Polycell-gel assay of water for spores of Saprolegniaceae (fungi), especially those of the *Saprolegnia* pathogen of fish

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Abstract

Large aliquot water samples (30 ml) were enriched with nutrients, then gelled in the cold with Polycell (wallpapering paste) and incubated to derive Saprolegniaceae colonies and hence deduced spore counts, from water collections of about 1 litre. The *Saprolegnia* pathogen of fish was a component of the total recoveries; it was recognised on the basis of its secondary zoospore cyst ornamentation. Windermere water entering The Ferry House fish hatchery gave spore assays of 2 to 28 l^{-1} for the pathogen, a significant component of total *Saprolegnia*, which was 6–73 spores per litre.

Introduction

In the years 1964–69 thousands of Atlantic salmon *Salmo salar* L. and sea trout *Salmo trutta* L., migrating back from the sea to spawn in fresh water, died in rivers in the north and west of the British Isles as a result of 'salmon disease', also termed ulcerative dermal necrosis (UDN). Brown trout *Salmo trutta* L. resident in fresh water were also affected. UDN has also occurred in mainland Europe and despite much study the disease is still not fully understood. Early stages feature circular blanched areas on the skin of the fish, up to several centimetres across and clearly visible to the naked eye. Apparently the fish cannot produce its protective external mucus in these areas, the skin epidermis then loses its integrity and destruction of the dermal melanophores soon follows (Roberts *et al*, 1970; Willoughby, 1972; Johansson *et al*, 1982). At this stage no pathogen is implicated, although bacteria and viruses have been repeatedly looked for. It is even possible that a chemical irritant, spreading from a point centre, is responsible. If *Saprolegnia* fungus does not intervene in the affected areas of the skin the fish can apparently recover. However, the recognition of the disease is based largely on the external fungal infection, which is very obvious and eventually lethal (Stuart & Fuller, 1968; Willoughby, 1968). Local opinion is that in some rivers in the north of England, e.g., the River Eden, there has been 'recovery' from UDN, while in others, e.g. the River Kent, this diseased condition of migrating salmonids still persists. Given a rapid and reliable assay method for spores of the *Saprolegnia* pathogen in water, comparative investigations of the infective loading in such rivers could be initiated.

At The Ferry House there are now very substantial outdoor facilities (termed the hatchery) for rearing and maintaining fish. The water supply, 1–2 million gallons of untreated water per day, is derived from Windermere which is immediately adjacent; it is pumped to a large holding tank and then enters each pond via a separate inflow pipe. Although the classical initial signs of UDN have never been seen in our hatchery fish (predominantly brown trout) these are vulnerable to *Saprolegnia* infection, especially in the autumn and winter spawning season. Malachite green fungicide is flushed through as a preventative at this time. It was

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logical therefore to initiate the spore assay development at The Ferry House.

In previous work on Saprolegniaceae spore assay (Willoughby, 1962; Willoughby & Collins, 1966; Hallett & Dick, 1981) determinations of the yields were mostly made to genera only. This is not satisfactory in investigations of fish pathology. When salmonid fish have been infected the sole fungi isolated have been particular strains of *Saprolegnia* which are characterised by groups of long, hooked hairs on the secondary zoospore cyst (Pickering *et al,* 1979). These strains have either remained sexually sterile over a wide range of temperature (*Saprolegnia* sp.) or have produced sparse elongated oogonia at 7 °C (but not at 20 °C or above), in which case they were identified as Type 1 strains of *S. diclina* Humphrey (syn *S.parasitica* Coker) (Willoughby, 1978). The two kinds of strains have been collectively termed by us the *Saprolegnia* 'salmonid pathogen'. The fact that diseased perch *Perca fluviatilis* L. in Windermere could become infected with *Achlya, Aphanomyces, Leptolegnia, Leptomitus* and *Pythiopsis* as well as *Saprolegnia* (Bucke *et al,* 1979) had biased us into thinking that coarse fish infections always had different features. It now appears that perch disease in Windermere was a very exceptional, perhaps unique case. The *Saprolegnia* pathogen alone has now been recovered by us from several other coarse fish, even including the eel *Anguilla anguilla* L. (Willoughby & Copland, in press). It now seems realistic therefore to up-date our working terminology and refer to it as the *Saprolegnia* 'fish pathogen' a terminology used in this present contribution. No species of *Saprolegnia* other than *S. diclina* is known to produce groups of long, hooked hairs on the secondary zoospore cyst. Thus, as it stands at present, *Saprolegnia* isolates from water with this character can be taken to represent the most important *Saprolegnia* pathogens of fish. In assaying for these it is not obligatory to evoke the sexual state in those strains capable of producing it, in order to produce a specific identification. Consequently the assay need not be conducted at low temperature. This is borne in mind in the present contribution. Type 2 strains of *S. diclina,* producing oogonia at 7 °C only, have single, short, hooked hairs on the secondary zoospore cyst (Pickering *et al,* 1979). These strains are known to be involved in the complex fungal infections of perch in Windermere (see above). However, in this current contribution such strains, possibly of minor importance in the wider context of fish pathology, have not been specifically looked for in water assays and are not included in the totals of fish pathogen obtained.

