3.2.2 Diplomonad (Hexamitid) Flagellates: Diplomonadiasis, Hexamitosis, Spironucleosis

Sarah L. Poynton

Department of Comparative Medicine
Johns Hopkins University School of Medicine
1-127 Jefferson Building, Johns Hopkins Hospital
600 North Wolfe Street
Baltimore, MD 21287
410/502-5065
fax: 443/287-2954
spoynton@jhmi.edu
spoynton@igb-berlin.de

A. Name of Disease and Etiological Agent

Diplomonadiasis or hexamitosis is infection by diplomonad flagellates (Order Diplomonadida, suborder Diplomonadina, Family Hexamitidae). If the exact genus is known, the infections may be reported as hexamitiasis (*Hexamita*), octomitosis (*Octomitus*), or spironucleosis (*Spironucleus*); of these, probably only the latter is applicable to fish (see below). Infections may be reported as localized (commonly in the intestine, and possibly also including "hole-in-the head disease" of cichlids (Paull and Matthews 2001), or disseminated or systemic (Ferguson and Moccia 1980; Kent et al. 1992; Poppe et al. 1992; Sterud et al. 1998).

Light microscopy studies have reported three genera from fish - namely *Hexamita*, *Octomitus*, and *Spironucleus*. However, transmission electron microscopy (TEM) is needed to confirm genus (Poynton and Sterud 2002), and light microscopy studies are therefore taxonomically unreliable. If TEM is not available, the organisms should be recorded as diplomonad or hexamitid flagellates. All recent comprehensive ultrastructural studies show only the genus *Spironucleus* infecting fish, and it is probable that this is the genus to which all diplomonads from fish belong (Poynton and Sterud 2002).

Some 15 to 20 species of diplomonads have been reported from fish (Poynton and Sterud 2002). However, most descriptions do not include comprehensive surface and internal ultrastructure and thus are incomplete. To date, only three species are well characterized: *S. barkhanus* (Sterud et al. 1997), *S. torosa* (Poynton and Morrison 1990), and *S. vortens* (Poynton et al. 1995) (possibly synonymous with *S. elegans*); the commonly reported "*H. salmonis*" is currently being redescribed, and will probably also be assigned to the genus *Spironucleus*.

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Diplomonad flagellates commonly infect freshwater and marine fish in many parts of the world, and occur in wild, farm, and aquarium environments (Kulda and Lom 1964a; Kulda and Nohýnková 1978; Woo and Poynton 1995). Some species may have a wide geographic range, for example *S. barkhanus* and *S. torosa* occur in freshwater and marine waters, and *S. vortens* occurs in the USA, England, and Norway.

2. Host Species

Infections occur in many families of fish including Acipenseridae, Anguillidae, Catostomidae, Centrarchidae, Cichlidae, Cyprinidae, Cyprinodontidae, Gadidae, Gasterosteidae, Mugilidae, Percichthyidae, Percidae, Salmonidae, Siganidae, and Sparidae. Two species appear to be host specific: *S. barkhanus* from salmonids, and *S. torosa* from gadids (Poynton and Sterud 2002). In contrast, *S. vortens* has been reported from cichlid and cyprinid fish. Pathogenic infections are most commonly reported from farmed salmonids (*S. barkhanus* and "*H. salmonis*") and cichlids (*S. elegans, S. vortens*).

C. Epizootiology

The reporting of disease from captive but not wild fish, and the absence of disease in wild fish with heavy infections suggests that: a) the stressors of captivity (particularly poor husbandry) may predispose fish to pathogenic infections, and b) certain species and stains of diplomonads are pathogenic, while others are not. Furthermore, in some instances pathogenic infections occur when infection passes to a novel host. For example, in Norway, *S. barkhanus* is a serious pathogen in sea-farmed Atlantic salmon, *Salmo salar*, yet is a commensal in feral Arctic char, *Salvelinus alpinus*.

The life cycle is believed to be direct, with oral or rectal acquisition of infection. Although trophozoites (motile feeding stage) and cysts are known, their relative roles in the life cycle have not yet been clearly demonstrated.

D. Disease Signs

Disease signs are not the same for each well-characterized species of diplomonad, thus each is treated separately below. No disease is associated with infection by *S. torosa*.

