans, it was found that the level of infection increased markedly following periods of maintenance of colonies in nutrient-rich culture media at 18°C. Okamura and Wood (2002) conducted a large survey of North American and European freshwater sites, examining 11 separate species of bryozoans representing potential hosts for *T. bryosalmonae*. A very low prevalence of *T. bryosalmonae* infection was found, although the specimens were examined using only dissecting microscopes which involved teasing the material apart to look for characteristic malacosporean stages. It was found in the current study that following collection from waterways, the long-term maintenance of bryozoans in laboratory systems coupled with regular examination using inverted microscopes allowed a higher sensitivity in detection of malacosporean infection than simple immediate inspection of freshly collected bryozoans. In some of the samples, infection was not evident until 38 days following collection; therefore, shorter periods of maintenance and scrutiny may have underestimated the prevalence of infection.

The suggestion that cryptic stages of *T. bryosalmonae* exist within the bryozoan peritoneal wall, subsequently leading to overt infection (Tops and Okamura 2003) would appear consistent with the current findings that a delay occurred between sample collection and observation of infection. Early in the course of infection, stages of *T. bryosalmonae* were observed adhered to the peritoneal wall of the bryozoans examined. Examination of sections of *F. sultana* infected with *T. bryosalmonae* by immunohistochemistry using MAbs developed against fish stages of the parasite revealed some cross-reaction with the bryozoan stages of *T. bryosalmonae*. Although much of the staining appeared non-specific in nature, localisation of reaction was evident in some focal areas, presumably representing parasitic developmental stages. Previously, the MAb B4 had been shown to cross-

react with the myxosporean *Myxobolus cerebralis*, identifying the release of antigen within fish tissue (Morris, El-Matbouli and Adams 2004). This MAb has proved valuable in the study of various species of myxozoan parasites, and in the current material has suggested dissemination of malacosporean material within the bryozoan host. As such, these findings support the suggestion of malacosporean stages developing within the peritoneal walls of bryozoan hosts (Canning et al., 2002).

Consistently, the first observable event in the cycle of *T. bryosalmonae* development in the bryozoans was the presence of numerous small swirling objects within the metacoel of the host. These stages were consistent with those witnessed as a precursor to overt infection of *P. repens* with *B. plumatellae* as described in Section 3.3.3. Initially, irregular stages were seen with *T. bryosalmonae* as described with *B. plumatellae*, although spheroid spore sacs developed with the former species as opposed to the vermiform spore sacs of the latter. Both species of malacosporean had initially apparently empty immature spore sac stages. With *T. bryosalmonae*, although the sacs were regular in outline, multiple irregularly positioned bulbous protrusions could be seen from some sacs, these features being evident in both infected specimens of *F. sultana* and *Plumatella* sp. The function of the protrusions is unknown, although they seem to be lost during the latter stages of sac maturation, resulting in regularly outlined bodies, possibly meaning that the protrusions were vestigial in nature.

No marked differences were seen between the morphology of *T. bryosalmonae* developing within different genera of bryozoan host, in contrast with *B. plumatellae* which reportedly adopts markedly different morphologies in *C. mucedo* and *P. repens* (Canning and Okamura 2004). As with *B. plumatellae*, the spore sacs of *T. bryosalmonae* became progressively filled with refractive objects (presumably

primordial spores) which formed aggregates, eventually coalescing to fill the lumen of the sac. This was consistent with an early description of the development of parasitic bodies within the metacoel of *Lophopus crystallinus* Pallas which might have represented *T. bryosalmonae* or a related species (Allman 1856), although neither of the recognised Malacosporea has been described in this bryozoan species. Gay *et al*. (2001) described the presence of spores within five days of identification of infection in *F. sultana*, findings which were corroborated in the current study. Within the current study, upon maturation of the spore sacs of *T. bryosalmonae*, large numbers of spores were released into the host metacoel as had previously been witnessed with *B. plumatellae*.

The high burden of developing spore sacs within some colonies meant that multitudinous spores were released into the coelomic cavity, their dissipation within 12-24 hours suggesting that they were subsequently released into the surrounding culture media. As with *B. plumatellae*, no intact mature spore sacs were ever witnessed exiting from any of the bryozoans within the current studies. The release of mature spores was exploited in the experimental exposure of rainbow trout to the media putatively containing spores. Fish were successfully infected following two exposures four days apart of 60 minutes duration. As the water volume had not been reduced excessively for each exposure, it could be interpreted that the spores were highly efficient at infecting their hosts as previously suggested (Feist *et al.* 2001). All 12 fish demonstrated gross nephromegaly and proved positive for the presence of *T. bryosalmonae* in kidney tissue by at least one diagnostic test, although with each of the assays only 11 out of the 12 tested positive. Tissue from one fish proved negative by immunohistochemistry, but weakly positive by PCR. This could be either attributed to a very low burden of infection which could not be discerned by immunohistochemistry with PCR proving more sensitive or possible contamination of the DNA sample resulting in a false positive result. Tissue from another fish proved

positive by immunohistochemistry but negative by PCR, possibly due to degradation of pathogen DNA during the storage or extraction stages. Alternatively, as separate biopsies of kidney were analysed in each assay, the parasite burden may have varied between them accordingly. Thus, neither assay appeared to be 100% sensitive in detecting infection, the combination of both appearing more rigorous. Regardless of the outcomes of these two fish, the aim of the experiment was to ascertain if trout could be experimentally infected by exposure to culture media from bryozoans, which was indeed shown to be possible. Feist *et al*. (2001) had successfully transmitted *T. bryosalmonae* infection to trout by the exposure of fish for 90 min to disrupted infected bryozoan material, and by long-term cohabitation with infected bryozoans, but not by short-term exposure to culture media alone. Similarly, Longshaw *et al*. (2002) successfully infected 99 rainbow trout following an exposure of 90 min in 10 L water, allowing a study of the entry sites for the parasite to be carried out. The current study demonstrated that infection could be elicited in trout without direct contact with infected bryozoans. Thus, the procedure was non-destructive for the alternate host of the parasite, allowing preservation of valuable infected colonies. In light of the observation of sequential waves of *T. bryosalmonae* infection within bryozoans, this implied that under optimal culture conditions, infection could be maintained long-term, representing a rewarding source of parasites.

From previous studies, it has been suggested that a low dose of *T. bryosalmonae* spores might be able to elicit infection in fish, although no quantitative data was available (Feist *et al.* 2001; Longshaw *et al.* 2002; Tops *et al.* 2004). Thus, in the current study, experiments were carried out to try to establish the minimum number of spores capable of leading to *T. bryosalmonae* infection in rainbow trout. In the first trial, only one fish demonstrated signs of infection, although the level of parasitism was relatively high, confirming that *T. bryosalmonae* was capable of multiplying

successfully upon entering a teleost host. However, that fish had been exposed to only one spore, while other fish exposed to greater numbers of spores did not exhibit detectable infection. This was attributed to possible degradation of some of the samples between collection of the spores from their bryozoan hosts and exposure to the fish; spores of *T. bryosalmonae* having been shown to be relatively labile in nature (de Kinkelin *et al.* 2002). Following dissection of the spores, they had been stored in a laboratory at ambient temperature (approximately 23°C) for up to one hour before being exposed to the fish. During the dissection procedure some of the spores were seen to have extruded their polar filaments, suggesting that degradation was already underway. Also, during the first trial, the quantification of spores was conducted as they were drawn into the pipettes rather than upon ejection. Thus, it would be possible that some spores may not have been successfully ejected into the bijoux containers, resulting in reduced number of spores to which some fish were exposed. Therefore, the limited success in eliciting infection in the fish may have resulted from a combination of degradation in the viability of spores in conjunction with fewer spores being exposed to the fish than planned. In order to address these issues, a second trial was conducted incorporating modifications in the methodology.

A similar fish tank system was employed in the second experimental trial aiming to establish the lowest dose of *T. bryosalmonae* spores capable of leading to infection in rainbow trout. However, the handling of the spores before exposure was modified. Following dissection of mature spores from infected colonies of *F. sultana*, the microscope slides were kept cool on ice or on a microscope stage cooled by ice packs. By following this protocol, no spores were seen to extrude their polar filaments due to degradation. In addition, it was decided to numerate the exact number of spores which were ejected from the micropipette on each occasion, rather than counting the intake and presuming consistency with the ejected number as was conducted during the first trial. This meant that there was a greater variability in the

number of spores to which the experimental fish were exposed, as it proved difficult and time consuming to try and count out exact predetermined numbers of spores. It was still considered, however, that a representative range of spore numbers was employed which would allow any resulting infection to be interpreted. Following ejection onto cavity slides, the spores were exposed to the experimental fish as quickly as possible. This trial proved more successful, with five fish testing positive for infection of *T. bryosalmonae*.

In the second trial, the PCR assay proved marginally more sensitive than immunohistochemistry, with one fish testing positive by PCR but only provisionally positive by immunohistochemistry. As an alternative diagnostic method, *in situ* hybridisation might have proved more robust in detecting various developmental stages of *T. bryosalmonae* within the tissue (Morris *et al.* 1999). The results of these trials confirmed previous suggestions that a low dose of *T. bryosalmonae* spores would prove infectious to fish (Feist *et al.* 2001; Longshaw *et al.* 2002; Tops *et al.* 2004). De Kinkelin *et al*. (2002) showed that spores of *T. bryosalmonae* were relatively fragile, and postulated that obtaining an enriched preparation would be useful, although they did not manage to do so despite mincing hundreds of *F. sultana* colonies which had tested PCR positive for *T. bryosalmonae*. The labile nature of the spores may explain why, in the current study, less than 100% of those fish exposed to spores developed infection, with spores potentially being degraded before successfully coming in contact with fish mucous membranes. Despite preventative measures, it seems possible that some of the spores may have become attached to substrates such as the glass cavity slides or tank apparatus, thus preventing exposure to fish. It therefore seems logical that although very low numbers of spores are capable of eliciting infection in rainbow trout, higher numbers would be more efficient in leading to PKD outbreaks. The success within the current study of obtaining sufficient spores to carry out investigative procedures has

emphasised the importance of being able to maintain infected bryozoan colonies for extended periods within laboratory systems and the value of regular examination by inverted microscopes to detect parasitic stages.

Gay *et al*. (2001) suggested that the severity of the immune response in trout kidney to extrasporogonic stages of *T. bryosalmonae* was dependent upon the amount of foreign material encountered rather than the ambient temperature. In the current studies, marked parasitic proliferation with resultant host immune reaction was seen in kidney sections of fish exposed to only one spore, with severe interstitial nephritis noted in a fish exposed to only six spores (Figure 4.20). This contrasts with findings of a linear dose response in rainbow trout exposed to triactinomyxon spores of *M. cerebralis* (Markiw 1992). It has been suggested that the developmental course of PKD in individual fish depends upon a combination of genetic and environmental factors (Morris, Ferguson and Adams 2005). Thus, the dose of *T. bryosalmonae* spores necessary to elicit infection and subsequent development of PKD may vary between individuals within a population of fish. Gay *et al*. (2001) stated that verification of their hypothesis would require the development of a quantitatively reproducible challenge method conducted at less than 10°C. The methodology developed within the current trials could potentially be utilised in future for such a study of the relationship between temperature and host immune reaction with quantifiable infection loads applied to the subjects.

Canning *et al*. (2000) suggested that upon entry of *T. bryosalmonae* stages into fish tissue, two haploid secondary cells released from the sporoplasms of the spore would fuse together resulting in fertilisation. It was speculated that due to the relatively small number of spores synchronously contacting fish, the process would result in autogamy, with two secondary cells from a single spore fusing. The current finding of development of infection in fish exposed to only one spore tends to support

this hypothesis. Whether cross-fertilisation occurred in those fish exposed to greater numbers could not be ascertained, although even during the maximum exposure dose of 16 spores it would seem unlikely that two spores would synchronously penetrate fish tissue proximately enough to result in cross-fertilisation. As the pathological appearance of *T. bryosalmonae* infection from such low spore numbers did not differ from established descriptions (Clifton-Hadley *et al.* 1987a), it would seem likely that, at least some, if not all clinical cases of PKD result from autogamous parasite reproduction. Future trials could look at wider differentials in spore challenge numbers if sufficient infected material could be obtained, as this has proven to be the limiting factor in such proposed studies in the past (de Kinkelin *et al.* 2002).

Whereas myxosporean spores are encompassed with hard valves formed from valvogenic cells that subsequently degenerate, the outer surface of the spore of *T. bryosalmonae* and its sister taxon *Buddenbrockia* comprises valve cells that retain their cellular integrity and lack hardened valve coats (Canning *et al.* 2000). While light microscopical and ultrastructural studies have revealed many developmental and mature features of these organisms, the precise morphology of the fully formed spores has remained elusive, with only basic features being discernable. Diagrams based on light microscopical examinations have added little to our understanding of how the cells are arranged to form spores (Canning *et al.* 1996; Morris *et al.* 2002a). Although spores were studied by conventional light microscopy during the current study, the limitations of resolution meant that little further information of spore morphology could be discerned by this method.

The development of CLSM techniques has facilitated the production of 3D reconstructions of biological specimens with little or no processing of tissue leading to minimal development of artefacts (Kubinova, Janacek, Karen, Radochova, Difato

and Krekule 2004) and has proved especially useful in the imaging of microorganisms (Martin-Cereceda, Alvarez, Serrano and Guinea 2001). The optical brightener Blankophor has been widely used in the staining of microorganisms, showing a high affinity for β-glycosidically linked polysaccharides (Ruchel and Schaffrinski 1999). Its use allowed structural details of released *T. bryosalmonae* spores to be visualised. In particular the junctions between adjacent valve cells and capsulogenic cells were evident, although little internal absorption of the dye within the spores occurred. The fluorophore DAPI has previously been found to be effective in staining nuclear accumulations of DNA in myxozoans (El-Matbouli, Holstein and Hoffmann 1998), and in the current study demonstrated the presence of nuclei of capsulogenic and valve cells in the *T. bryosalmonae* spores. However, no sporoplasmic nuclei were imaged convincingly by this technique, presumably due to insufficient penetration of the spore interior by the fluorophore. The lipophilic qualities of non-polar BODIPY fluorophores have been shown to make them highly suitable for investigating membrane structures (Yamada, Toyota, Takakura, Ishimaru and Sugawara 2001). From the scans conducted, internal components including the germinative sporoplasms of the spores could easily be visualised. Within the sporoplasms, non-fluorescent spheres of approximate diameter 500 nm were observed, while highly fluorescent bodies of a similar size were also seen throughout the cytoplasm of cells forming the spore, presumably representing previously described lipid vacuoles (Canning *et al.* 1999, 2000). The demarcation of primary and secondary cells composing each sporoplasm was clearly seen using the lipophilic fluorophore.