Type 3 strain of *S. diclina,* producing prolific, spherical oogonia at both 7 °C and 20 °C are not, in our experience, involved in fungal infection of either salmonid or coarse fish (Willoughby, 1978). These strains again have only single, short, hooked hairs on the secondary zoospore cyst (Pickering *et al,* 1979).

Previous methods for Saprolegniaceae assay involve one or more steps which may be destructive of spores. Water samples have been incorporated into warm nutrient agar, either directly (Willoughby, 1962; Willoughby & Collins, 1966) or following centrifugation (Hallett & Dick, 1981). On the assumption that incorporated spores give rise to colonies on incubation these have been counted and then manipulated for identification. If potentially destructive steps are evaded, by plating out water samples onto solidified nutrient agar, then enormous replication is required to assay a large volume of water. This current method has no step which is obviously potentially destructive of spores; furthermore a large volume of water can be assayed easily.

Materials and methods

The assay method is based on a domestic observation (L.G.W.) that Polycell (regular, wallpaper paste, manufactured by Polycell Products Limited, 30 Broadwater Road, Welwyn Garden City, Herts, England), made up for wallpapering and then left for some weeks, showed mould colonies within it. These moulds had obviously developed from spores in the added tap water and in the atmosphere. This suggested that a water sample for Saprolegniaceae assay might be converted directly into a gel by the addition of Polycell in the cold and that distinct, separate colonies, derived from viable spores in the water, would develop within it. In using Polycell for spore assay the products which contain added fungicides (clearly stated on the package labels) are obviously to be avoided.

The spore assay is made as follows. The constituents of glucose-peptone broth are prepared in two separate flasks and each is sterilized by steam auto-

claving (20 min at 103.5 kPa). The first flask contains 0.18 g ml^{-1} glucose; the second flask contains g ml^{-1}: peptone 0.06, $MgSO_4 \cdot 7 H_2O$ 7.6 × 10^{-3}, KH_2PO_4 8 × 10^{-4} together with trace micronutrients (mg l^{-1}: Ca 24, Fe 1.5, Mn 1.5, Cu 0.3 and Zn 0.3). Immediately prior to the assay the contents of the two flasks, each of 50 ml, are mixed. Separate autoclaving of the glucose ensures that it is not caramelized excessively in the sterilization. An antibiotic mixture is also prepared. This contains g ml^{-1} sodium benzylpenicillin, 7.5 × 10^{-3}, streptomycin sulphate 7.5 × 10^{-3}.

The water for assay is collected in a sterile 1 l stoppered glass bottle, which is almost completely filled at the site. On return to the laboratory 30 ml aliquots of the water are dispensed into sterile polystyrene Petri dishes, 9 cm diam, using a sterile measuring cylinder. Between each dispensation the bottle is re-stoppered and inverted once; this is aimed at ensuring that the negatively geotropic behaviour of any included zoospores does not lead to their non-random distribution in the dishes. The whole of the water sample is used and a note is made of the volume in the final dish, which may contain less than 30 ml. Addition of 1 ml of the glucose-peptone broth per dish results in a final concentration which is the same as that previously recommended for the growth of *Saprolegnia* (Willoughby & Pickering, 1977). Addition of 1 ml of the antibiotic mixture per dish results in a final concentration which is now used routinely (in glucose-peptone agar) for isolating *Saprolegnia* from diseased fish, i.e. g l^{-1}: Sodium benzylpenicillin 0.25, streptomycin sulphate 0.25. Approximately 0.09 g of Polycell granules are then sprinkled on the surface of each sample aliquot and after a few seconds the dishes are gently rotated by hand on the bench to ensure thorough mixture of the gel which has formed. At this level of addition the gel is semi-solid only.

Incubation of Polycell-gel dishes was always at 20 °C for 72 h in initial assays but experience has shown that this temperature is not critical and there can be some flexibility to suit the investigator. For example, assays running at day room temperature of 22–25 °C can be harvested at 48 h and assays can also be left at room temperature for 24 h and then transferred to 5 °C incubators for several days to slow down colony growth. Incubation is completed when the largest colonies have diameters of 40–50 mm. All colonies are examined in the dishes at low magnification and fungi with fine septate mycelium or with the aseptate and richly-branched mycelium typical of *Mucor* are not considered further. All other colonies with aseptate mycelium are transferred complete, using scissors to free them if they are contiguous, to separate polystyrene Petri dishes containing approx. 20 ml of paper-filtered, steam-autoclaved (20 min at 103.5 kPa) Windermere water (SL). Experience showed that *Aphanomyces* colonies could generally be distinguished in the Polycell-gel dishes because of their small size (mostly 5–10 mm diam, 72 h at 20 °C) and mycelium of a more uniform and narrow diameter, 5–10 μm, than that in *Saprolegnia,* where it measures 8–21 μm. When *Aphanomyces* is very numerous it is convenient to transfer to SL only those larger presumptive colonies for which the identification might be in doubt. Almost without exception these prove to be *Aphanomyces. Saprolegnia* (including the fish pathogen and 'filter paper' strains, see below) and *Leptolegnia* colonies for transfer to SL are usually large while *Achlya* colonies are often smaller (Table 1).