1. Behavioral Changes Associated with the Disease

a. *S. barkhanus*
In Atlantic salmon: sluggish behavior (inconsistent finding).
In chinook salmon: none reported.

b. *S. vortens*
Variable: in light infections, none; in very heavy infections, decreased appetite, anorexia, lethargy, occasionally floating on side yet continuing to eat.

c. *"H. salmonis"*
Fish may be anorexic, weak, and excessively nervous.

2. External Gross Signs

a. *S. barkhanus*
In Atlantic salmon: diseased fish significantly smaller than other fish in same age group, morbidity, and mortality.
In chinook salmon: anemia, swollen abdomen, and mortality.

b. *S. vortens*
Variable: none to weight loss, malaise, poor growth rate and mortality in juveniles; "hole-in-the-head" lesions.

c. *"H. salmonis"*
Emaciation, dull and dark color, red vent, pale shiny feces, abdominal distension, and mortality.

3. Internal Gross Signs

a. *S. barkhanus*
In Atlantic salmon: whitish granulomatous nodules in kidney and liver; red-brown putrid boils in muscle; hemorrhage in cerebral meninges, contents of posterior gut more fluid than normal with a greenish color, fibrinous perihepatitis, severe muscular degeneration, multifocal encephalitis, and suppurative meningitis.
In chinook salmon: serosanguinous ascites, enlarged mottled and congested liver, and enlarged kidney and spleen.

b. *S. vortens*
Variable: none to fluid-filled intestine.

c. *"H. salmonis"*
Gastrointestinal infections may be accompanied by enteritis, and yellow and watery or jelly-like gut contents.

4. Histopathological Changes Associated with the Disease

a. *S. barkhanus*
In Atlantic salmon: multifocal coagulation and caseous necrosis in liver and kidney.
In chinook salmon: liver edema, congestion and inflammation; renal interstitium hyperplastic.

b. *S. vortens*
Sometimes intestinal dilation, in "hole-in-the-head disease" flagellates present in kidney, liver, spleen, head lesions, and intestine.

c. *"H. salmonis"*
Gastointestinal infections exhibit excess mucus, cytoplasmic blebbing, and apoptotic bodies in epithelial cells.

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

a. Isolation/Detection of Pathogen

Live diplomonad flagellates can be readily recognized in fresh wet mounts, usually made from material from the gastrointestinal tract, and covered by a cover slip (Figure 1 and Figure 2). The trophozoites are highly motile, pyriform to elongate, 5 to 20 μm long (excluding flagella), with six anterior flagella and two posterior recurrent flagella. Detection may be improved if the preparation is diluted with tap or distilled water, or 0.9% NaCl, and the motility of the organisms can be reduced by the addition of a viscous medium such as Protoslo (Carolina Biological Supply Company). Preparations should be examined under bright field, phase contrast, or Normarski illumination at 400x magnification or higher.

Stains may be used to show general features of a diplomonad flagellate (most notably the paired nuclei) and species-specific features (see "Confirmatory Diagnosis" below and Figures 3 and 4).

If a systemic infection is suspected, blood smears stained with Giemsa may be examined (Figure 6). The characteristic paired nuclei and two recurrent flagella passing through the cell can be seen.

Preparations may also be stained by DAPI (4'-6-diamidino-2-phenylindole) and exposed to bright field and UV illumination; the characteristic paired nuclei of the diplomonad appear blue/violet (Figure 7) (Sterud 1998).

b. Clinical Signs

See "Disease Signs" above.

c. Histopathological Examination

Hematoxylin and eosin and the Feulgen reaction (nuclei magenta pink, cytoplasm turquoise) may be used to show the characteristic paired nuclei (Figure 8 and Figure 9). The diplomonads may be seen in the gastrointestinal lumen, aligned along the brush border of the intestine, and present in viscera and somatic musculature.

2. Confirmatory Diagnosis

For determination of genus, transmission electron microscopy is required (Brugerolle 1974, 1975; Brugerolle et al., 1973, 1974; Poynton and Sterud 2002). Of particular importance are: the shape of the nuclei, location of kinetosomes (flagellar bases), and presence or absence of a flagellar pocket (Figure 10), (Table 1). Keys for identification to genus are given in Poynton and Sterud (2002) (for diplomonads from fish), and in Brugerolle and Lee (2000) (all diplomonads).