Previous morphological studies of malacosporean spores identified the presence of four spherical polar capsules sited within capsulogenic cells, two internal sporoplasms (each composed of primary and secondary cells), and four external valve cells (Canning *et al.* 1996, 1999, 2000). Previously, polar capsules were stated

to have a diameter of 1.6-1.8 µm which proved consistent with the current findings. However, the dimensions of sporoplasms had not been formerly described, presumably due to deformations in morphology from processing procedures in the preparation of material for ultrastructural analysis. While the CLSM findings confirmed the previous descriptions of polar capsules and sporoplasms; eight valve cells – rather than the previously reported four – were identified within each spore of *T. bryosalmonae*. These cells were evident both in Blankophor and DAPI scans, with the nuclei being eccentrically positioned in those valve cells not contiguous with capsulogenic cells. An attempt was made to synchronously stain a spore with both DAPI and BODIPY to allow direct comparison of the various organelles, but unfortunately only fluorescence attributable to the latter dye could be detected, possibly due to interaction between the fluorophores. The CLSM software analysis package allowed the dimensions of spores and their components to be measured in the material examined, as well as allowing 3D models to be developed from the multiple image sections captured.

The 3D models allowed integration of the information obtained from the various CLSM scans using the range of fluorophores. Although in the computer models the cells were represented schematically as spheres, the relative position of the cells composing the outer layer of the spore appeared consistent between the models and the 3D CLSM images, even although the model images appeared slightly more bulbous. The use of CLSM in the current study represents the first documented 3D visualisation and representation of the cellular components of malacosporean spores. Although perhaps as a function of their lack of hard outer valves, there was some variation in diameter of the spores examined, the *T. bryosalmonae* spore has been shown to be highly organised in structure, with consistency in architecture noted between the examined spores.

Observations by Morris *et al*. (2002a) of malacosporean development within specimens of *P. repens* revealed distinct ornamentation of spores, allowing the authors to formulate a schematic representation. They described a divergence of the bottom corners of the spores, which may have represented an inward folding of the layer of valve cells furthest from the polar capsules. In previous studies of malacosporeans, only four valve cells per spore have been numerated (Canning *et al.* 1996, 1999, 2000) which may have been due to invagination of the distal valve cell layer in immature spores. In the current study, the spores were released spontaneously within the bryozoans having fully matured within spore sacs and were convex in appearance, possibly resulting from swelling of the valve cells as has been described in some actinosporean species (Lom, McGeorge, Feist, Morris and Adams 1997). Future examination of other malacosporean spores such as *Buddenbrockia plumatellae* and sporogonic stages of *T. bryosalmonae* released from fish, utilising similar CLSM techniques would represent a powerful comparative method between the species of the class Malacosporea.

This study has demonstrated that *T. bryosalmonae* could be maintained and examined within its invertebrate hosts for relatively prolonged periods. The development of successful experimental challenge methods for rainbow trout which did not compromise the bryozoans could be implemented in future vaccine and chemotherapeutant trials. The quantification of the infectious dose of *T. bryosalmonae* spores to rainbow trout could have implications in understanding the epidemiology of PKD, while the study by CLSM has elucidated new details of the spore morphology. Ultimately, it would be hoped that such new findings could play an integral role in the development of successful control measures against PKD.

Chapter 5: Development of prevention and control methods for proliferative kidney disease

5.1. Introduction

Despite intensive research, no satisfactory prophylaxis or treatment has been licensed for use in combating PKD as yet. Husbandry measures including the use of cooler water from subterranean sources, altering transfer times of stock to endemic waters, reducing feeding rates, secondary pathogen control and minimising environmental stressors have been shown to have some impact on disease manifestation, but at considerable cost (Hedrick *et al.* 1993). Similarly, chemotherapeutants including malachite green, fumagillin and TNP-470 have shown some efficacy, but at the price of toxicity to fish, environmental concerns and lowered profit margins (Higgins and Kent 1998; le Gouvello *et al.* 1999; Morris *et al.* 2003a). Therefore, an effective vaccine or chemotherapeutic protocol to alleviate the losses attributable to annual outbreaks of PKD would be highly desirable.

Since the initial realisation that following their recovery from clinical PKD, fish can exhibit acquired immunity to subsequent *T. bryosalmonae* challenge, there has been much interest in the production of an effective vaccine. While experimental vaccination trials using various preparations of infected kidney extract have conferred some limited protection, implementation of such methodologies would not be practical on a commercial scale (Petchsupa 2002). Therefore, an alternative vaccination strategy is required that would be both efficacious and sustainable. Potentially, this could be based on a whole parasite preparation from infected bryozoans.

Screening of *T. bryosalmonae*-infected trout tissue has elucidated the presence of numerous carbohydrate terminals on the parasite (Morris and Adams 2004). Three lectins which led to positive parasite staining have previously been found to bind to human blood-group precursor antigens (Cao, Stosiek, Springer and Karsten 1996;

Gilboa-Garber and Sudakevitz 2001). These structures, known as the Thomsen-Friedenreich (TF) and Tn antigens (Ags) are expressed in various tissues including carcinomatous mammalian tissue, gram-negative bacteria such as *Escherichia coli* Migula, 1895 strain O86 (Springer and Desai 1977a), and the parasites *Echinococcus granulosus* (Batsch, 1786) (Errico, Medeiros, Míguez, Casaravilla, Malgor, Carmona, Nieto and Osinaga 2001) and *Schistosoma mansoni* (Cummings and Kwame Nyame 1999). Much research has centred on the production of anti-cancer vaccination protocols involving the administration of TF and Tn Ags prepared from degraded blood cells and from commercial typhoid vaccine preparations (Springer and Desai 1977a; Springer 1997). Examination of the presence of TF and Tn Ags in *T. bryosalmonae* tissue has not been previously documented. However, such findings could potentially allow development of a vaccination strategy based on commonly available preparations.

With no vaccine available, and present management practices being costly and limited in effectiveness, the discovery of a novel effective drug to combat PKD would be desirable. While some were relatively efficacious, none of the previously studied agents have been licensed for use in fish. Findings from previous innovative treatment trials for myxosporean and protozoan parasitic fish diseases could potentially be implemented in the development of strategies to address PKD (Dohle, Schmahl, Raether, Schmidt and Ritter 2002; Shinn, Wootten, Côté and Sommerville 2003; Athanassopoulou, Karagouni, Dotsika, Ragias, Tavla and Christofilloyanis 2004).

The aim of this study was to explore the potential of developing novel prophylactic or chemotherapeutic agents against PKD. This entailed the following objectives:

- The screening of *T. bryosalmonae* for the presence of TF and Tn antigens.

- Development and efficacy testing of experimental vaccine preparations potentially based on TF and Tn specificities.

- Development and efficacy testing of a vaccine preparation composed of *T. bryosalmonae*-infected bryozoan tissue.

- Efficacy testing of novel in-feed drug preparations at varying doses.

Success with such studies could potentially have implications for the future control of PKD.

5.2. Materials and Methods

5.2.1. Screening of PKD-affected trout kidney tissue and strains of *E. coli* for the presence of Thomsen-Friedenreich (TF) and Tn antigens

5.2.1.1. Immunohistochemistry using anti-TF and Tn monoclonal antibodies

Sections of rainbow trout kidney from a fish naturally affected with PKD on an endemic farm were examined by immunohistochemistry following the method outlined in Section 3.2.8.2. In addition to incubation with P01 MAb as positive controls, sections were incubated with commercially available MAbs targeting the TF and Tn antigens (Biogenesis, Poole, UK) diluted to 10 µg ml^{-1} in PBS.

5.2.1.2. Immunogold electron microscopy

Sections of rainbow trout kidney from a fish naturally affected with PKD on an endemic farm and control sections of uninfected rainbow trout were examined by immunogold electron microscopy following the method of Morris *et al*. (1997). Kidney sections were cut into 1 mm^3 cubes, and fixed in 2.5% glutaraldehyde fixative for 2-4 hours at 4°C. They were rinsed in cacodylate rinse buffer overnight. The tissue was then rinsed three times in cacodylate buffer and twice in distilled water. The tissue was then dehydrated through a graded alcohol series to 70% ethanol and embedded in LR white (London Resin Company, Reading, UK). Ultra-thin sections were cut and mounted on nickel grids.

The grids were then floated section-side down on drops of wash buffer containing 10% (v/v) goat serum in wash buffer [PBS (Appendix 2), with 1% (v/v) Tween 20, and 1% (w/v) bovine serum albumin (BSA, Sigma), pH 7.2], and incubated for two hours at room temperature to block non-specific binding sites. The grids were then transferred to drops of anti-Tn MAb diluted to 10 µg ml^{-1} in PBS and incubated overnight at 4°C, with negative control grids floated on PBS. The grids were then

washed by floating them on wash buffer in a 96-cell well plate and agitated occasionally for 90 min. The buffer was then changed and the wash procedure repeated for a further 90 min. The grids were then incubated overnight at 4°C on drops of anti-mouse biotin diluted 1:50 in wash buffer, followed by a wash as before. They were then incubated overnight at 4°C on drops of anti-biotin conjugated to 20 nm gold colloid diluted 1:50 in wash buffer. The grids were then held with fine forceps under a stream of distilled water and washed again in the cell wells of a 96-cell well plate. Finally, they were counter-stained with uranyl acetate and lead citrate before being viewed using a Philips 301 transmission electron microscope at 80 kV. Photographs were taken with an integral flat plate camera using black and white Kodak 4489 EM film.

5.2.1.3. Culture of *E. coli*

Eight strains of *E. coli* were cultured for future analysis. From frozen stocks, the following library strains from the Institute of Aquaculture were revived: 9481, V517, 25922, ATCC 35218, ATCC 49106 and NCTC 11560. In addition, strain NCIMB 86 (National Collection of Industrial and Marine Bacteria) was purchased and strain O86 K61 was obtained from G. Foster, Veterinary Science Division, Scottish Agricultural College, Inverness. This strain had originally been cultured from a greenfinch, *Carduelis chloris* (L.).

The strains were cultured in sterile Petri dishes on tryptone soya agar (TSA, Oxoid, Appendix 1) or in sterile tryptone soya broth (TSB, Oxoid, Appendix 1) at 20°C.

5.2.1.4. Preparation of *E. coli* for SDS-PAGE

The strains of *E. coli* were grown in TSB for 48 hours at 20°C. The broth was then centrifuged (Mistral 2000R, Sanyo) at 3000 × *g* for 10 min. As heat treatment was found to be ineffective for killing the bacteria, formalin treatment was used. The pellet

was resuspended in 10 ml 0.2% (v/v) formaldehyde in sterile PBS, and incubated overnight at 4°C. To neutralise the formalin, 1 ml of 5% (w/v) sodium metabisulfite solution in PBS was added. The sample then underwent two rounds of centrifugation and was resuspended in sterile PBS as before. The samples were then pelleted by centrifugation at 3000 × *g* and resuspended to a concentration of 1×10^9 CFU ml^{-1} as discerned by comparison of OD_{610} readings against a standard curve. These samples were diluted in sample buffer (Appendix 2) for use in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

5.2.1.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-PAGE was conducted on the eight strains of *E. coli* and samples of kidney from PKD-affected and uninfected control rainbow trout. The method of Laemmli (1970) was used with modifications.

5.2.1.5.1. 12% Gel formation protocol

A gel holder apparatus (SE245 Dual Gel Caster, Hoefer Scientific Instruments, Staffs., UK) was assembled. The separating gel (Appendix 2) was prepared: firstly, separating gel buffer was added to distilled water and acrylamide solution. This mixture was placed in a Buchner flask and attached to a pump to facilitate degassing for 15 minutes. Then, 10% (w/v) ammonium persulphate and N,N,N,N-tetramethylethylenediamine (TEMED, Appendix 2) were added and the solution was immediately poured between the mounted plates of the gel casting apparatus. One hundred µl of water saturated butanol was layered over the separating gel to prevent evaporation from the free surface. The gel was allowed to polymerise for 60 minutes at room temperature. The butanol was washed away with distilled water, and then the stacking gel (Appendix 2) was poured over the solidified separating gel. Placing plastic combs in the stacking gel made sample wells. This was allowed to set for 60

minutes at room temperature, after which the combs were removed, allowing sample addition.

5.2.1.5.2. Electrophoresis procedure

The gel apparatus (SE250, Hoefer Scientific Instruments) was filled carefully with reservoir buffer (Appendix 2), avoiding the entrapment of any air bubbles. Kidney samples were homogenised in PBS in an Eppendorf tube using a sterile pestle and then centrifuged (IEC Micromax, Thermoquest, UK) at 1000 × *g* for five min. The supernatant and samples of *E. coli* were added to sample buffer (Appendix 2) at a ratio of four parts sample to one part buffer. They were heated to ~98°C for four minutes in a boiling water bath before being centrifuged at 13,000 × *g* for five minutes. Approximately 20 µl of sample was then added to each well, known molecular weight standards were also applied: broad range protein markers (P7702S, New England BioLabs Inc.) for gels subsequently stained; recombinant protein molecular weight markers (Rainbow RPN 800, Amersham Life Science) for gels used for Western blot assays. An electrical potential difference of 100 volts was then applied across the gel until the dye front (visible due to the presence of bromophenol blue) approached the bottom of the separating gel. The gel was then either stained or subsequently used for Western blot analysis.

5.2.1.5.3. Coomassie blue staining

The gel was placed in a glass dish and covered with Coomassie blue stain (Appendix 2). Overnight incubation at room temperature with gentle automated rotation (Gyro-Rocker, Stuart Scientific) followed. The next morning, successive changes of de-stain solution (Appendix 2) were used until reasonable contrast could be achieved. The gel was then stored in a Petri dish containing distilled water.

5.2.1.6. Western blot analysis

The products were examined by Western blot immunoassay, using the method of Wiens, Turaga and Kaatari (1990) with modifications. Samples of *E. coli* and PKD-affected kidney were processed by SDS-PAGE as described in Section 5.2.1.5.2, but the gel was not stained. Instead, the gel was placed into transblot buffer (Appendix 2) for 20 minutes. Nitrocellulose paper, filter papers and filter pads were also equilibrated in the buffer at the same time. The gel and nitrocellulose paper were then mounted in the transblotting device (Fisher) in the following order: outer envelope, negative electrode, filter pad, three sheets of filter paper, gel, nitrocellulose paper, three sheets of filter paper, filter pad, positive electrode, outer envelope. Any air bubbles were carefully removed by rolling each layer with a Pasteur pipette. The cassette was placed into the transblotting chamber and this was filled with transblot buffer. The buffer was kept well mixed by placing the device over a magnetic stirrer which rotated a magnet sited in the bottom of the chamber.