In the initial stages of the work sterilized hemp seeds were added to transferred colonies to induce sporangium and possible oogonium formation. This was later found to be unnecessary. The transferred colonies in SL alone at room temperature usually have swollen sporangial apices within a few hours if they are of *Saprolegnia;* the exceptions are discussed below. On the other hand *Achlya* and *Leptolegnia* colonies are largely inert at this stage. Dehiscence of *Saprolegnia* sporangia is usually prolific overnight and during the next day but it then declines and oogonia or chlamydospores begin to form. Generic identification is based on the nature of the mycelial branching and the sporangial dehiscence. Specific identification of *Saprolegnia* colonies is based traditionally on characteristics of the oogonia (Seymour, 1970). In this work these characteristics distinguish *S. ferax* (Gruith.) Thuret, *S. hypogyna* (Prings.) de Bary and *S. diclina* Type 3. The latter gives colonies which are very strongly sexual and bear predominantly spherical oogonia.

In the assay for the Saprolegniaceae, *Achlya* usually produces a reasonable number of dehisced sporangia in the SL dishes but *Leptolegnia* is more difficult. Dehiscence, when it occurs, is always

Table 1. Combined figures obtained from two preliminary hatchery inflow water assays, both of 990 ml. Following incubation in Polycell-gel for 3 days at 20 °C individual colonies were measured and then transferred to SL, to induce sporangium and oogonium formation. The counts of *Aphanomyces* are incomplete since many presumptive colonies were eliminated by inspection in the Polycell-gel assay dishes.

Colony diam (mm)	Saprolegniaceae identifications							
	Achlya	Aphanomyces	Leptolegnia	S. diclina Type 3	S. ferax	S. hypogyna	Saprolegnia sp.	Unidentified
2		1						
3	1	1						1
5	14	9	1					
7	12							
8	9							
9	9	1						
10	32	5						
12	4							
13	3	1						
15	24	2	3					2
18	7	1					1	
20	6	1	9				4	3
25	1		6		2	2	3	
30			8		1		3	
35			5				4	
40			2	1			3	
Totals	122	22	34	1	3	2	18	6

sparse and it may not be observed at all. However, the long, largely unbranched hyphae of fairly uniform diameter (8–11 μm) are quite characteristic.

Identification of the fish pathogen

Light microscopy

The zoospore cysts of *Saprolegnia* colonies in SL, at 20 °C or at room temperature, which are not identified to species on oogonial characters within a few days are examined by light microscopy. The *Saprolegnia* colony, together with its SL is poured into another Petri dish and previously flamed coverslips are laid on the water film left behind. Examination is made with a ×40 negative phase (Anoptral) objective and particular attention is given to the empty cases of zoospore cysts. Experience with the electron microscope has suggested that ornamentation is especially obvious where indirect germination (Willoughby *et al*, 1983) or repeated emergence of secondary zoospores has occurred. For strains which are considered to be of the fish pathogen, long fine 'hairs' are observed (Fig. 18 & 19); each 'hair' represents a bundle of long, hooked hairs (see below). This interpretation was checked and confirmed by examination of reference cultures previously studied with the electron microscope (Pickering *et al*, 1979). If the material in the SL dishes has very few empty cysts the coverslips are carefully removed and the whole colony and its water replaced. Another examination can be made the next day. However, with the passage of time and repeated handling of open dishes, bacterial growth inevitably occurs and this eventually prevents fruitful examination.

Electron microscopy

The zoospore cysts of *Saprolegnia* colonies which are not identified to species on oogonial characteristics are also examined by transmission electron microscopy. Carbon-stabilized, formvar-coated, copper grids are placed, carbon side uppermost, on the bottoms of the dishes of SL which contain the colonies. These additions are made just at the stage when swollen sporangial apices have distinguished presumptive *Saprolegnia* colonies (see above). Zoospores are released from the sporangia over the next 24 h, at the end of which period