For determination of species, scanning and transmission electron microscopy are required (Poynton and Sterud 2002). Important taxonomic features are: surface architecture (especially at the posterior end of the body), pattern of the bands of microtubules that accompany the recurrent flagella (when viewed in transverse section though the middle of the cell), and cytoplasmic organelles (Table 2). Special light microscopy stains such as protargol silver protein may be used to demonstrate the cytoskeleton, particularly at the posterior end of the body (Figure 3, Figure 4, and Figure 5).

Suitable electron microscopy fixatives are glutaraldehyde or a combination of glutaraldehyde/paraformaldehyde (Ferguson 1979; Poynton and Morrison 1990; Poynton et al. 1995; Sterud et al. 1997). Samples may be processed as tissue pieces or as a cell suspension (in the case of lumen infections).

The molecular taxonomy and protein code sequencing of diplomonads from fish is still in its infancy because few species are well recognized by classical methods, and few species have been isolated and maintained in *in vitro* culture.

Table 1. Ultrastructural features used to distinguish three genera of diplomonads reported from fish (from Poynton and Sterud 2002).

Character	*Spironucleus*	*Hexamita*	*Octomitus*
Flagellar pockets (cytostomal canals)	+	+	-
Central axis formed by recurrent axonemes, microtubular bands, endoplasmic reticulum	-	-	+
Two terminal spikes	-	-	+
Shape of nuclei	S- shaped	spherical	reniform
Location of kinetosomes relative to nuclei	sub-apical	external surface	between
Position of recurrent flagella relative to nuclei	medial	lateral	medial
Supra-nuclear microtubular band	+	+	reduced
Infra-nuclear microtubular band	+	+	reduced

F. Procedures for Detecting Subclinical Infections

Since the relationship between prevalence and density of infection and disease are not well established, no recommendations are given.

G. Procedures for Determining Prior Exposure to the Etiological Agent

No methods are currently available to detect previous infections with diplomonad flagellates affecting fish.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Samples should be collected within minutes from a freshly killed fish. Flagellates may survive for 15 to 30 minutes in freshly killed fish kept in the refrigerator.

For long term studies, diplomonad flagellates can be successfully maintained in *in vitro* culture. Protocols are available for "*H. salmonis*" (Buchmann and Uldal 1996), *S. barkhanus* (Sterud 1998), and *S. vortens* (Sangmaneedet and Smith 2000).

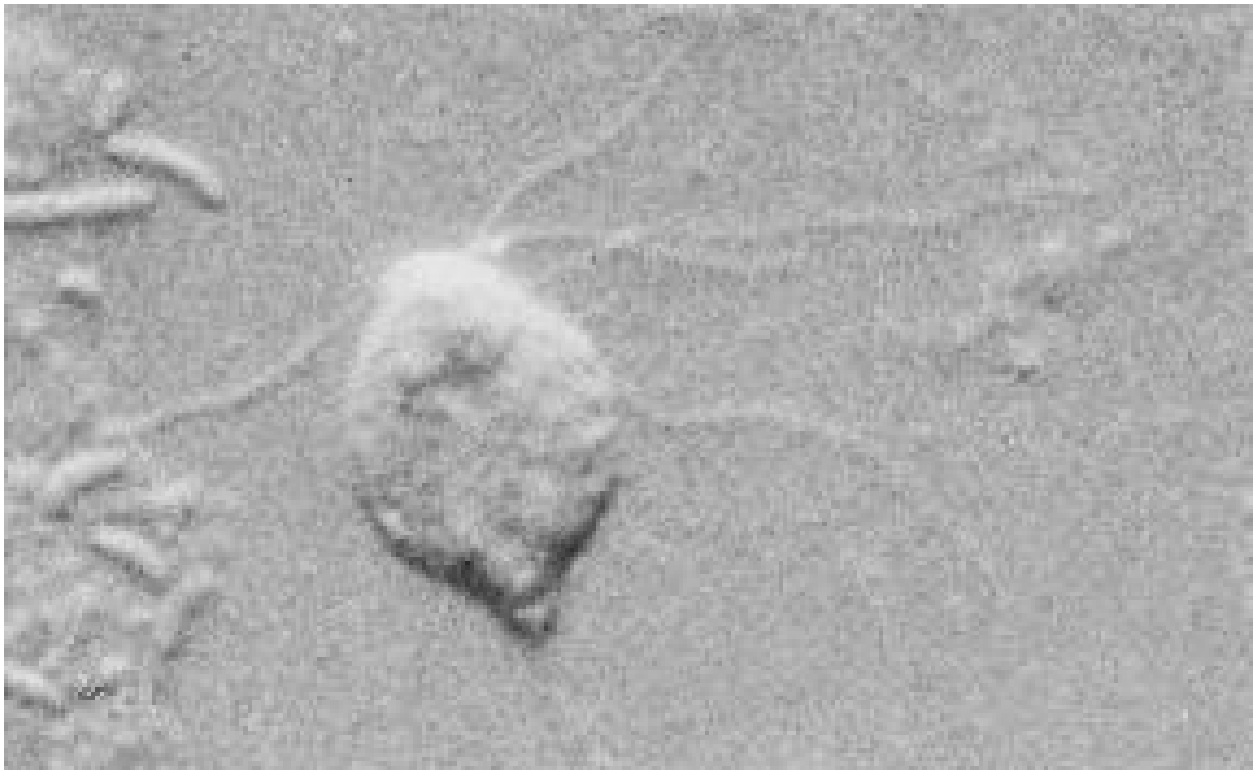
Table 2. Ultrastructure of currently recognized diplomonad species from fish (from Poynton and Sterud 2002).