The transfer was achieved by applying a potential difference across the apparatus of 60 volts for 60 minutes. The device was then taken apart and non-specific binding sites were blocked by soaking the nitrocellulose paper in 1% (w/v) BSA in TBS (Appendix 2) for 60 minutes at room temperature. The nitrocellulose paper was washed three times for five minutes each with HSWB and placed in a mini-Protean II multiscreen (Bio-Rad). To each lane, 900 µl of one of the following was added and incubated for two hours at room temperature with gentle automated rotation with one of the following: anti-Tn MAb diluted to 44 µg ml^{-1} in PBS; anti-TF MAb diluted to 38 µg ml^{-1} in PBS; the biotinylated lectin VVL (Vector) from *Vicia villosa* Roth diluted to 20 µg ml^{-1} in HEPES buffer (10 mM HEPES, 0.15 M NaCl, 0.1 mM Ca^{2+}, 0.04% NaN_3, pH 7.5); biotinylated Jacalin lectin (Vector) from *Artocarpus integrifolia* L. diluted to 20 µg ml^{-1} in HEPES buffer. Three washes as above followed. For only those lanes incubated with MAbs, conjugate in the form of one ml of biotin anti-

mouse immunoglobulin-G diluted 1/100 in antibody buffer (1% BSA in PBS) was added to each lane for 90 minutes at room temperature, followed by washing as above. Next, one ml of streptavidin peroxidase (1/100 in antibody buffer) was added to each lane, and left for 90 minutes at room temperature. Washing as above was followed by a one minute soak in TBS. The blot was developed by adding chromogen and substrate (two ml of stock substrate solution (Appendix 2) plus 10 ml of PBS and 10 µl of H_2O_2) until the bands materialised. Soaking the strips in distilled water for 10 minutes stopped the reaction. The relative electrophoretic migration of the products compared with the standard markers was calculated using TotalLab v2003.03 (Nonlinear Dynamics Ltd.).

5.2.2. Development and efficacy testing of experimental vaccine preparations

5.2.2.1. Vaccination of rainbow trout with typhoid vaccine

Six experimental groups of six rainbow trout (mean weight 38 g) were obtained from a source known to be free of PKD and were kept in the ARF at 18°C in a flow-through system using dechlorinated water. Four of the groups were anaesthetised in 4 mg L^{-1} benzocaine and vaccinated by intramuscular (i.m.) injection of 0.1 ml of 15 µg ml^{-1} Vi polysaccharide typhoid vaccine (Typherix, Smith Kline Beecham Pharmaceuticals) in PBS. The two remaining groups were anaesthetised and sham-vaccinated with 0.1 ml PBS injected intramuscularly. The fish were identified by clipping of the adipose fins, with two vaccinated and one sham-vaccinated group being allocated to each of two 100 L tanks.

5.2.2.1.1. Intraperitoneal injection of rainbow trout with homogenised PKD-affected kidney tissue

Rainbow trout were experimentally exposed to *T. bryosalmonae* following the method of Clifton-Hadley *et al*. (1984b). Fish from a source naturally affected by PKD were euthanased by exposure to 10 mg L^{-1} benzocaine followed by severing of the

spinal cord. The abdominal cavity was opened and the kidney observed for signs of enlargement indicative of PKD. From any kidneys graded as ≥ 2 according to the classification of Clifton-Hadley *et al*. (1987a), the kidney was removed aseptically and an impression smear was made and stained with Rapi-Diff (Lamb Laboratory Supplies, London, UK). If the smear exhibited numerous distinctive extrasporogonic stages of *T. bryosalmonae*, the kidney was placed in 5 ml chilled sterile PBS and homogenised with a sterile glass mortar and pestle.

Forty two days after vaccination, all six groups of fish from Section 5.2.2.1 were anaesthetised in 4 mg L^{-1} benzocaine. Two of the vaccinated groups and the two sham-vaccinated groups of fish were injected i.p. with 0.2 ml of the *T. bryosalmonae*-infected kidney homogenate. The two remaining vaccinated groups were injected i.p. with 0.2 ml PBS and maintained to check for adverse side effects of the vaccine alone. All of the fish were then be maintained at 18°C for 54 days.

5.2.2.1.2. Sampling and analysis

At the end of the experimental period, the fish were overdosed in 10 mg L^{-1} benzocaine and euthanased by severing the spinal cord. For fixation, samples of kidney, spleen and liver were placed in universal tubes containing 10% neutral buffered formalin for 24 hours. The tissue samples were then processed for histology as outlined in Section 3.2.8.1 and subsequently examined by immunohistochemistry as described in Section 3.2.8.2. *Tetracapsuloides bryosalmonae* burden within the kidney sections was estimated using the method of Higgins and Kent (1998) with modifications. Using an inverted microscope (Olympus CK2) at ×400 magnification, numbers of extrasporogonic stages present were counted within 12 random fields (470 µm diameter) of the kidney section for each specimen. Thus, the mean parasite burden mm^{-2} could be estimated for each kidney section. Statistical analysis was conducted using Minitab 13.32 (Minitab Inc.), GraphPad InStat 3.01 (GraphPad

Software Inc., San Diego, Ca., USA) and Quantitative Parasitology 2.0 (Rózsa, Reiczigel and Majoros 2000). Parasite counts were subjected to normality tests (Kolmogorov-Smirnov, Anderson-Darling). As the data were not normally distributed, non-parametric tests were conducted (Kruskal-Wallis, Mann-Whitney, Mood's median test).

5.2.2.2. Vaccination trials using bryozoan tissue or *E. coli*

5.2.2.2.1. Formulation of vaccines

Colonies of *F. sultana* were collected and maintained as described in Section 4.2.1 and Section 4.2.2. Upon recognition of the production of characteristic mature spore sacs and released *T. bryosalmonae* spores, excised fragments of infected colonies were rapidly frozen in liquid nitrogen before being stored in a freezer at -70°C. As no accredited uninfected *F. sultana* material was available as negative control material, *Plumatella* sp. statoblasts were collected from a source known to be free of *T. bryosalmonae* and reared as described in Section 2.2.1. When colonies had become established, samples were excised and cryopreserved as described above. Colonies of *E. coli* O86 were cultured in TSB as described in Section 5.2.1.3 and inactivated in formalin as described in Section 5.2.1.4.

For formulation of the experimental vaccines, each of the preparations was reconstituted in sterile PBS and homogenised with a sterile plastic pestle in an Eppendorf tube. The concentration of *E. coli* was calculated as described in Section 5.2.1.4 while the protein concentration of the bryozoan preparation was estimated using a commercial protein assay (Bio-Rad). Each formulation was mixed 1:1 with an adjuvant (Montanide ISA 563, Seppic), vortexed, and stored overnight at 4°C to ensure that an emulsion had formed.

5.2.2.2.2. Vaccination of fish

One hundred and forty four rainbow trout (mean weight 40 g) from a source known to be free of PKD were acclimatised in a 250 L stock tank at 18°C using dechlorinated water in the ARF. The fish were anaesthetised in 4 mg L^{-1} benzocaine and vaccinated by i.p. injection with 0.1 ml of one of the following formulations. Three groups of 12 fish were given 1×10^8 CFU of *E. coli* O86, three groups were given 1.4 µg of *T. bryosalmonae*-infected *F. sultana*, three groups were given 1.4 µg of *Plumatella* sp., and three control groups were sham-vaccinated with PBS and adjuvant. Each fish was injected i.m., ventral to the dorsal fin with a sterile passive integrated transponder (PIT) tag (Trovan) at the time of vaccination and the details scanned with a Trovan Scanner and uploaded via Trovan Database 1.33 software to a PC. Each group was allocated to a separate 100 L tank and maintained at 18°C.

5.2.2.2.3. Challenge of fish with *T. bryosalmonae*

Following vaccination, the fish were kept in the 12 tanks at 18°C for 10 weeks. After this period, all of the groups were allocated to a single 250 L stock tank. After acclimatisation, the water flow was stopped, the volume reduced to 100 L with ongoing vigorous aeration and culture fluid from *T. bryosalmonae*-infected *F. sultana* colonies was added to the tank as described in Section 4.2.3. After one hour the water supply was recommenced. The exposure procedure was repeated after 48 hours. The fish were maintained at 18°C for a further 9 weeks. The fish were then overdosed in 10 mg L^{-1} benzocaine and euthanased by severing the spinal cord. The designation of each fish was ascertained using the PIT scanner and software. For fixation, samples of kidney, spleen and liver were fixed in 10% neutral buffered formalin for 24 hours. The samples were then processed for histology as outlined in Section 3.2.8.1 were subsequently examined by immunohistochemistry as described in Section 3.2.8.2 and the *T. bryosalmonae* burden within the kidney sections was estimated as described in Section 5.2.2.1.2.

5.2.3. Efficacy testing of selected in-feed chemotherapeutic products

5.2.3.1. Formulation of preparations

Three experimental diets were formulated for each of two drug trials. For the first trial, commercial trout pellets (2.5 mm, Biomar, Grangemouth, UK) were coated with either 100 mg kg^{-1} active ingredient of a 12% premix of salinomycin sodium ($C_{42}H_{69}NaO_{11}$, Bio-Cox 120G, Alpharma), 100 mg kg^{-1} of amprolium hydrochloride ($C_{14}H_{19}N_4^+Cl^-HCl$, Sigma), or were left untreated. For the second trial, the diets were formulated as described above but the drugs were included at the higher dose of 10 g kg^{-1}. For each diet, the pellets were then coated with 40 ml kg^{-1} of 5% (w/v) gelatine solution and stored at -20°C until use.

5.2.3.2. Drug trial 1

Two hundred rainbow trout (mean weight 41 g) were obtained from a PKD-endemic fish farm on the River Test, Hampshire, UK, where they were believed to have been exposed naturally to *T. bryosalmonae*. Following transfer to the ARF, the fish were treated prophylactically against ectoparasites and furunculosis with a 200 parts per million (ppm) formalin bath and i.m. injection with 25 mg kg BW^{-1} oxytetracycline (Engemycin LA, Intervet) respectively. The fish were then allocated randomly into 10 groups of 20 fish and acclimatised at 18°C in 100 L tanks in the ARF. In addition, 160 uninfected rainbow trout (mean weight 39 g) were obtained from a source known to be free of PKD and randomly allocated into eight groups of 20 fish in 100 L tanks. To allow acclimatisation, the fish were all fed unmedicated gelatine-coated pellets for 10 days prior to the initiation of the drug trial. At the end of this period, the fish from one group of unexposed and one group of exposed fish were each weighed individually to act as sample weight indicators for the other groups. The fish from the infected group were euthanased and sampled as described in Section 5.2.2.1.2 to assess the prevalence and severity of PKD before commencing drug administration.

Following acclimatisation, feeding with the experimental diets (as described in Section 5.2.3.1) commenced at 1% BW day^{-1} and continued for a 10 day period. If the fish ate 100% of their ration, they would receive 1 mg kg BW^{-1} day^{-1}. Of the previously exposed fish, three groups of 20 were fed with each of the test diets. To gauge any adverse feeding reactions to the test diets, three groups of the unexposed fish were fed each of the medicated diets, and one group was fed with the unmedicated diet. The daily ration was split into two meals and fed by hand and feeding behaviour was observed. Before onset of the trial, the average weight of a pellet of each food was calculated by weighing 200 pellets of each diet. Thirty minutes after feeding, any uneaten pellets were siphoned from the tanks and counted to allow calculation of the weight of waste feed, and thus estimation of the percentage ingestion of each diet.

Thirty days after the initiation of feeding experimental diets, the fish were sampled as previously described. All of the samples were processed and analysed to allow quantification of the renal burden of *T. bryosalmonae* as described in Section 5.2.2.1.2. In addition, liver and spleen samples were histologically examined for evidence of toxicopathology.

5.2.3.3. Drug trial 2

This trial followed the protocol of the first trial with the following modifications. The mean weights of the PKD-affected and uninfected fish were 41 g and 69 g respectively. The drugs were delivered at the higher doses described in Section 5.2.3.1. If the fish ate 100% of their ration, they would receive 100 mg kg BW^{-1} day^{-1}. In an effort to reduce the number of experimental subjects, only one group of 20 uninfected fish was fed on each of the experimental diets to gauge for adverse feeding behaviour.

5.3. Results

5.3.1. Screening of PKD-affected trout kidney tissue and strains of *E. coli* for the presence of Thomsen-Friedenreich (TF) and Tn antigens

5.3.1.1. Immunohistochemistry using anti-TF and Tn monoclonal antibodies

Specific staining of renal *T. bryosalmonae* interstitial extrasporogonic and tubular sporogonic stages was observed in the sections incubated with the anti-TF MAb (Figure 5.1). No immunological staining was seen using the anti-Tn MAb.

5.3.1.2. Immunogold electron microscopy

An association of gold particles with vacuolar areas adjacent to electron-dense bodies was seen in the infected rainbow trout sections incubated with anti-Tn MAb (Figure 5.2). Examination of similar areas of control sections which were incubated with PBS did not show similar accumulations of gold particles (Figure 5.3). Within uninfected renal tissue, gold particles were found to be non-specifically associated with areas of connective tissue (Figure 5.4).

5.3.1.3. SDS-PAGE analysis of *E. coli* and *T. bryosalmonae*

Screening of the *E. coli* strains by SDS-PAGE showed the presence of multiple products (Figure 5.5). Further analysis of infected and uninfected renal tissue and selected strains of *E. coli* also revealed bands, although little consistency between kidney and bacterial profiles could be seen using this assay (Figure 5.6).

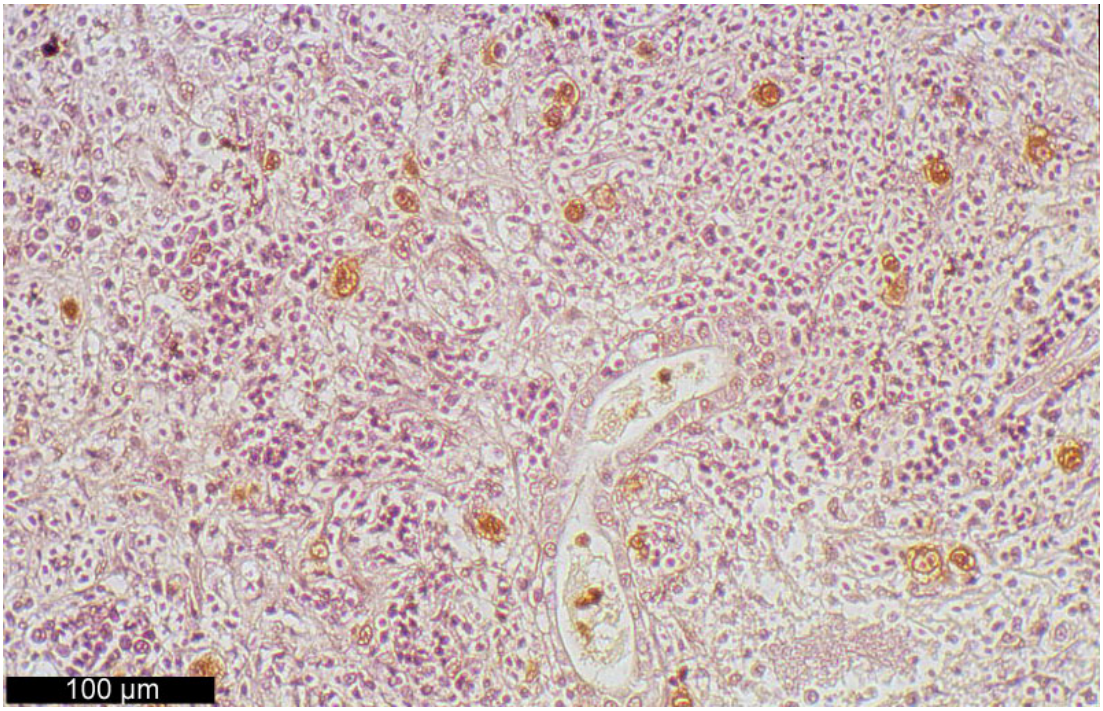

Figure 5.1: Immunohistochemistry of a section of PKD-affected rainbow trout kidney incubated with anti-TF MAb