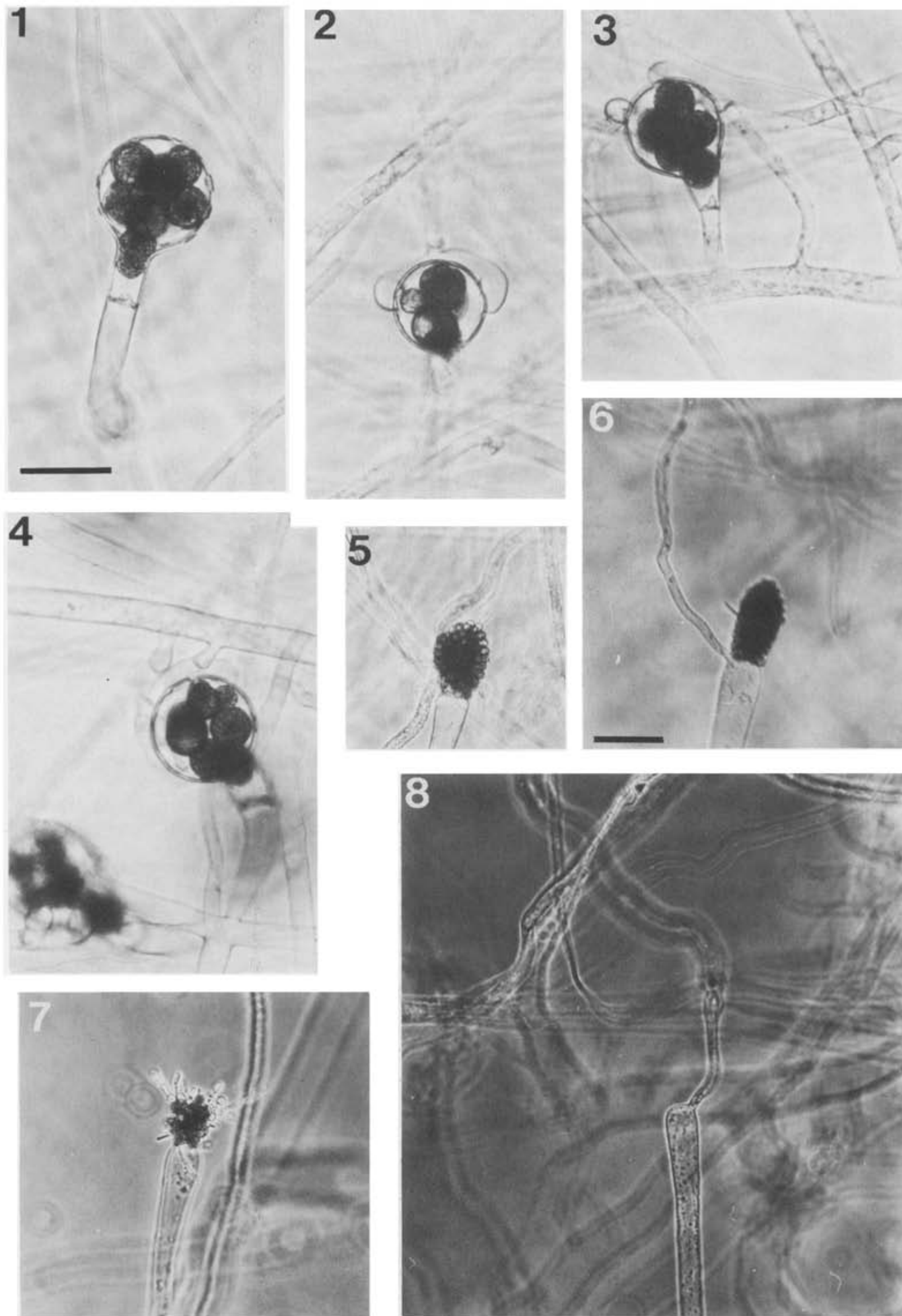

Plate I. Figs. 1–4, *Saprolegnia ferax* from Windermere lake centre surface water assay (Table 3) showing antheridia on a single colony which are, Fig. 1, absent; Figs. 2, 4, diclinous; Fig. 3, monoclinous. Figs. 5–8, *Thraustotheca* sp. from Smooth Beck water assay (Table 3) showing sporangia which are, Figs. 5, 6, mature; Fig. 7, with zoospore germination in situ; Fig. 8, dehisced. Sympodial branching from below in Figs. 6, 8. Scale lines or equivalents: 1–4; 50 μm: 5–8; 100 μm.