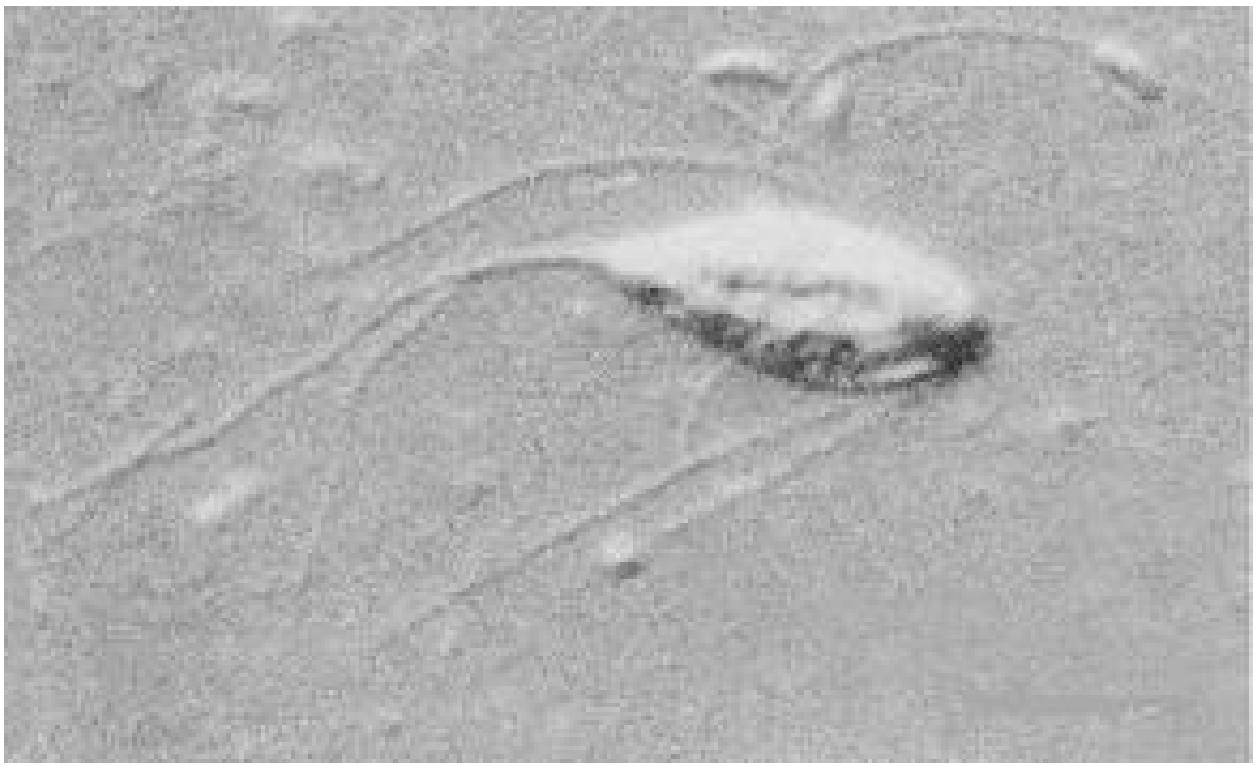
	External	Internal	
Species	surface and ornamentation	pattern of microtubular bands accompanying flagellar pocket (a)	other cytoskeletal elements
S. barkhanus	unadorned body, rf emerge from barhans (b)	3 radiate	-
S. torosa	unadorned body, rf emerge from tori (c) caudal projection, trophozoites can attach to host microvilli	3 concentric	mt + lamellae support tori and flanges
S. vortens	adorned body with compound lateral ridges (d) counter-crossing at posterior end with papillae, rf emerge from counter-crossing ridges	3 staggered	mt support right peripheral ridge, striated fibrous structures supports central ridge
*S. elegans**	adorned body with compound lateral ridges (e), rf emergence not known	staggered?	mt support peripheral ridge, striated fibrous structure supports central ridge