T. bryosalmonae stages have been stained brown. (haematoxylin & DAB substrate)

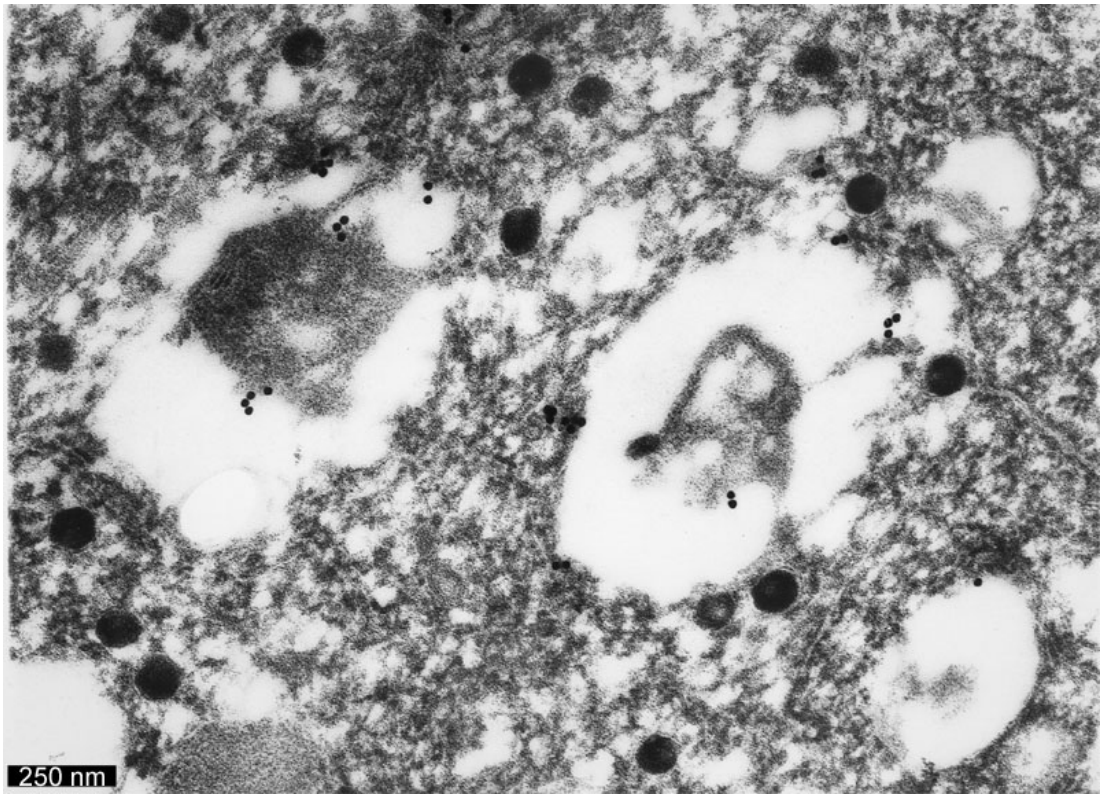

Figure 5.2: Immunogold section of *T. bryosalmonae*-infected rainbow trout kidney incubated with anti-Tn MAb

Gold particles are associated with vacuoles close to electron-dense bodies.
(uranyl acetate & lead citrate)

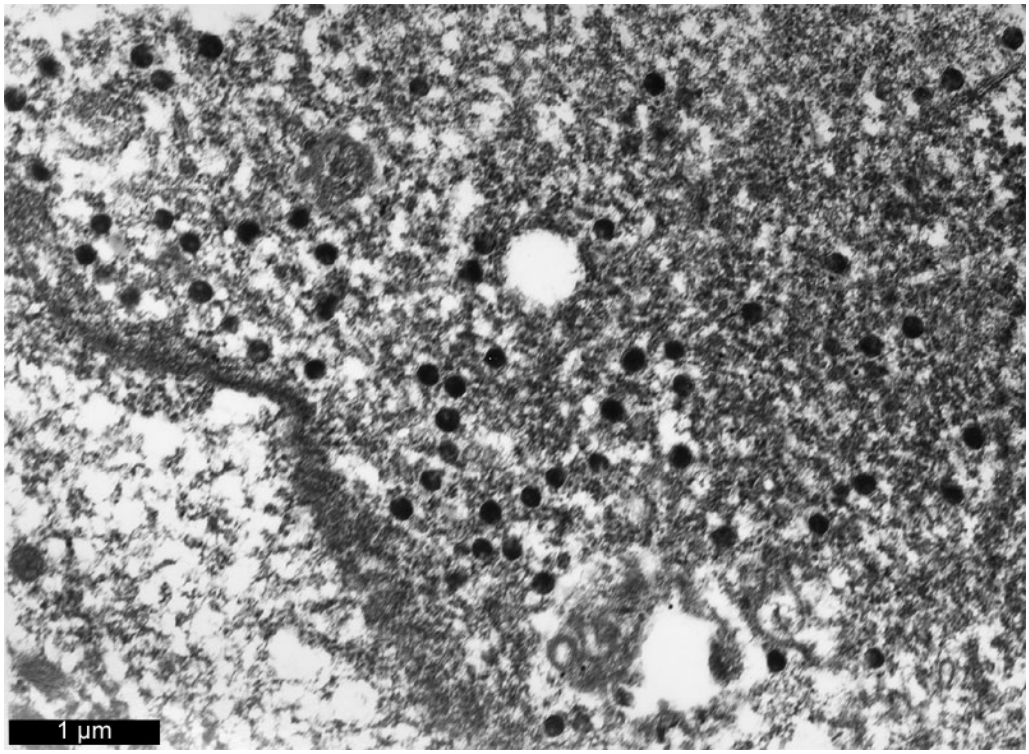

Figure 5.3: Immunogold section of *T. bryosalmonae*-infected rainbow trout kidney incubated with PBS

Gold particles are not associated with electron-dense bodies in this control section. (uranyl acetate & lead citrate)

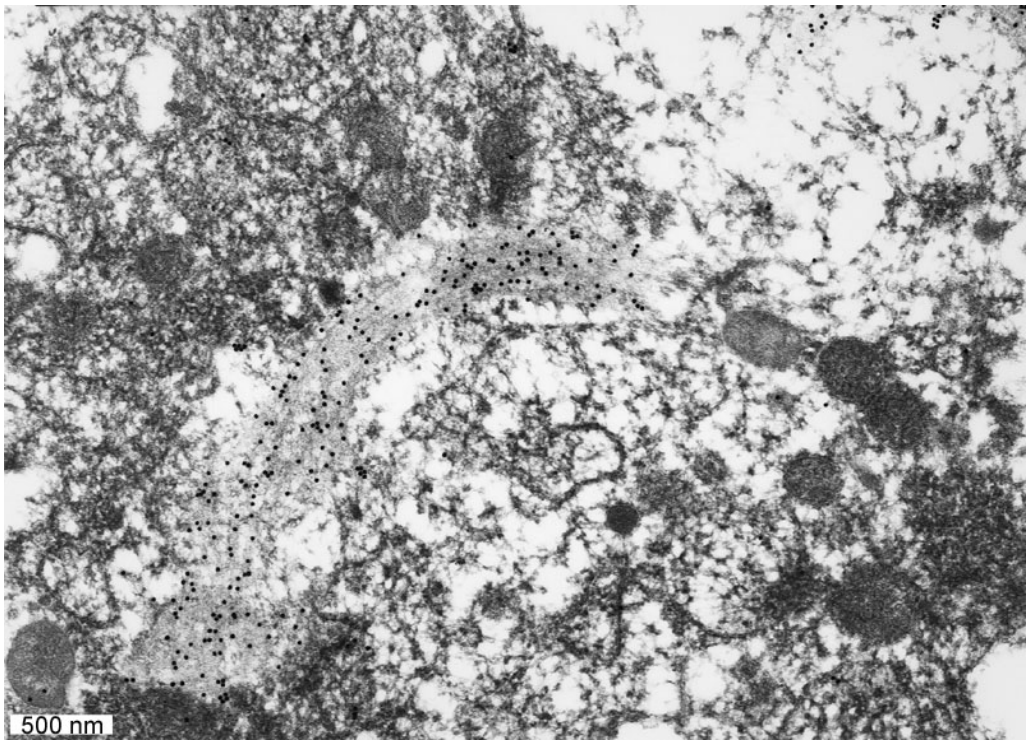

Figure 5.4: Immunogold staining of uninfected rainbow trout kidney incubated with anti-Tn MAb

Gold particles are associated with zones of connective tissue. (uranyl acetate & lead citrate)

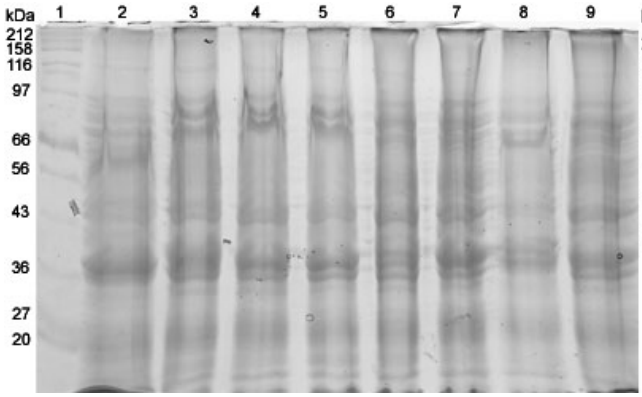

Figure 5.5: SDS-PAGE of *E. coli*

Lane 1 = standard marker; 2 = V517; 3 = O86; 4 = 35218; 5 = 49106; 6 = 11560; 7 = 86; 8 = 25922; 9 = 9481. (Coomassie blue)

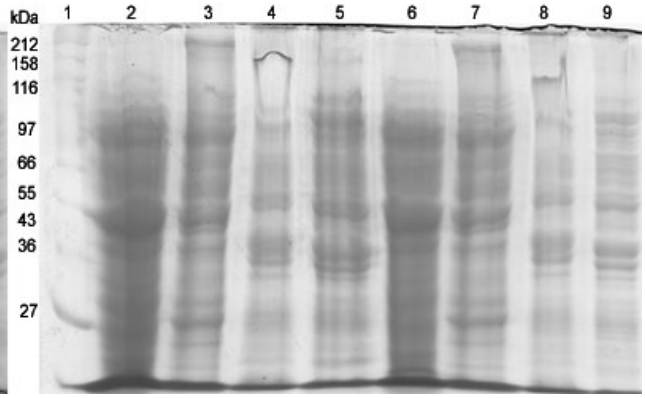

Figure 5.6: SDS-PAGE of rainbow trout kidney and selected *E. coli* strains

Lane 1 = standard marker; 2 = *T. bryosalmonae*-infected kidney; 3 = uninfected kidney; 4 = *E. coli* O86; 5 = *E. coli* 9481; 6 – 9 = replicates of lanes 2 – 5. (Coomassie blue)

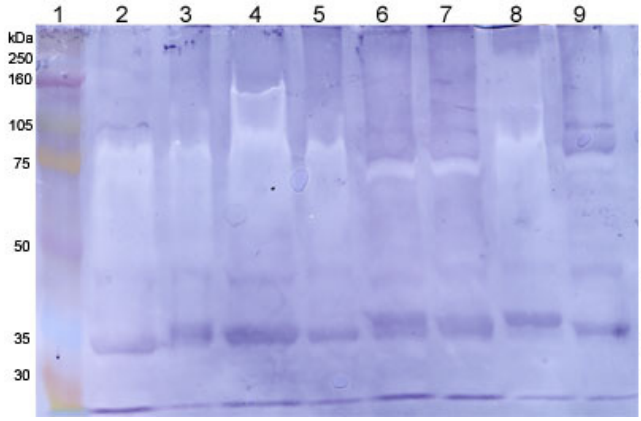

Figure 5.7: Western blot of rainbow trout kidney and selected *E. coli* strains incubated with VVL

Lane 1 = standard marker; 2 = *T. bryosalmonae*-infected kidney; 3 = uninfected kidney 4 = V517; 5 = O86; 6 = 11560; 7 = 86; 8 = 49106; 9 = 9481.

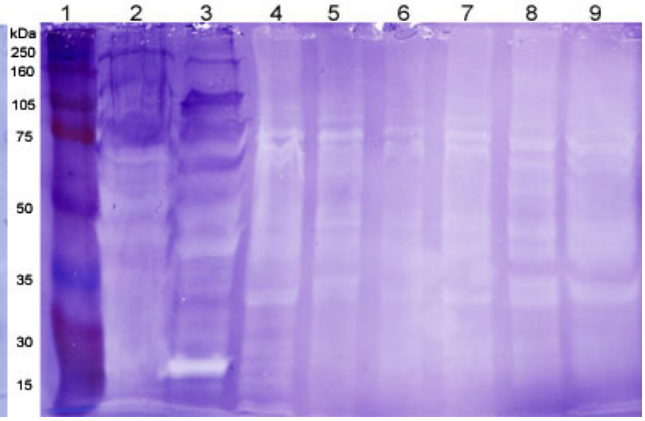

Figure 5.8: Western blot of rainbow trout kidney and selected *E. coli* strains incubated with Jacalin

Lane 1 = standard marker; 2 = *T. bryosalmonae*-infected kidney; 3 = uninfected kidney 4 = V517; 5 = O86; 6 = 11560; 7 = 86; 8 = 49106; 9 = 9481.

5.3.1.4. Western blot analysis

Incubation of rainbow trout kidney and *E. coli* with VVL lectin showed staining of multiple bands in each lane; in particular a low molecular weight band (33 kDa) was common to every sample (Figure 5.7). However, a band of weight 105 kDa was seen to be common only to the *T. bryosalmonae*-infected kidney and *E. coli* strain 9481, with another band of 75 kDa from strains 11560, 86 and 9481. Incubation with Jacalin lectin showed few consistencies between *T. bryosalmonae* and the strains of *E. coli* (Figure 5.8).

Incubation of kidney samples and selected *E. coli* strains with anti-Tn and TF MAbs revealed common bands of 16 kDa in the *T. bryosalmonae*-infected kidney samples and *E. coli* strains O86 and 9481, but not seen with the uninfected kidney samples (Figure 5.9). In addition, another strong band at 33 kDa was common to both bacterial strains incubated with anti-Tn MAb.

5.3.2. Development and efficacy testing of experimental vaccine preparations

5.3.2.1. Vaccination of rainbow trout with typhoid vaccine

The descriptive statistics are shown in Table 5.1. The data were not normally distributed. At the time of sampling, eight fish were present in each group, with 100% prevalence of PKD detected. No adverse signs were witnessed in the group that was administered the vaccine alone. Although the mean and median parasite counts were less in the vaccinated than unvaccinated fish, no statistically significant difference was detected between the groups.