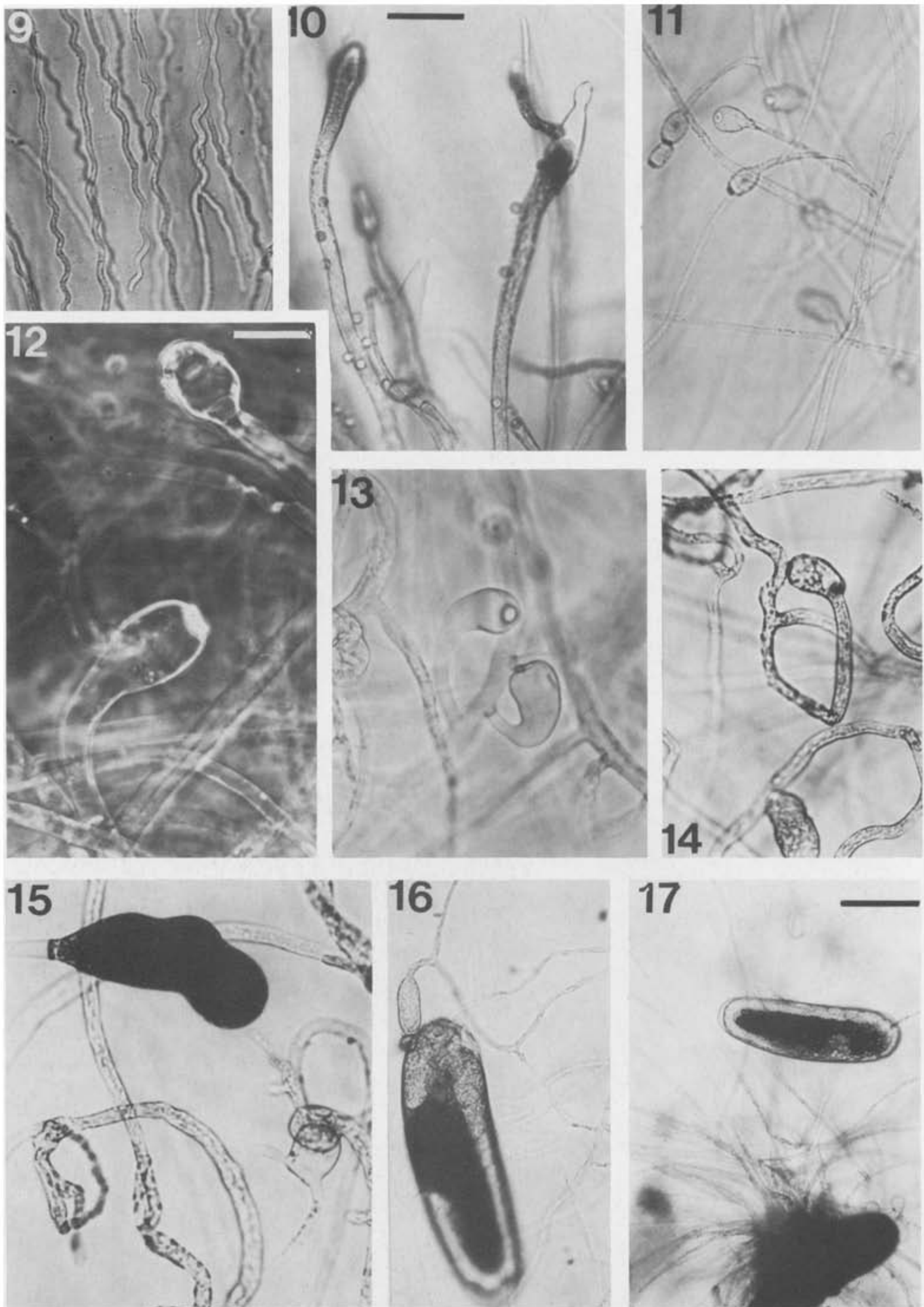

Plate II. Saprolegnia 'filter paper' colonies from various water assays. Mycelium in: Fig. 9, GP agar; Figs. 10–15, water alone; Figs. 16, 17, water plus chironomid eggs. Fig. 10: Dehisced sporangia and proliferation. Figs. 11–14, 16: 'Appressoria'. Fig. 15: Chlamydospore. Note adjacent colonized and uncolonized chironomid eggs in Fig. 17. Scale lines or equivalents: 9, 11, 16, 17; 200 μm: 10, 13–15; 100 μm: 12; 50 μm.