(a) viewed in transverse section through the middle of the body
(b) barkhans are crescent shaped ridges
(c) tori are raised ring-shaped areas, surrounded by flanges
(d) compound lateral ridges comprise a smooth central part, and a rope-like peripheral ridge narrower on the left than on the right, peripheral ridges bear tufts of microfibrillar material
(e) compoud lateral ridges comprise a smooth central part, and peripheral ridges bearing tufts of microfibrillar material

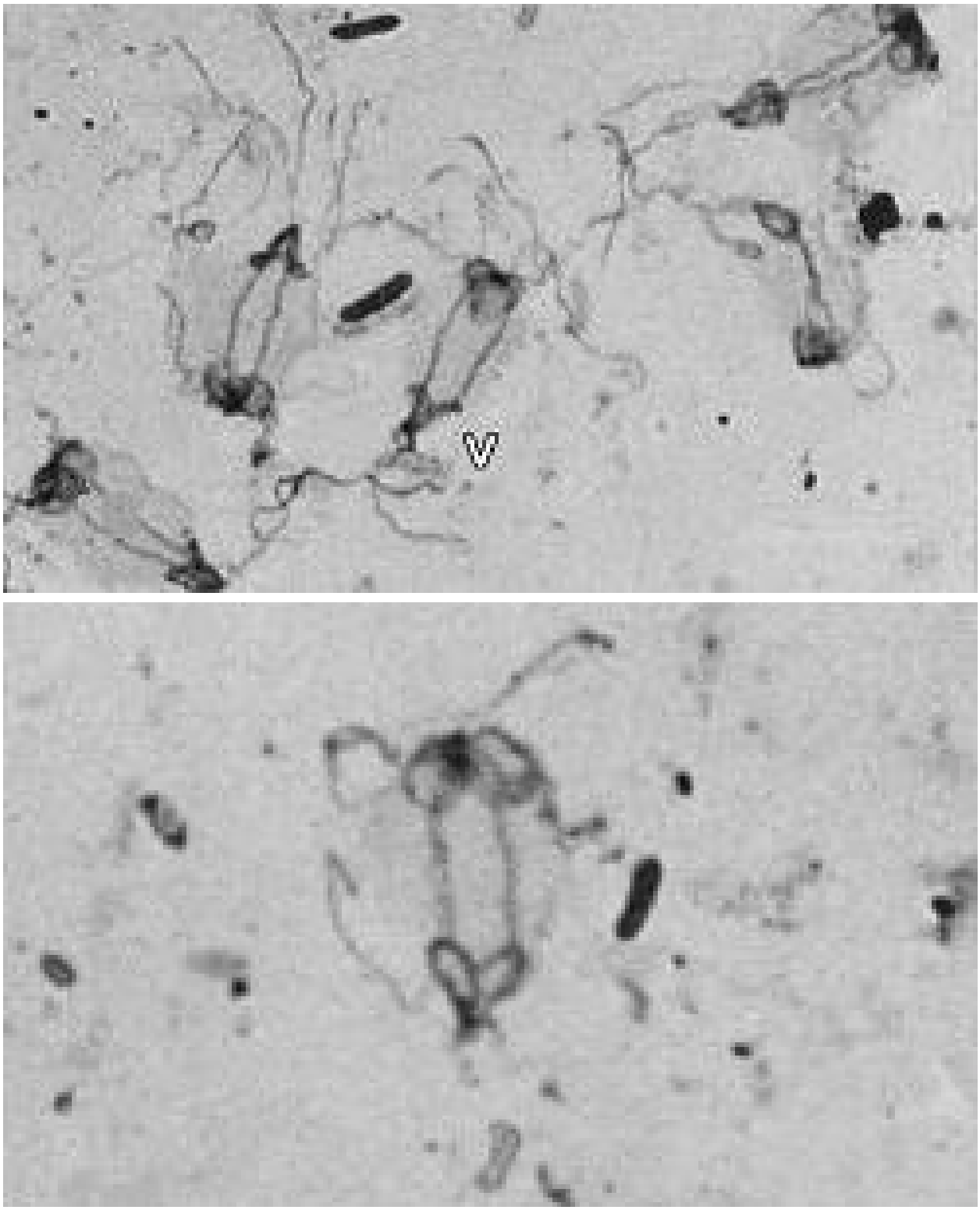
mt = microtubules
rf = recurrent flagellum