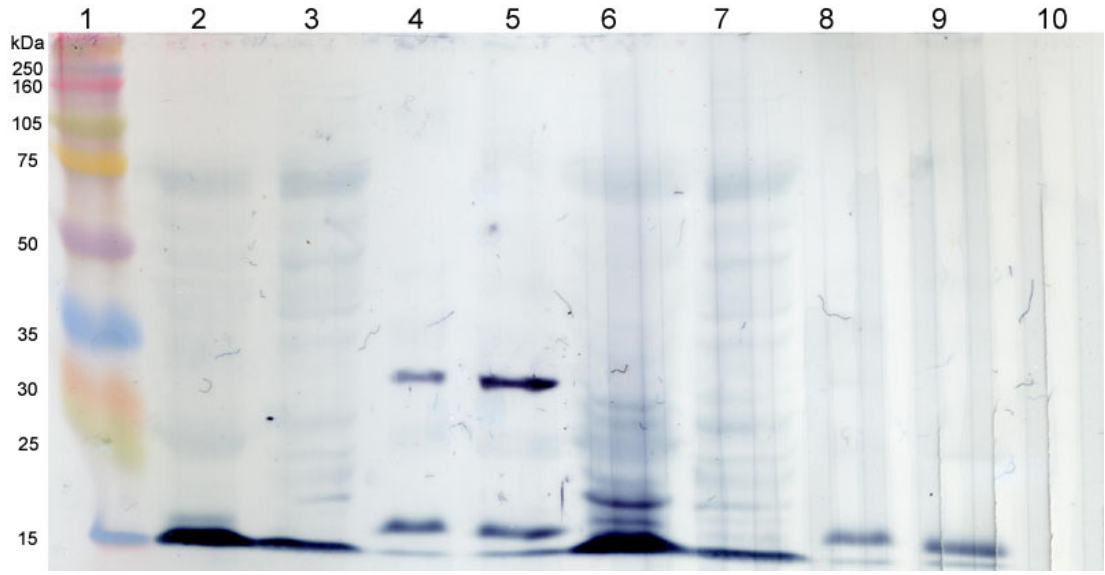

Figure 5.9: Western blot of rainbow trout kidney and selected *E. coli* strains incubated with anti-Tn and TF MAbs

Lane 1 = standard marker; 2 = *T. bryosalmonae*-infected kidney; 3 = uninfected kidney; 4 = *E. coli* O86; 5 = *E. coli* 9481; 6-9 = replicates of lanes 2-5; lane 10 = *T. bryosalmonae*-infected kidney (control). Lanes 2-5 were incubated with anti-Tn MAb; lanes 6-9 were incubated with anti-TF MAb. Lane 10 was incubated with PBS.

Table 5.1: Experimental trial of typhoid vaccine in rainbow trout: *T. bryosalmonae* counts (mm^{-2}) in kidney sections

	unvaccinated	vaccinated
mean (n)	33.2 (8)	24.5 (8)
minimum	1.9	2.4
maximum	72.1	73.1
median	36.8	16.1

5.3.2.2. Vaccination trials using bryozoan tissue or *E. coli*

Following the vaccination procedure which included the implantation of PIT tags, four fish developed skin lesions at the site of tag implantation and subsequently died. Following the challenge protocol, there was a temporary failure of the cold water supply resulting in an elevation of the tank temperature to 25°C. Mortalities resulted, reducing the number of experimental fish which were sampled at the conclusion of the trial. The descriptive statistics are shown in Table 5.2. No reduction in severity of PKD was found in the vaccinated groups. Indeed, the sham-vaccinated group had the lowest PKD prevalence and parasite burden. No statistically significant differences were detected between the parasite burdens of the unvaccinated and vaccinated groups.

Table 5.2: Experimental trial of novel vaccine preparations in rainbow trout: *T. bryosalmonae* counts (mm^{-2}) in kidney sections

	unvaccinated	***E. coli***	***F. sultana***	***Plumatella***
mean (n)	16.6 (11)	30.2 (13)	16.8 (10)	24.9 (4)
minimum	0	0	0	0
maximum	50.4	83.1	31.7	58.6
median	8.2	36.0	21.2	20.4
prevalence of PKD (%)	72.7	84.6	80	75

5.3.3. Efficacy testing of selected in-feed chemotherapeutic products

5.3.3.1. Drug trial 1

Feed intake was high in each group: approaching 100% of the ration offered. The data were not normally distributed, with a wide range of values (Table 5.3, Figure 5.10). Assuming a level of significance of $p < 0.05$, comparison of neither the three treatment classes nor individual tank results showed statistical differences.

Table 5.3: Drug trial 1: intake of medication and renal parasite burden

* represents intake of medication expressed as mg kg BW^{-1} day^{-1}; † tank designation number

			dose*		*T. bryosalmonae* count mm^{-2}			PKD
treatment	**tank†**	**n**	**mean**	**s.d.**	**mean**	**range**	**median**	**prevalence (%)**
pre-treatment	C24	20			19.58	0-107	11.54	91
amprolium	C9	18	0.99	0.01	25	0-106	9.62	50
amprolium	C13	17	0.99	0.02	25.17	0-189	11.06	59
amprolium	C15	18	0.99	0.01	11.54	0-74	3.37	67
salinomycin	C14	20	1.0	0.00	25.75	0-193	0.48	50
salinomycin	C16	20	0.99	0.01	34.11	0-131	12.26	75
salinomycin	C21	20	0.99	0.01	12.72	0-98	0	40
unmedicated	C8	17			21.44	0-78	5.77	65
unmedicated	C17	20			21.92	0-161	1.92	60
unmedicated	C23	20			16.83	0-95	0	40

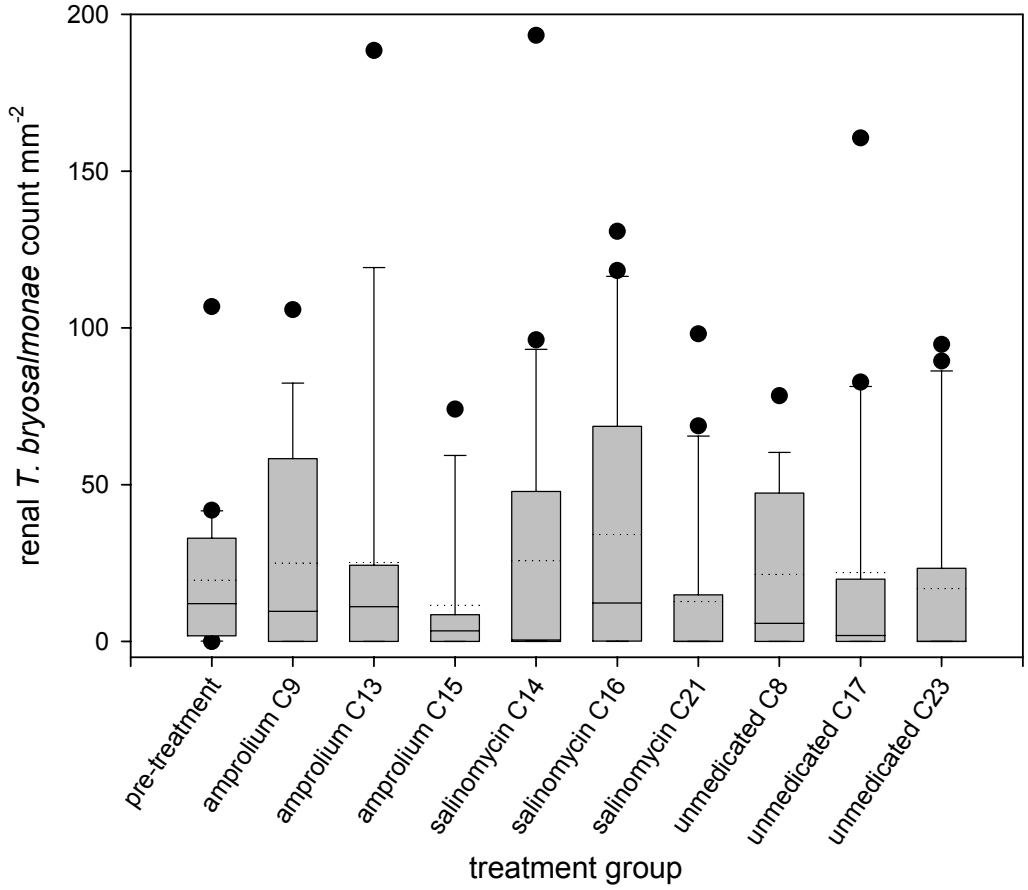

Figure 5.10: Box plot of parasite burden data from drug trial 1

Solid line = median; dotted line = mean; margins of box = quartiles;
whiskers = 10^{th} and 90^{th} percentiles; circle = outlier.

5.3.3.2. Drug trial 2

Although appetite levels were depressed in comparison with the previous trial, there was an overall increase in the dose of drugs ingested due to the increase of concentration of the formulations (Table 5.4). The parasite burden data were not normally distributed, requiring non-parametric analysis. Assuming a level of significance of $p < 0.05$, overall comparison of the three treatment classes showed no differences. Upon comparing individual tanks, the only significant result was C14 *vs.* C16 ($p = 0.0128$). A lesser level of significance was seen with C14 *vs.* C22 ($p = 0.0845$). Both of these represented comparison between a group administered salinomycin and unmedicated control groups. Tank C14 showed the highest level of ingestion of this drug that proved less palatable than amprolium. However, marginally significant results were also seen in comparison of C21 *vs.* C15 ($p = 0.0891$) and C9 *vs.* C15 ($p = 0.1147$) which both represented higher parasite counts from medicated groups (respectively amprolium and salinomycin) than a control tank (Figure 5.11). In both trial 1 and trial 2, no abnormal pathology was seen in the examined organs of the uninfected fish which were fed medicated diets.

Table 5.4: Drug trial 2: intake of medication and renal parasite burden

* represents intake of medication expressed in mg kg BW^{-1} day^{-1}; † tank designation number

			dose*		*T. bryosalmonae* count mm^{-2}			PKD
treatment	**tank†**	**n**	**mean**	**s.d.**	**mean**	**range**	**median**	**prevalence (%)**
pre-treatment	C13	19			49.37	5-106	50.91	100
amprolium	C11	16	61.32	23.04	60.43	0-248	51.63	88
amprolium	C20	13	57.77	15.47	55.24	0-180	33.62	92
amprolium	C21	14	28.32	11.16	71.26	5-209	64.60	100
salinomycin	C9	13	7.93	4.44	96.29	0-257	77.33	85
salinomycin	C14	15	15.23	12.10	21.42	0-66	7.69	67
salinomycin	C19	16	7.75	4.86	81.74	0-409	31.94	69
unmedicated	C15	14			40.35	0-136	7.20	86
unmedicated	C16	12			86.46	0-184	81.18	92
unmedicated	C22	14			55	0-191	35.30	86

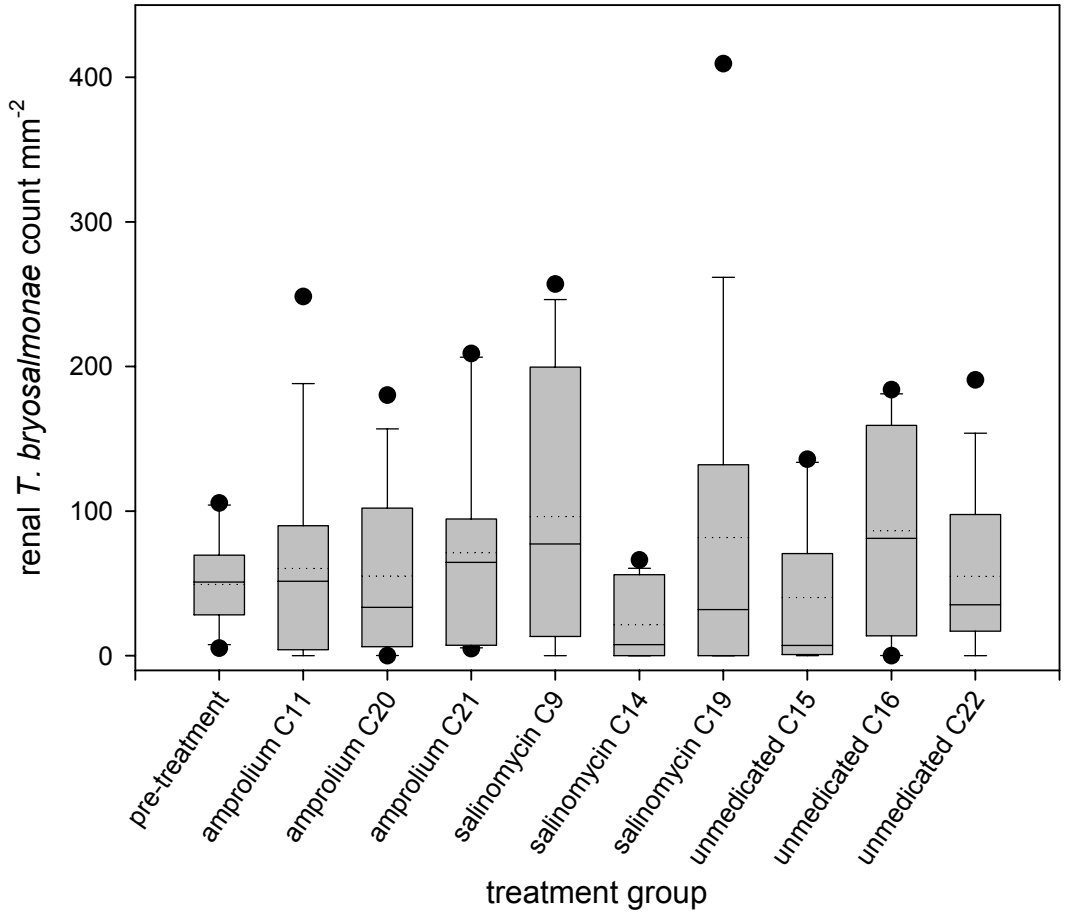

Figure 5.11: Box plot of parasite burden data from drug trial 2

Solid line = median; dotted line = mean; margins of box = quartiles;
whiskers = 10th and 90th percentiles; circle = outlier.

5.4. Discussion

Human cystic hydatidosis caused by the canine tapeworm *E. granulosus* is a chronic potentially fatal condition. It has been demonstrated that parasite glycoconjugates play a crucial part in this parasite-host interaction (Errico *et al.* 2001). In neoplastic mammalian cells, aberrant glycosylation of cell surface glycoproteins is linked with malignant transformation resulting in the expression of glycoconjugates including TF and Tn (Springer, Desai, Murthy, Tegtmeyer and Scanlon 1979). Experimentally, the TF structure (Galβ1-3Gal*N*Acα1-R) is uncovered by complete sialic acid depletion of blood-group precursors known as the M and N Ags (Springer and Desai 1975). Treatment with β-D-galactosidase transforms the TF Ag into one possessing Tn activity (Gal*N*Acα1-R). The Tn immunodominant sugar has been found to be the same monosaccharide responsible for human blood-group A specificities (Springer and Desai 1977a). While the TF and Tn antigens, or more accurately epitopes, are present in ordinary mammalian cell membranes they are always masked by covalent sugars and thus do not appear terminally. TF and Tn motifs have also been reported in the trematode *S. mansoni* (Cummings and Kwame Nyame 1999), while the Tn determinant (normally cryptic in mucin-type *O*-glycans) has been described in *E. granulosus* (Errico *et al.* 2001). Analysis of *T. bryosalmonae*-infected tissue has demonstrated binding of lectins exhibiting TF and Tn binding specificities (Morris and Adams 2004).