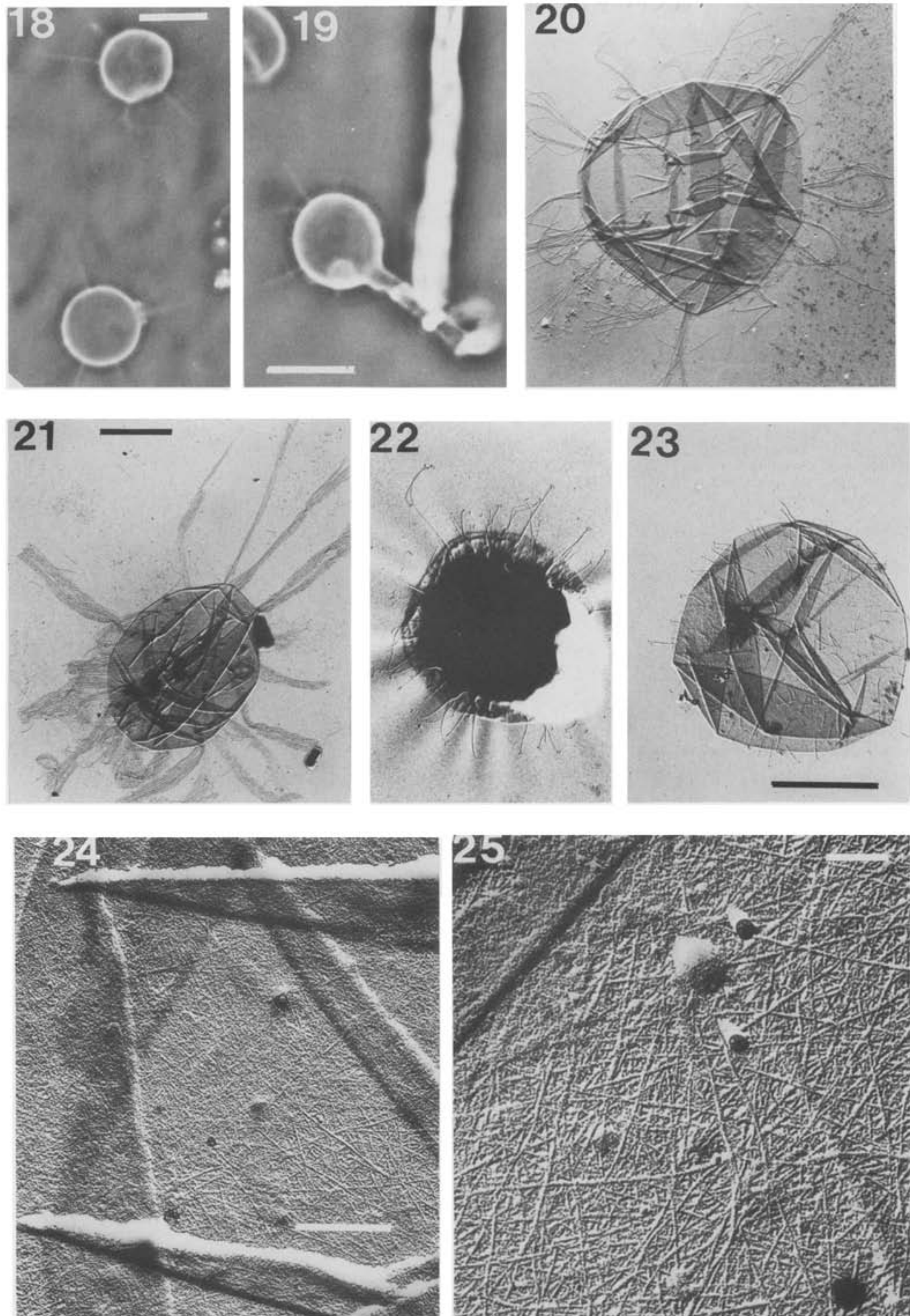

Plate III. Surfaces of secondary zoospore cysts of *Saprolegnia* from various water assays. Figs. 18, 19, using light microscopy; Figs. 20–25, using electron microscopy. Diagnoses: Figs. 18, 19 (zoospore cyst germinated), 20, 21: Fish pathogen. Fig. 22: Possible *S. hypogyna*, Fig. 23: Possible *S. ferax*. Figs. 24, 25: *Saprolegnia* 'filter paper'. Note dense amorphous layer in Fig. 24 (left) and Fig. 25 (top left) apparently eroding away from a fibrillar layer below. Scale lines or equivalents: 18; 10 μm: 19; 10 μm; 20, 22, 23; 5 μm: 21; 5 μm: 24; 500 nm: 25; 200 nm.

the grids are removed, dried down on filter paper and stored. Grids which had been placed in dishes containing *Saprolegnia* colonies which went on to produce oogonia of *S. ferax, S. hypogyna* or *S. diclina* Type 3 are not processed further. The remaining grids are shadowed with gold/palladium and examined with a Corinth 500 transmission electron microscope. The presence of groups of long hooked hairs on the secondary zoospore cyst cases is taken as diagnostic for the fish pathogen (Figs. 20 & 21). Where only numerous, single, hooked hairs are present it is inferred that the fungus, although it is asexual under the conditions used, is not the fish pathogen (Figs. 22 & 23). Attempts were made to alter the assay conditions so that such strains could be induced to form oogonia in the SL dishes and hence be eliminated from consideration with the electron microscope, which is very time consuming. These attempts are discussed below.

When the same colonies were examined for the fish pathogen by both light and electron microscopy there was never any disagreement over positive identifications. However, the electron microscope distinguished a low percentage (about 8%) of positive identifications which had been missed with the light microscope.

Results

In preliminary assays of hatchery inflow water (derived from Windermere, see above) *Saprolegnia ferax* was recovered only rarely from the standard assay. This was unexpected since *S. ferax* is a common species in local waters, readily obtained from them using hemp seed baits. The question arose whether, in this species, the nutrient food base stored in the colonies was always sufficient to induce oogonium formation following transfer to SL. Clearly it is desirable to make as many specific determinations as possible, to reduce the number of strains which must be examined as potential fish pathogens for the secondary zoospore cyst character. A general exploration of this aspect was therefore made using zoospores from cultures (Table 2).

Of the cultures selected for use the first four, i.e. TP 41 (ATCC 42062), 848, 824 and A/17/82 had the E.M. characteristics (groups of long, hooked hairs) of the *Saprolegnia* pathogen (Table 2). Isolates A/10/82 and A/24/82 had the E.M. characteristics of *Saprolegnia* 'filter paper' strains (see below). Zoospores from cultures were placed in 30 ml SL together with the glucose-peptone nutrient addition at the standard (×1) or at higher levels. The usual antibiotic and Polycell additions were also made and following incubation for 72 h at

Table 2. Oogonia and chlamydospores produced on *Saprolegnia* colonies in water at two different temperatures, after transfer from Polycell-gel plus varying levels of nutrients.

			20 °C			7 °C		
Isolate	Origin	Identity	×1	×3	×10	×2	×4	
TP41	Trout	S. diclina	A–	A–	A–	A–	A–	Oogonia
(ATCC 42062)		Type 1	A–	A–	A–	SC	PC	Chlamydospores
848	Char	S. diclina	A–	A–	A–	NC	NC	Oogonia
		Type 1	A–	A–	NC	A–	NC	Chlamydospores
824	Char	Saprolegnia sp.	A–	A–	A–	A–	A–	Oogonia
		fish pathogen	A–	A–	SC	A–	PC	Chlamydospores
A/17/82	Water	Saprolegnia sp.	A–	A–	A–	A–	A–	Oogonia
		fish pathogen	A–	PC	PC	A–	PC	Chlamydospores
OG2A1(5)	Water	S. ferax	A–	SC	NF	SCF	NF	Oogonia
(ATCC 36146)			SC	PC	PC	PC	PC	Chlamydospores
805	Water	S. hypogyna	SC	PCF	PCF	PCF	PCF	Oogonia
			A–	A–	A–	A–	A–	Chlamydospores
A/10/82	Water	Saprolegnia sp.	A–	A–	A–	A–	A–	Oogonia
		'filter paper'	A–	A–	SC	NC	SC	Chlamydospores
A/24/82	Water	Saprolegnia sp.	N.D.	A–	A–	A–	A–	Oogonia
		'filter paper'	N.D.	SC	SC	SC	SC	Chlamydospores

A, absent; C, produced at colony centre; F, produced at colony fringe; S, sparse; N, fairly numerous; P, prolific; N.D., not determined.