Morris and Adams (2004) described the staining of elliptical *T. bryosalmonae* primary cell structures and secondary cells by lectin histochemistry with VVL, peanut agglutinin (PNA) and Jacalin. By immunogold electron microscopy, each of the lectins was seen to bind to lysosomal-like vacuolar areas of *T. bryosalmonae*, with PNA and Jacalin also accumulating in the cytoplasm. The VVL has been shown to bind to the Tn epitope (Gilboa-Garber and Sudakevitz 2001), while both PNA and

Jacalin bind to the TF specificity (Cao *et al.* 1996). Morris and Adams (2004) proposed that the *T. bryosalmonae* structures that were stained were rich in the *N*-acetylgalactosamine glycoconjugate. This was confirmed in the current study with both TF and Tn specificities being displayed in *T. bryosalmonae* within trout kidney by immunohistochemistry and immunogold electron microscopy. Both extrasporogonic and sporogonic stages were stained by anti-TF MAb, with consistent staining patterns to those reported with PNA and Jacalin (Morris and Adams 2004). The lack of staining with anti-Tn MAb by immunohistochemistry may be due to the same factors which led to variability in lectin-binding (Morris and Adams 2004) or may represent assay restriction of this probe (Eryl Liddell and Cryer 1991). However, specific staining was seen using immunogold electron microscopy following incubation of infected kidney tissue with anti-Tn MAb. The staining pattern bore similarities to that previously seen with VVL (Morris and Adams 2004), with localisation of gold particles in vacuolar parasitic regions. Morris and Adams (2004) suggested that the wide range of lectins that recognised lysosomal structures left the relevance of the carbohydrate residues of *T. bryosalmonae* open to further investigation.

Expression of the TF and Tn epitopes has been found in 90% of carcinomata in humans, but they are shielded in healthy tissue or benign tumours and thus unavailable for interaction with the immune system (Springer and Desai 1977a; Springer *et al.* 1979; Springer 1997). It was discovered that while cellular immune responses to TF and Tn are only induced in carcinoma or lymphoma patients, every individual has pre-existing circulating anti-TF and Tn antibodies which are thought to be induced by exposure to intestinal flora. Screening of members of the family Enterobacteriaceae Rahn, 1937 displayed TF and Tn specificities, apparently accounting for natural antibody production in infants (Springer and Desai 1977b). Subsequent analyses displayed the presence of such specificities in 14 strains of

gram-negative bacteria, 17 lipopolysaccharides and five vaccine preparations including those against *Salmonella typhi* (Schroeter, 1886) Warren and Scott, 1930, *Vibrio cholerae* Koch, 1883 and *Clostridium tetani* (Flügge, 1886) Bergey *et al.*, 1923 (Springer *et al.* 1979). Experimental exposure of humans and chickens, *Gallus gallus* (L.) to *E. coli* O86 was found experimentally to result in immune responses, demonstrating TF and Tn specificities of the bacterium (Springer and Horton 1969; Springer and Tegtmeyer 1981).

In relation to *E. granulosus*, Errico *et al*. (2001) suggested that the Tn epitope could be involved in several interactions between parasite and host as multiple Tn-binding proteins were characterised in a range of cells including hepatocytes and macrophages. The antigen identified by the immune system was considered to be more complex than the biochemical definition of Tn. It has been proposed that in addition to being crucial in the development of diagnostic techniques, the TF and Tn glycoconjugates are highly immunogenic, immunomodulatory and may be crucial in the pathogenesis of parasitic disease due to *E. granulosus* and *S. mansoni* (Cummings and Kwame Nyame 1999; Errico *et al.* 2001). In light of the discovery of TF and Tn specificities of *T. bryosalmonae*, it was decided to prepare experimental vaccines expressing TF and Tn specificities.

Springer (1997) developed a therapeutic vaccine which proved efficacious in treating patients suffering from advanced breast cancer. The vaccine consisted of TF and Tn components derived from degraded human blood-group O erythrocytes, typhoid vaccine preparations (phosphoglycolipid A) and calcium phosphate as an adjuvant. After his death in 1998, “Springer’s Vaccine” was no longer available, but it was suggested that using typhoid Vi polysaccharide vaccine alone may be an appropriate substitute (D'Adamo 2001). Thus, the first experimental vaccine trial of the current study involved the administration to rainbow trout of a commercial typhoid vaccine

expressing the Vi polysaccharide. As a challenge method for exposure to fish of released *T. bryosalmonae* spores was not available at that time, the fish were challenged by the established method of i.p. injection of *T. bryosalmonae*-infected kidney homogenate. The challenge method was evidently successful, resulting in 100% prevalence of disease. As this was a preliminary experiment, the number of subjects was low. Although no significant statistical difference was found between the different vaccination groups, the trend of lower parasite burden in the typhoid vaccinated group justified further development of a PKD vaccine based on TF and Tn epitopes.

As obtaining TF and Tn epitopes by the degradation of human blood cells would not be appropriate for injection into fish due to regulatory concerns, an alternative approach to vaccine development from that of Springer (1997) was adopted. As coliform bacteria, including *E. coli* O86 (K61) specifically, had been found to display the required properties (Springer *et al.* 1979; Springer and Tegtmeyer 1981), several strains were screened with lectins and MAbs to gauge the presence of the epitopes. A similar strategy had previously been attempted in developing experimental vaccines against *I. multifiliis* based on the free-living ciliate *Tetrahymena pyriformis* (Ehrenberg) which was thought to be transformable into an efficient immunogen of the parasite (Dickerson and Clark 1997; Buchmann, Sigh, Nielsen and Dalgaard 2001). All of the strains of *E. coli* examined produced recognisable bands by Western blot, with particularly clear binding with anti-Tn MAb. These findings were consistent with previous reports of a high prevalence of expression of the epitopes in strains of *E. coli* (Springer and Desai 1977b; Springer *et al.* 1979). In light of previous reports and findings from the screening techniques, strain O86 was selected for use in the preparation of an experimental vaccine. Springer (1997) stated that an adjuvant was necessary to allow sufficient persistence of the TF and Tn immunogens to allow development of cell-mediated immunity. This was satisfied by

the use of a commercial veterinary water-in-oil adjuvant which did not lead to subsequent development of any noticeable peritoneal adhesions.

The fish were challenged with *T. bryosalmonae* spores in this trial, replicating natural exposure. This challenge method proved effective with resulting PKD prevalence levels of over 70% despite an accidental fluctuation in water temperature post-exposure. Although there were mortalities following this incident, sufficient numbers survived to allow appreciation of the general trend of PKD burden. Higher parasite load was found in the *E. coli*-vaccinated group than the control group suggesting that this vaccine was not efficacious. The increased burden may have been attributable to immunosuppression of the fish in response to the injection of bacteria. The findings of this experimental trial suggest that the TF and Tn epitopes may not be protective antigens in the development of *T. bryosalmonae* within fish tissue. The difference in result between the typhoid vaccine and *E. coli* vaccine trials may be a result of differing levels of epitopes in the preparations or the different challenge method used in each. A future approach could be a vaccine preparation based on the combination of a previously produced synthetic TFα hapten (MacLean, Bowen-Yacyshyn, Samuel, Meikle, Stuart, Nation, Poppema, Jerry, Koganty, Wong and Longenecker 1992) with a typhoid vaccine preparation, potentially allowing higher levels of the epitopes to be delivered.

Buchmann and Lindenstrøm (2002) described efficacious experimental vaccines against monogenean parasites of fish. Preparations of *Dactylogyrus vastator* Nybelin, 1924 and *Microcotyle sebastis* Goto, 1894 were homogenised and i.p. injected respectively into carp and rockfish, *Sebastes schlegeli* Hilgendorf, conferring protection to subsequent challenge. Li and Woo (1995) outlined the production of a live vaccine of *Cryptobia salmositica* Katz, 1951 attenuated by continuous *in vitro* culture which protected rainbow trout against cryptobiosis. Petchsupa (2002) found

that i.p. vaccination of live *T. bryosalmonae* cells conferred greater protection to future challenge than vaccination with formalin- or UV-treated cells. It was hypothesised that such treatments altered the surface immunogens of *T. bryosalmonae*, reducing subsequent immune recognition. However, these preliminary vaccination trials were conducted using *T. bryosalmonae* cells harvested from infected trout kidneys for both vaccination and subsequent challenge which did not emulate natural infection routes.

There are no previous reports of an experimental PKD vaccine being developed from *T. bryosalmonae* spores, the infective stage to fish. Feist *et al*. (2001) injected rainbow trout with live disrupted spore sacs of *T. bryosalmonae* harvested from *F. sultana*. However, as the fish were all later sampled for assessment of PKD development, there was no opportunity to subsequently challenge the fish with *T. bryosalmonae* to gauge any development of protective immunity. In the current study, *F. sultana* tissue including mature spores of *T. bryosalmonae* was harvested for experimental vaccine preparation. As insufficient fresh parasitic material was synchronously mature, infected bryozoans were harvested upon recognition of spores, rapidly frozen and stored at -70°C until sufficient infected material was collected to be pooled. To avoid potential attenuation of surface epitopes as previously reported (Burreson and Frizzell 1986; Petchsupa 2002), no formalin- or UV-treatment of the tissue was conducted. The fish were successfully challenged by subsequent exposure to live *T. bryosalmonae* spores. However, the *T. bryosalmonae*-vaccinated fish did not show reduced parasite burdens compared to the sham-vaccinated group.

It has been demonstrated using MAb probes that *T. bryosalmonae* expresses contrasting antigens at different stages of the life cycle (Morris *et al.* 1997), explaining the differences in response between the current vaccination with spores

and previous vaccination with kidney-derived cells (Petchsupa 2002). The kidney preparation contained predominantly extrasporogonic *T. bryosalmonae* cells which have long been recognised to lead to development of a profound immune response in fish (Ferguson and Ball 1979). However, the previously reported finding that myxozoan spores of *M. cerebralis* can be recognised as self-antigens by rainbow trout (Pauley 1974) could explain the current apparent lack of protection facilitated by vaccination with *T. bryosalmonae* spores. Stages of *T. bryosalmonae* have previously been observed by *in situ* hybridisation entering fish tissue via the gills and skin (Morris *et al.* 2000b; Longshaw *et al.* 2002). The findings of Feist *et al*. (2001) that spores directly injected i.p. did not elicit infection would seem to support the hypothesis that potentially protective antigens of *T. bryosalmonae* are not unmasked until the parasite has entered its host through a natural portal. This would imply that an inactivated vaccine based on the spore stages of *T. bryosalmonae* might not be efficacious, explaining the findings of the current study. While the exposure of live spores to fish, with modulation of subsequent water temperatures, has been used as a crude PKD vaccination method (de Kinkelin and Loriot 2001), the inherent risk of the development of clinical disease remains. A successful vaccination protocol could theoretically be developed if a method could be found to attenuate spores in such a way that the parasite could enter fish tissue, subsequently expose protective antigens, but be incapable of causing clinical disease. The lack of an efficacious vaccine leaves a requirement for other control strategies.

Previously, several chemotherapeutants have been demonstrated to be efficacious against PKD, but with concerns over toxic side-effects. Alderman and Clifton-Hadley (1988) described difficulties in implementing a flush treatment of rainbow trout using malachite green, which utilised a starting concentration of 3.2 ppm and led to observed toxicity in the fish. Chinook salmon fed 5-10 mg kg BW^{-1} day^{-1} of fumagillin for seven weeks showed increased mortality levels and depletion of haematopoietic

tissues (Hedrick *et al.* 1988). Anorexia and cumulative mortalities of 92% were seen in rainbow trout fed at 20 mg kg BW^{-1} day^{-1} (Wishkovsky *et al.* 1990). Similar toxicity patterns were seen in rainbow trout treated with TNP-470, a synthetic analogue of fumagillin (Morris *et al.* 2003a). Field trials demonstrated that the fish were immunocompromised, being unable to combat opportunistic bacterial challenge. Thus, a need exists for novel safe drugs which are efficacious against PKD.

Salinomycin sodium and amprolium hydrochloride are both in-feed medications licensed for use against coccidial diseases in food-producing animals including pigs, poultry and rabbits. Salinomycin is a monocarboxylic polyether ionophore produced by *Streptomyces albus* (Rossi Doria, 1891) Waksman and Henrici, 1943 with both antimicrobial and anticoccidial effects. It acts as a chelator of monovalent cations, disturbing the intracellular balance of monovalent ions, allowing excess water to penetrate parasitic cells. Oral medication with salinomycin has been shown experimentally to protect tapir fish, *Gnathonemus petersii* Günther, 1862 against *Henneguya* sp. Thélohan, 1892, a myxosporean gill parasite (Dohle *et al.* 2002). The drug was ingested by water fleas, *Daphnia* spp. Müller which were in turn fed to tapir fish for up to 9 days, delivering a dose of approximately 18 µg kg BW^{-1} day^{-1}. Ultrastructurally, deleterious effects were observed in the trophozoite cytoplasm and the extrasporogonic and pansporoblastic parasitic stages. Amprolium is a structural analogue of thiamine that causes competitive inhibition of thiamine utilisation preventing coccidian merozoite production (Athanassopoulou *et al.* 2004). Experimental oral dosing at 10 mg kg BW^{-1} day^{-1} was found to confer limited protection to rainbow trout challenged with the microsporidian gill parasite *Loma salmonae* Putz, Hoffman and Dunbar, 1965, although poor absorption from the intestine may have limited the efficacy (Speare, Athanassopoulou, Daley and Sanchez 1999). Shinn *et al*. (2003) described the use of a range of drugs including salinomycin and amprolium against *I. multifiliis*, the former proving the most

efficacious of those tested. These oral preparations were delivered separately at 100 ppm for 10 days. However, Athanassopoulou *et al*. (2004) recently described an effective regime against the myxosporean *Polysporoplasma sparis* Sitjà-Bobadilla and Álvarez-Pellitero, 1985 infection in sea bream, *Sparus aurata* L. This involved the combined oral administration of salinomycin and amprolium at 100 mg kg feed^{-1} day^{-1} of each.

In light of these previous reports, experimental trials of salinomycin and amprolium against PKD were undertaken in the current study. Due to the variation of dose rate of the drugs administered in the previous reports, a low initial dose of 1 mg kg BW^{-1} day^{-1} was given in the first trial. Although the diets were well tolerated, no relationship was seen between parasite burden and treatment group. For the second trial, a higher dose rate of 100 mg kg BW^{-1} day^{-1} of each drug was presented orally. Inclusion at this level led to reduced ingestion, with two of the salinomycin groups taking less than 8% of their ration, although no signs of toxicity were detected. This was consistent with previous reports of poor palatability of salinomycin (Shinn *et al.* 2003). Gelatine had been used to top-coat the experimental diets in the current trial in the hope that it would more effectively mask inappetent traits than the previously reported use of cod liver oil. This proved successful only at the lower dose of inclusion of the drugs. Although a trend was seen between increased salinomycin intake and reduced parasite burden, the only medicated group to show a statistically significant difference from an untreated group was that which had ingested the highest mean dose of salinomycin (15 mg kg BW^{-1} day^{-1}). This group also showed the lowest prevalence of PKD. Even if a true inverse relationship between ingestion of salinomycin and severity of disease exists, the depression of appetite witnessed could detrimentally affect growth rates if salinomycin was used in a commercial setting (Shinn *et al.* 2003). Dosing with amprolium alone did not lead to any reduction in the renal *T. bryosalmonae* burden of the rainbow trout. Investigation of

the efficacy of dual dosing with the agents was not carried out, but could be an interesting future study in light of the previous report of successful treatment of *P. sparis* infection (Athanassopoulou *et al.* 2004).

However, other factors could compromise the potential use of salinomycin, amprolium or other novel treatments for the control of PKD. Both salinomycin and amprolium are rapidly metabolised, with short half-lives of elimination, meaning that the duration of action would be limited (Hamamoto, Koike and Machida 2000; Anonymous 2004a). Thus, any potential treatment plan would have to take into account duration of action of the medication in combination with the season of infectivity and the incubation period of infection. Although such protocols are routinely instigated in the control of intestinal and respiratory endoparasitic infections by anthelminthic dosing in ruminant farm animals (Urquhart, Armour, Duncan, Dunn and Jennings 1991), the nature of the pathogenesis of PKD, in particular the impact of variables such as temperature, could make robust strategies difficult to plan in advance. If individual fish were to be maintained on endemic premises for more than one season, it would be important to ensure that they were either suitably exposed to *T. bryosalmonae* to allow protective immunity to develop, or treated medically every year. Although such management practices might be costly and inconvenient, the potential reduction in the economic impact of PKD could justify such an approach in larger units. As salinomycin is currently classified as a growth promoter, it has been announced (after the current trials were conducted) that its licence for use in food-producing animals within the European Union will be withdrawn on 1st January 2006 (Anonymous 2004b). Thus, due to the apparent limited efficacy of both amprolium and salinomycin, and regulatory concerns over the latter, it seems unlikely that it would prudent to pursue further investigations into the use of these agents for the control of PKD in the near future.

Although the presence of TF and Tn specificities was detected in samples of *T. bryosalmonae* from trout kidney, the limited success of the subsequent vaccine trials suggests that the protective role of these epitopes in the development of the parasite is equivocal. Similarly, vaccine preparations composed of *T. bryosalmonae* spores did not elicit reduction in parasite load following subsequent challenge, implying that fish do not mount an effective acquired immune response against this stage of parasitic development. Despite showing some effects against PKD at high doses, salinomycin may not prove to be a strong candidate for chemotherapy due to limited palatability and regulatory considerations. The future of PKD prevention and control may rely upon the development of novel agents by non-traditional means.

Chapter 6: Final discussion and conclusions

Longshaw *et al*. (2002) concluded that the development of a robust laboratory based culture system maintaining malacosporean parasites would be intrinsic in the study of prospective vaccine and chemotherapeutants to combat PKD in farmed fish. During the current project, a culture system was designed which was shown to be capable of maintaining bryozoans infected with malacosporean parasites for extended periods. This achievement facilitated study of the development of *B. plumatellae* and *T. bryosalmonae* within their invertebrate hosts. Large numbers of spores of the latter species were harvested from infected bryozoans and employed in the production of a replicable experimental challenge method for rainbow trout. These developments allowed the production of a vaccine preparation made from *T. bryosalmonae* spore material and subsequent efficacy testing, thus fulfilling the primary aim of the overall project.

Despite their pathological effects upon their hosts, malacosporean infections were maintained within bryozoans within the new system. The mixture of protozoa and algae developed as a result of the feeding trials has proved successful in the long-term culture of both *Plumatella* sp. and *F. sultana*. The culture system is currently being utilised in a new research project studying the life cycle of *T. bryosalmonae* with particular emphasis on the prospective fish to bryozoan transmission phase. The flexibility of this system has been further demonstrated, as *F. sultana* colonies collected in December 2004 from the River Cerne were maintained within the system at 10°C before being brought to 21°C and manifesting overt *T. bryosalmonae* infection. Thus, until a supply of spores is required, infected bryozoan colonies could be maintained at lower temperatures (inhibiting malacosporean development (Gay *et al.* 2001)). The culture temperature of the material could then be raised upon demand, encouraging parasitic proliferation. The water temperature of the bryozoan culture tank can be readily modified by altering that of the surrounding water sleeve. Thus, it would be possible to maintain separate genera of bryozoans at their

individual optimal temperatures. Experience drawn from the current project and previous studies suggests that *Fredericella* colonies thrive better at lower temperatures than *Plumatella* (Toriumi 1972). It is planned to construct a larger scale permanent system based on the prototype with some additional modifications. Therefore, the bryozoan culture system developed within the current project may yet aid in fulfilling the suggestion that the best chance of closing the life cycle of *T. bryosalmonae* rests on the development of robust bryozoan culture systems (Canning and Okamura 2004; Tops *et al.* 2004).

Fundamental to the success of achieving the objectives of this project was the successful collection of bryozoan colonies infected with *T. bryosalmonae*. The placement of naïve colonies within PKD-endemic waters did not lead to observed infection either in the current study or by Tops and Okamura (2003). However, an alternative strategy of collecting bryozoans directly from endemic sources and rearing them in the laboratory for extended periods did lead to the positive identification of two malacosporean parasites. Although the initial objective was to obtain colonies infected with *T. bryosalmonae*, the unexpected discovery of *Buddenbrockia* proved highly valuable. The previously demonstrated close relationship between *B. plumatellae* and *T. bryosalmonae* implied that in-depth examination of the former could be justified (Canning *et al.* 2002). Indeed, it was considered possible that the 'worm' stages observed might have represented an alternative manifestation of *T. bryosalmonae* as previously described with *B. plumatellae* (vermiform forms in *Plumatella* and spheroid "*T. bryozoides*" sacs in *Cristatella*) (Canning *et al.* 1996, 2002; Monteiro *et al.* 2002). The subsequent failure to infect rainbow trout with released spores and morphological similarities with previous descriptions (Okamura *et al.* 2002) suggested that the vermiform stages observed herein corresponded to *B. plumatellae* and did not represent a *Buddenbrockia* form of *T. bryosalmonae* (Canning *et al.* 2002).

The biology of the *P. repens* colonies infected with *B. plumatellae* allowed intimate study of malacosporean development. Rapid growth of translucent colonies and a predilection for colonisation on the surface of Petri dishes meant that proliferating infected bryozoan populations could be repeatedly examined. These characteristics made the study of *B. plumatellae* within *P. repens* more fruitful than the more troubled examination of *T. bryosalmonae* within *F. sultana* whose colonies were both less translucent and amenable to colonisation of the Petri dish surface. The observation of sequential growth of the former malacosporean meant that when *T. bryosalmonae* infection was subsequently witnessed within colonies of *F. sultana*, it was possible to recognise characteristic malacosporean developmental traits at an early stage. The primary indicator witnessed with both species was the presence of numerous minute particles swirling within the currents of the bryozoan metacoel. This finding proved highly consistent within the malacosporeans, although failure to examine the morphology at an ultrastructural level precluded identification of the particles. It could not be concluded if they were either primordial parasitic cells (possibly acting to disseminate infection throughout the metacoel) or host-derived coelomic corpuscles (Mano 1964). If the latter origin is the case, then the size and nature of the particles would make them most likely to be lymphocytes, the most primitive of the described bryozoan coelomic corpuscles.

Developmental peritoneal stages were less conspicuous within *T. bryosalmonae*-infected *F. sultana* colonies than had previously been seen in *P. repens* infected with *B. plumatellae*. With the former species they appeared round in shape as opposed to the pyriform manifestation of the latter. The growth of *T. bryosalmonae*-infected *F. sultana* colonies maintained using the previously established culture methods led to limited proliferation of translucent colonies on Petri dish surfaces. As the design of the low maintenance culture system did not reach fruition until later in the project, the use of sub-optimal systems for the culture of the infected colonies studied might

have compromised recognition of the full spectrum of parasitic development at that time. Recently, examination of the *F. sultana* colonies described above which were kept within the new culture system following collection in December 2004, has confirmed the presence of *T. bryosalmonae* stages adhered to the peritoneum. In both malacosporean species, the peritoneal stages acted as developmental precursors to larger freely-circulating bodies. Possibly, some parasitic cells could also remain viable in association with the host peritoneum, being consistent with previous suggestions from molecular evidence that cryptic stages exist within bryozoan tissue (Canning *et al.* 2002). If so, the presence of such latent stages could explain the manifestation of discrete waves of infection first witnessed with *B. plumatellae* and subsequently with *T. bryosalmonae*. These findings further reinforced the relevance of studying the development of *B. plumatellae* with reference to *T. bryosalmonae* infection.

Unlike previous observations of malacosporeans by Canning *et al*. (2002), intact mature spore sacs of neither species were observed directly exiting the bryozoans, but instead malacosporean spores matured fully within their sacs while still contained within the bryozoan metacoel. Confocal microscopy analyses revealed morphological features of *T. bryosalmonae* spores that were previously unknown. It had been hoped to conduct similar analysis of *B. plumatellae* spores as part of this study to aid comparison of the two species, but no suitable material was obtained successfully following establishment of the methodology. However, comparison with the TEM analysis of *B. plumatellae* showed morphological consistencies between the malacosporean species. A similar pattern of articulation was seen between the four capsulogenic cells at the front of both spores (Figure 3.41 and Figure 4.28a). Rather than all four cells meeting at an apex, a line of apposition was formed between two of the cells, with each of the remaining two cells contacting these (Figure 6.1). A similar pattern had previously been shown in early sporogonic stages

of *B. plumatellae* ("*T. bryozoides*") within *C. mucedo* (*cf.* Figure 19 in Canning *et al*. (1996)). This consistency between the species is interesting in light of fact that Canning *et al*. (2000) reported contrasting modes of development between the species. *Buddenbrockia plumatellae* spore cells result from division of a single sporoplasmogenic cell into 10 cells, whereas *T. bryosalmonae* spores develop from the aggregation of eight stellate cells around a single sporoplasmogenic cell. Future CLSM analysis of *B. plumatellae* could potentially determine if the pattern of valve cells is also consistent between the genera *Buddenbrockia* and *Tetracapsuloides*.

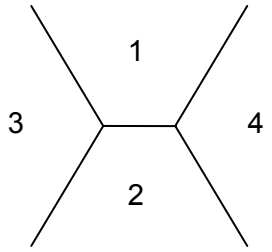

Figure 6.1: Representation of the pattern of articulation between the four capsulogenic cells of *T. bryosalmonae* and *B. plumatellae*.

Whereas cells 1 and 2 are in contact with all of the remaining three cells, cells 3 and 4 make contact with only two others (cells 1 and 2).

Despite the multiple CLSM scans of *T. bryosalmonae* conducted, the aspect of the spore diametrically opposite to the polar capsules was never directly imaged due to attenuation of the fluorescent signal. Future examination of malacosporean spores using CLSM could use a modified technique to address these issues (Heintzmann, Kreth and Cremer 2000). The diameter of mature spores and the length of extruded polar filaments were found to be similar between *B. plumatellae* and *T. bryosalmonae*. Their respective mean polar filament lengths represented 68% and 65% of the diameter of intact spores, being relatively short in comparison to some myxosporean species in which filaments measuring up to 10 times of the length of the spore have been reported (Cannon and Wagner 2003). In at least some myxosporeans, the polar filament is thought to be able to act as an attachment

mechanism between hatching spores and the intestinal surface of the host (Lom and Dyková 1992). The delay before extrusion in *B. plumatellae* following exposure to mucus, in conjunction with the diminutive length of the polar filaments suggests that they might not act as highly evolved anchoring methods for the parasites upon contact with a potential host. As malacosporeans have previously been shown to enter fish via the gills or skin (Morris *et al.* 2000b; Longshaw *et al.* 2002) – unlike many myxosporeans which are ingested (Lom and Dyková 1995) – it may be that a longer filament would act as a hindrance in the ability for malacosporeans to successfully enter host tissue. Upon eversion of polar filaments, the diameter of these spores was not seen to significantly differ from unaffected specimens, with no external migration of sporoplasmic material observed as had been described with some actinosporean spores (Xiao and Desser 2000). However, the morphology of spores was seen to alter, becoming less spherical in shape, with the outer valves and internal sporoplasms showing discernable changes.

Canning *et al*. (2000) suggested that infection of fish with *T. bryosalmonae* entailed entry of sporoplasms followed by release and subsequent fusion of their secondary cells. Therefore, these stages would represent the formative cells of *T. bryosalmonae* to which fish would be exposed. As parasites acquire unique strategies to evade immune destruction, any successful vaccine candidate must break their cycle of infection (Jenkins 1998). Despite these considerations, the vaccine preparation produced from *T. bryosalmonae* spore material (which included sporoplasmic cells) did not prove protective. This would suggest that rainbow trout might not mount an effective immune response against the earliest stages of *T. bryosalmonae* which are present at the sites of entry in gill or skin tissue. Further studies could explore this observation by trying to assess exactly which stages of the parasite are targeted successfully by fish with acquired immunity to *T. bryosalmonae*. As it has been demonstrated that challenge with very low numbers of

spores can lead to PKD, the ideal solution might be presented by a vaccine that could neutralise the limited numbers of gill, skin or haematogenous parasitic stages before they have a chance of reaching the kidney and establishing the positive feedback loop of aberrant immune-mediated host response. Experiments of this type could utilise the challenge method developed during the current study.

Immunohistochemical analysis of *F. sultana* infected with *T. bryosalmonae* showed extensive staining patterns with MAbs (including MAb B4) raised against *T. bryosalmonae*. The B4 antigen has been found to be highly significant within the Myxozoa, being expressed in at least 35 different myxozoan species (Morris, Longshaw and Adams 2004). Passive immunisation of fish with the MAb B4 has been shown to delay the onset of PKD development (Petchsupa *et al.* 1999). The staining of both bryozoan and *T. bryosalmonae* tissue by immunohistochemistry suggested that the antigen may be expressed within these stages. It was thus hoped that the vaccination preparations containing *T. bryosalmonae* spores may elicit a protective response. The resultant lack of efficacy of the preparations suggests that the staining by immunohistochemistry may have been non-specific. It has been proposed that the B4 antigen represents a strong candidate for development of a sub-unit recombinant vaccine against PKD.