20 °C colonies were transferred to separate Petri dishes of SL at 20 °C or at 7 °C. The following day they were examined for dehiscence of sporangia. Dehiscence was best from the ×1 colonies and worst, or even absent, from the ×10 colonies. Pre-growth of the colonies at increasing nutrient levels clearly affected other aspects of their performance following transference to water. At the ×1 nutrient level small growth fringes only were produced by the transferred colonies. However, at the ×2 level and above growth fringes of increasing amount were produced. At the ×10 level growth fringes were very considerable and tended to fill the whole dish. Again, another effect of the increasing food base reservoir carried over in the mycelium was to enhance subsequent chlamydospore or oogonium production (Table 2). In isolated A/10/82 and A/17/82 this reservoir was channelled into chlamydospore production. In the *S. hypogyna* isolate it was channelled into oogonium production. In the *S. ferax* isolate it was channelled into both chlamydospore and oogonium production. There was some variation in the location of these reproductive cells. In *S. ferax* for example, at the ×3 nutrient level, sparse oogonia were present at the colony centre; at the ×10 nutrient level fairly numerous oogonia were present at the colony fringe only. *Saprolegnia diclina*, isolate 848, showed induction of oogonia at low temperature in a pathogenic isolate, as previously described (Willoughby, 1978).

In general, therefore, addition of higher levels of nutrients in the Polycell-gel assay may have advantages in enhancing the prospect of oogonium recovery and full specific identification but on the other hand it is clearly essential not to jeopardise the prospects of obtaining dehiscence, in order to make the initial generic separations. As a compromise nutrient addition at the ×2 level was tested in a hatchery inflow water assay. In this assay difficulty in observing dehiscences and zoospores was experienced immediately and in addition bacteria were more troublesome. The option of enhanced nutrient addition was therefore abandoned. The position of *S. ferax* in the standard assay is thought to be as follows. Judging from the morphological variability of *S. ferax* in regard to sexuality, with purely parthenogenetic strains, monoclinous strains and even strains showing a range of sexual performance in a single colony (Figs. 1–4), it seems likely that there is also considerable physiological variability. This would explain why some *S. ferax* strains will be distinguished as such in the SL dishes while others will go through as *Saprolegnia* sp. for secondary zoospore cyst examination. Here they

Table 3. Polycell-gel assays of Saprolegniaceae made on seven separate dates in the spring and summer of 1982 for hatchery inflow water and one date only for the other two waters. Hatchery inflow assay 7 was made with ×2 nutrients (see text). In assays 1 and 2 *Saprolegnia* was not determined to species (N.D.).

	Hatchery inflow							Windermere, lake centre, surface	Smooth Beck
	1	2	3	4	5	6	7		
Dates	19.3.82	5.4.82	9.7.82	12.7.82	19.7.82	30.7.82	17.8.82	14.7.82	23.7.82
Achlya	0	1	1	0	0	1	2	0	2
Aphanomyces	45	12	5	8	3	3	8	1	0
Leptolegnia	1	2	6	2	3	0	6	1	2
Pythiopsis	1	0	0	0	0	0	0	0	0
Saprolegnia diclina Type 3	N.D.	N.D.	0	0	0	0	0	0	35
Saprolegnia ferax	N.D.	N.D.	0	0	0	1	0	1	0
Saprolegnia hypogyna	N.D.	N.D.	22	7	29	19	1	3	0
Saprolegnia sp. – fish pathogen	N.D.	N.D.	4	3	26	28	2	1	0
Saprolegnia sp. – 'filter paper'	N.D.	N.D.	0	1	0	2	3	1	4
Saprolegnia sp.	9	5	5	5	15	13	26	5	83
Thraustotheca	0	0	0	0	0	0	0	0	1
Unidentified	0	0	0	0	0	0	0	0	5
Volume Sampled (ml)	540	831	1050	1020	960	1000	960	1020	990

will show the single, short, hooked hairs typical of the species (Fig. 23).

Colonies named as *S. hypogyna* on their oogonial structure were well represented in the standard assays (Table 3). However, the secondary zoospore cyst ornamentation typical of this species, i.e. single, long, hooked hairs (Pickering *et al,* 1979) was seen for several colonies which were asexual only in the assay dishes (Fig. 22). It seems likely therefore that *S. hypogyna* also, but to a lesser extent than *S. ferax,* is not fully accounted for in these assays.

In preliminary assays of hatchery inflow water the main objective was to survey and distinguish the forms encountered rather than to make accurate counts. This preliminary work suggested that the *Achlya* and *Aphanomyces* loadings varied widely and could be very heavy (see Tables 1, 3) and drew attention to an unusual *Saprolegnia,* possibly previously undescribed. This also was often very numerous. The morphological characters of this *Saprolegnia* are largely negative. In pure culture, on hemp seeds, few sporangia and zoospores are produced at 20 °C but more at 7 °C. No oogonium has been seen at either temperature but there are peculiar inflations of the mycelium which are apparently not developing chlamydospores (Figs. 11–14, 16). These are often suggestive of appressoria, especially when they are borne on the tips of recurved branches which make contact with their own mycelium (Fig. 14). The 'appressorium' apex often has a circular ring of thickening (Figs. 11–14). Under the electron microscope (>30 strains were examined) the secondary zoospore cysts have no spines of any kind. There is a dense, amorphous outer layer, readily eroding away to reveal a fibrillar layer below (Figs. 24 and 25). From the character of the latter the laboratory designation of 'filter paper' isolates has been made. Since colonies of this fungus were asexual in the SL dishes in the assay they had, at least initially, to be considered as possibly of the fish pathogen. Their small production of sporangia and zoospores, usually nil overnight at room temperature, despite the presence of a large colony, is now taken to be fairly diagnostic. Confirmation follows in the next few days when the 'appressoria' are formed.