Although the bryozoan hosts of *T. bryosalmonae* can now be cultured for prolonged periods, their fastidious nature and the laborious general requirements of harvesting large quantities of parasites for potential vaccine production (Buchmann *et al.* 2001) might make such a strategy unrealistic in a commercial setting. Therefore, the opportunity of expressing large quantities of protective antigen by easily cultured organisms such as bacteria could present a more tenable solution. Reports of the importance of the TF and Tn epitopes in the pathogenesis of mammalian carcinomata and the protective effect bestowed by vaccine preparations expressing

their specificities (Springer 1997), coupled with their proposed role in the pathogenesis of parasitic diseases (Cummings and Kwame Nyame 1999; Errico *et al.* 2001) made their discovery in *T. bryosalmonae* highly intriguing. The predominant mammalian immune reaction seen in response to TF and Tn stimulation is cell-mediated (Springer and Desai 1977a). Such cellular responses have also been shown to be fundamental in the response of fish to *T. bryosalmonae* exposure (Klontz *et al.* 1986; Chilmonczyk and Monge 1999). Expression of these epitopes within strains of *E. coli* represented a simple method of producing large quantities of potential vaccine preparation. Although the resultant vaccine did not prove efficacious in the experimental trials, the role of the TF and Tn epitopes within the pathogenesis of *T. bryosalmonae* may warrant further investigation.

In addition to expressing TF and Tn epitopes (Cummings and Kwame Nyame 1999), the trematode *S. mansoni* has also been found to remodel host antigens for subsequent expression on its outer surface (Abath and Werkhauser 1996). The ultrastructural morphological similarities between bryozoan peritoneum and *B. plumatellae* spore sac wall, and the intimate association of analogous basement membrane tissue within both may be of significance. There is some evidence in the consistent conformational structure of both basal lamina and muscle layers between bryozoan and malacosporean specimens that the latter utilises and remodels tissue of the former during the formative development of vermiform spore sacs. Such a proposition could have fundamental impacts on the taxonomic placement of the Myxozoa. Although nematodes do not exhibit remodelling of host tissue during their development (Anderson 2000), the current consensus seems to place the Myxozoa as their degenerate ancestors (Smothers *et al.* 1994; Okamura *et al.* 2002). However, this classification is not settled, with recent findings suggesting that the Myxozoa may not be related closely to any other phylum (Kelley, Beauchamp and Hedrick 2004). If the preliminary findings of the current study could be confirmed,

and true remodelling of host tissue does occur in malacosporean development, then the proposed affinity between myxozoans and nematodes would be eroded in light of their contrasting developmental pathways.

It had previously been suggested that reducing potential bryozoan habitats such as submerged vegetation and wood debris for several hundred metres upstream of the inlets to PKD-endemic farms could act as an effective control measure against PKD (de Kinkelin *et al.* 2002). While this proposal seems logical in theory, findings from the present study suggest that there could be wide variation between different sites in the effectiveness of such an approach. In a location such as the River Cerne, where a large heavily infected bryozoan mass releases vast numbers of spores into a trout farm inlet, such actions could be appropriate. Nevertheless, the premise relies upon successfully identifying potential bryozoan habitats and then removing or compromising all infected colonies. However, during the current study, despite thorough examination of the inlet waters of certain farms known to be endemic for PKD, no bryozoan colonies could be identified. It appears that bryozoan colonies can remain elusive, with *T. bryosalmonae* infection having been found in an experimental recirculation system although no bryozoan colonies were ever discovered despite extensive scrutiny (de Kinkelin *et al.* 2002). The finding from the current work that massive numbers of *T. bryosalmonae* spores can be released from small numbers of bryozoans, in conjunction with observation that a single spore is capable of infecting a fish, would imply that bryozoan control would have to be meticulously successful to reliably prevent PKD outbreaks. Difficulties in being able to eradicate bryozoans from inlet screens, filters and pipe work could prove a major obstacle to such approaches. These findings suggest that it does not appear to be feasible to universally prevent exposure of fish to *T. bryosalmonae* in endemic waters, but emphasises the importance of developing alternative control measures such as vaccination or chemotherapy.

The success in developing a robust challenge method for infecting rainbow trout with *T. bryosalmonae* that was non-destructive to the bryozoan hosts has been used effectively within vaccine trials of the current study. Future experiments should ascertain if the established methodology would be successful in infecting other salmonid fish. The use of electronic PIT tags within the trials allowed the tracking of individual fish and proved highly useful in identifying their vaccination status while allowing them to be pooled together for the challenge protocol, thus standardising exposure. Unfortunately, the challenge methodology was not established in advance of the drug trials being conducted. Use of such an exposure protocol may have helped to accentuate any efficacious properties of the compounds examined, which might have been masked by the range in the stage of infection seen within the subjects which participated in the non-parametric distribution of parasite burden data. If also shown to be useful against PKD, the efficacy of salinomycin against *I. multifiliis* infection (Shinn *et al.* 2003) would make it highly attractive for use on trout farms that suffer from both PKD and ichthyophthiriasis. However, both palatability and regulatory considerations may curtail further research into its use. The challenge method which was developed in the current trial should represent a powerful tool in future efficacy testing of potential vaccines and chemotherapeutants.

By comparing levels of mortality between different treatment groups, the calculation of relative percent survival has previously been used in efficacy testing of PKD prevention and treatment protocols (le Gouvello *et al.* 1999; Petchsupa 2002). However, mortality levels during PKD outbreaks on farms are related to extrinsic factors such as the presence of secondary pathogens and stress factors (Bucke *et al.* 1981). The facility within which the current vaccine and drug trials were conducted (the ARF) is a specified pathogen free (SPF) environment with high standards of animal husbandry minimising stress factors. Therefore, PKD-affected fish show nominal levels of mortality within the ARF despite potentially high parasite burdens.

To assess the level of infection, *T. bryosalmonae* counts in kidney sections were measured as described by Higgins and Kent (1998) in their trial of TNP-470 in sockeye salmon. Morris *et al*. (2003a) used a similar methodology in their efficacy trials of TNP-470 in rainbow trout which were also conducted in the ARF. However, such a method would merely give an estimate of overall parasite load as only a 5 µm section of tissue from each fish was actually analysed. Future research could examine the feasibility of developing novel quantitative assays, perhaps using real-time PCR, as has been employed successfully in malaria vaccine efficacy testing (Witney, Doolan, Anthony, Weiss, Hoffman and Carucci 2001). However, this method would still only analyse a relatively small quantity of tissue, so rigorous standardisation of the sampling protocol would be necessary to ensure a consistent representation of parasite burden between subjects. Nevertheless, as PKD outbreaks on farms are multifactorial in causation, any experimental trial of a PKD control method conducted in a SPF facility would have to be followed by extensive on-farm field trials. This requirement was exemplified by the study of TNP-470 for treating rainbow trout (Morris *et al.* 2003a). Although it was exquisitely successful in lowering parasite burdens within experimental SPF aquarium systems, it was discovered through field trials to compromise the ability of fish to combat secondary opportunistic infections, and thus offered limited commercial potential.

There are many exciting facets to the future of research into PKD. The bryozoan culture system and challenge method developed herein can hopefully be implemented in future experiments. Elucidation of the life cycle of *T. bryosalmonae* by demonstrating the route of infection to bryozoans would be crucial in understanding the epidemiology of PKD. Morphological and developmental studies of malacosporeans should further elucidate their relationship to each other as well as to other myxozoans. Further insights into the role of PKD within wild populations of salmonids, and its impact on salmon farming may become prioritised, especially if

average annual water temperatures are seen to rise. Mitigation of the impacts of PKD may finally lie in the development and examination of promising potential vaccine candidates to provide a long-term solution to this most damaging of disease of salmonid fish.

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Appendix 1: Culture media

Chalkley's medium

NaCl	100 mg L^{-1}
KCl	4 mg L^{-1}
$CaCl_2$	6 mg L^{-1}

Constituents dissolved in distilled water

Jaworski's medium

$Ca(NO_3)_2.4H_2O$	20 mg L^{-1}
KH_2PO_4	12.4 mg L^{-1}
$MgSO_4.7H_2O$	50 mg L^{-1}
$NaHCO_3$	15.9 mg L^{-1}
EDTA FeNa	2.25 mg L^{-1}
EDTA Na_2	2.25 mg L^{-1}
H_3BO_3	2.48 mg L^{-1}
$MnCl_2.4H_2O$	1.39 mg L^{-1}
$(NH_4)_6Mo_7O_{24}.4H_2O$	1 mg L^{-1}
Cyanocobalamin	0.04 mg L^{-1}
Thiamine HCl	0.04 mg L^{-1}
Biotin	0.04 mg L^{-1}
$NaNO_3$	80 mg L^{-1}
$Na_2HPO_4.2H_2O$	17.8 mg L^{-1}

Tryptone soya agar

Tryptone	15.0 g L^{-1}
Soya peptone	5.0 g L^{-1}
Sodium chloride	5.0 g L^{-1}
Agar	15.0 g L^{-1}

Tryptone soya broth

Pancreatic digest of casein	17.0 g L^{-1}
Papaic digest of soybean meal	3.0 g L^{-1}
Sodium chloride	5.0 g L^{-1}
Di-basic potassium phosphate	2.5 g L^{-1}
Glucose	2.5 g L^{-1}

Appendix 2: Buffers

Phosphate buffered saline (PBS, pH 7.2)

0.02M Phosphate; 0.15M NaCl

$NaH_2PO_4.2H_2O$	0.876 g L^{-1}
$Na_2HPO_4.2H_2O$	2.56 g L^{-1}
NaCl	8.77 g L^{-1}
5M NaOH	used to adjust pH

Immunohistochemistry buffers

Tris buffered saline (TBS)

Trisma base	2.42 g L^{-1}
NaCl	29.24 g L^{-1}
Adjust pH to 7.6 using 12M HCl	

3,3'-diaminobenzidinetetrahydrochloride (DAB) substrate

Dissolve 1 tablet of DAB (Sigma) in 6.67 ml TBS.
Place 0.5 ml aliquots of the solution into bijoux bottles, store at -20°C.
For use: add 5 ml of TBS and 100 µl of 1% hydrogen peroxide to 0.5 ml aliquot

Electrophoresis buffers and gels

Separating gel buffer (pH 8.7)

1.5 M Tris; 0.4% (w/v) Sodium dodecyl sulphate (SDS)

Trisma base	182 g L^{-1}
SDS	4 g L^{-1}
12M HCl	used to adjust pH

Stacking gel buffer (pH 6.8)

0.5 M Tris; 0.4% (w/v) SDS

Trisma base	60.5 g L^{-1}
SDS	4 g L^{-1}
12M HCl	used to adjust pH

Sample buffer *(x5)*

0.5M Tris-HCl pH 6.8	2.5 ml
Glycerol	2.0 ml
SDS (10% w/v)	4.0 ml
Dithiothreitol (DTT)	0.31 g
Bromophenol blue	2.0 mg
Distilled water	to 19 ml

Reservoir buffer *(x5)* (pH 8.3)

Tris	15 g L^{-1}
Glycine	43.2 g L^{-1}
SDS	5 g L^{-1}
12M HCl	used to adjust pH

Acrylamide stock solution

30% (w/v) Acrylamide; 0.8% (w/v) Bis-acrylamide
Stored in darkness at 4˚C.

Separating gel (12% Acrylamide)

Separating gel buffer	5 ml
Distilled H_2O	7 ml
Acrylamide	8 ml
N,N,N,N-tetramethylethylenediamine (TEMED)	0.015 ml
10% Ammonium persulphate	0.07 ml

Stacking gel

Stacking Gel Buffer	2.5 ml
Distilled H_2O	6.1 ml
Acrylamide	1.34 ml
TEMED	10 µl
10% Ammonium persulphate	50 µl

Coomassie blue solutions

Coomassie blue stain

0.25% (w/v) Coomassie brilliant blue R-250
50% (v/v) methanol
10% (v/v) acetic acid

Coomassie blue de-stain

40% (v/v) methanol; 10% (v/v) acetic acid

Western blot buffers

TBS with Tween (TTBS, pH 7.5)

Tween 20	0.5 ml added L^{-1} of TBS

Transblot buffer (pH 8.3)

Glycine	14.4 g L^{-1}
Tris base	3.03 g L^{-1}
Methanol	200 ml L^{-1}
Distilled H_2O	800 ml L^{-1}

Stock substrate solution *(x 10)*

4-chloro-naphthol	0.15 g
Methanol	50 ml

Stored in darkness at -20°C.

Appendix 3: Instructions for DVD and CD ROM

This appendix comprises video files included on a digital versatile disc (DVD) and compact disc (CD).

The CD should be readable via a PC; the DVD should be playable with a standard DVD player or with a PC DVD drive.

The CD ROM also includes a digital copy of the thesis text as a .pdf file.

THE SAME VIDEO MATERIAL IS CONTAINED ON BOTH THE CD and DVD (DVD offering higher resolution).

If using a DVD player, press the 'menu' button to access the menu screen.

For optimum performance if using the CD, please copy the video files (*.avi) to your computer's local hard drive (normally denoted as C:\) before playing them.

The CD videos contain audio commentary which requires an active sound card.

The CD videos should be compatible with any current computer media player; if problems result then updating the software may help (links are available in the "Media resources" folder). Troubleshooting recommendations can be found within the file "READ ME.txt" on the CD.

Appendix 4: Presentations and publications from the project

McGurk C., Morris D.J. and Adams A. (2003) The relationship between PKD & Bryozoa: an inside story. Oral Presentation, *British Trout Farming Conference,* 3-5 September 2003, Sparsholt College, England.

McGurk C., Morris D.J. and Adams A. (2003) The development of *Buddenbrockia* sp. in Bryozoa with comparison to *Tetracapsuloides bryosalmonae*, the cause of salmonid proliferative kidney disease. Oral presentation, *European Association of Fish Pathologists - 11th International Conference: "Diseases of Fish & Shellfish",* 21-26 September 2003, Malta. Book of Abstracts p. O-10.

McGurk C. (2003) Developmental stages of myxozoan parasites in freshwater bryozoans, studied by light microscopy & transmission electron microscopy. Oral Presentation, *Departmental Seminars,* 8 October 2003, Institute of Aquaculture, University of Stirling, Scotland.

McGurk C., Morris D.J. and Adams A. (2003) The development of myxozoan parasites in freshwater bryozoans, with reference to salmonid proliferative kidney disease. Oral Presentation, *Alpharma Aquaculture Conference,* 14 October 2003, Inverness, Scotland.

McGurk C. (2003) Development of prevention or control measures against salmonid proliferative kidney disease. *European Network for the dissemination of Aquaculture RTD information*, Aquaflow Technical Leaflet TL2003-UK-003. http://www.aquaflow.org/home/showtl.asp?aunid=4272&lg=en.

McGurk C., Morris D.J. and Adams A. (2004) Microscopic studies of the link between PKD & bryozoans. Oral Presentation, *Scientific Meeting of the Fish Veterinary Society,* 19 May 2004, SECC, Glasgow, Scotland.

McGurk C., Morris D.J., Bron J.E. and Adams A. (2004) A study of the bryozoan spore stages of *Tetracapsuloides bryosalmonae*, the causative agent of salmonid proliferative kidney disease, using confocal microscopy techniques. Poster presentation, *Conference of the European Aquaculture Society - Aquaculture Europe '04: "Biotechnologies for Quality",* 20-23 October 2004, Barcelona, Spain. Book of Extended Abstracts and Short Communications pp. 550-551.

McGurk C. (2004) Proliferative kidney disease. *Intervet Aquatic Animal Health Newsletters* **9,** 7-9. http://www.intervet.com/aah/newsletters.asp.

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