By the time the assay was being handled with sufficient confidence to make counts, initially to genera only (Table 3, hatchery inflow 1, 2) and later to species and strain types (Table 3, hatchery inflow 3–7), *Saprolegnia* 'filter paper' isolates were encountered much less frequently (Table 3).

The picture emerging from the hatchery inflow water assays so far, made with the standard (×1) level of nutrient addition in the spring and summer of 1982, is that there seems to be a basic *Saprolegnia* component of about 6–73 spores per litre of which 2–28 are of the fish pathogen (Table 3). This tentative analysis does not include the *Saprolegnia* 'filter paper' component.

At Smooth Beck, a small Lakeland stream, several preliminary assays indicated the presence of a very heavy spore load, especially of *S. diclina* Type 3. In assaying Smooth Beck therefore (Table 3), Polycell-gel incubation was carried out at room temperature overnight, then at 5 °C for 48 h. This made it possible to transfer relatively small colonies to SL, before their growth could result in numerous fusions between them.

Discussion

In the knowledge that most *Pythium* species are heterotrophic for vitamin B_1 (Cantino & Turian, 1959), the assay growth medium does not include this, either alone or in yeast extract. This is a deliberate attempt to exclude *Pythium* from the assays and seems so far to be successful. Attempts to sterilize Polycell without loss of its relevant characteristics have so far proved unsuccessful but controls have suggested that it does not contain propagules of Saprolegniaceae. The active gelling ingredient is probably carboxymethylcellulose but this has not been substituted with success. Bacterial growth in the assay dishes has not always been completely controlled by the antibiotic additions but there is a reluctance to move to higher levels because these may inhibit Saprolegniaceae growth (Beakes & Gay, 1981). Experience has shown that the bacteria in the assay dishes can develop a resistance to penicillin-streptomycin. Therefore to make pure *Saprolegnia* sub-cultures for detailed examination, glucose-peptone-chloramphenicol agar is used. Chloramphenicol is at 200 mg l^{-1}.

No experimental tests of the percentage recovery of viable Saprolegniaceae spores in water, using the Polycell-gel system, have been made. However, the fact that all the main genera and one of the rarer ones (*Thraustotheca,* Table 3; Figs. 5–8) have been

recovered from natural waters suggests that there is no qualitative selectivity and so far as the *Saprolegnia* fish pathogen is concerned there is no obvious reason why the recovery in the Polycell-gel dishes should not be 100% (see Introduction).

The electron microscope method for identification, used on isolates other than those of the 'filter paper' *Saprolegnia,* has the advantage that a positive observation is always made on the secondary zoospore cyst, even though it may be negative for the fish pathogen and only single, hooked hairs are seen. In some of the more difficult isolates success in recording the fish pathogen with the light microscope may depend on the amount of time which can be spent in observation. On the other hand the fact that the assay can be accomplished in situations where only relatively simple apparatus is available has obvious appeal.

In a previous comparative study of the *Saprolegnia* fish pathogen (as defined in this current contribution) it was established that different isolates could have differing temperature ranges for growth and zoospore production. Furthermore, these ranges were often related to the particular kind of fish host sampled. Isolates from salmonid fish could release zoospores at 3.3 °C but not at 29 °C; isolates from coarse fish could release zoospores at 29 °C but not at 3.3 °C (Willoughby & Copland, in press). Although this has been borne in mind in this present investigation no attempt at segregation has been made so far. Working in the temperature range of 20–25 °C it is assumed that pathogenic strains of both kinds will be included in the assay.

In considering an explanation for the large numbers of *Saprolegnia* 'filter paper' isolates obtained in the preliminary assays there is the possibility that the fungus may be growing on materials trapped in 'dead spots' in the hatchery water intake system. This remains to be investigated. The fungus has no connection with fish pathology that we are aware of but it may be a specialized parasite on other fungi or on invertebrate animals. In considering the latter possibility there are certain similarities with *Couchia circumplexa* gen. nov., sp. nov., recently described by Martin (1981) as a parasite on eggs of chironomids. Attempts to infect live chironomid eggs with our own isolates have so far been indecisive. Only some of the chironomid eggs added to isolates in water have been permeated by the fungus while others have hatched out into larvae. This suggests that the permeated eggs were not viable. However, the mode of attachment to chironomid eggs, through the appressorium-like structure, may possibly be significant (Fig. 16).

In side-by-side comparisons following addition of *S. diclina* Type 1 zoospores to glucose-peptone broth, with or without added Polycell, it was clear the colony growth was greatly stimulated in the former condition. This seems largely be due to the physical support, which may make the system valuable in other connections where gentle resuscitation of viable cells is required.

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