* Although *S. elegans* has been described from fish only by light microscopy, the internal ultrastructure has been described for diplomonads from amphibians. In the absence of SEM, the morphology of the taxonomically important posterior end is not known, so its affinity to other species in this table cannot be fully assessed. Current evidence suggests that *S. elegans* and *S. vortens* may be conspecific. Cross infection studies (Kulda and Lom 1964b) have confirmed that freshwater angelfish, *Pterophyllum scalare*, can be successfully infected with diplomonads from amphibians, *Triturus alpestris*.





Figures 1 and 2. Diplomonad flagellates prepared by different techniques and viewed by light microscopy. Fresh preparation of spherical and elongate trophozoites of *Spironucleus torosa* viewed by Normarski illumination. Originally published in the Journal of Protozoology 1990 (**37,** 369-383) and reproduced with permission of the Society of Protozoologists.



Figures 3 and 4. Smear preparations of *S. torosa* stained by Protargol silver protein, note V-shaped structure (v) at the posterior end of the body [which TEM shows to be two rings of microtubules supporting the flanges around the tori (compare with Figure 4)], note also the passage of recurrent flagella near centre of the body. Originally published in the Journal of Protozoology 1990 (**37,** 369-383) and reproduced with permission of the Society of Protozoologists.

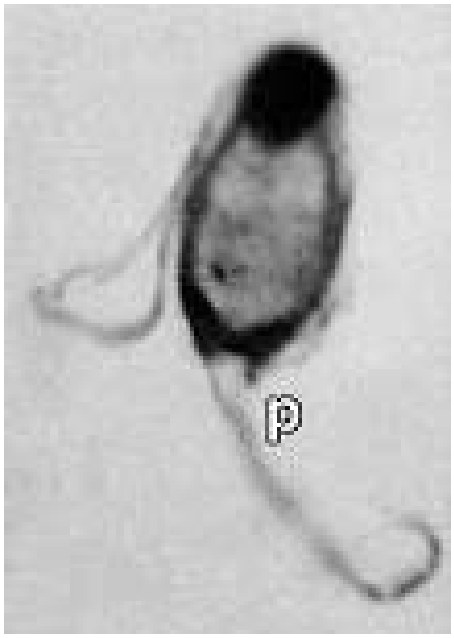


Figure 5. Preparation of *S. vortens* stained by Protargol silver protein (filter method); note dark area across posterior end of body (which TEM shows to be an extensive symmetrical system of microtubules supporting the elaborate architecture of the counter-crossing ridges at the posterior end of the body), the papillae (p), and passage of recurrent flagella just beneath the surface of the cell. Originally published in the Journal of Eukaryotic Microbiology 1995 (**42,** 731-742) and reproduced with permission of the Society of Protozoologists.

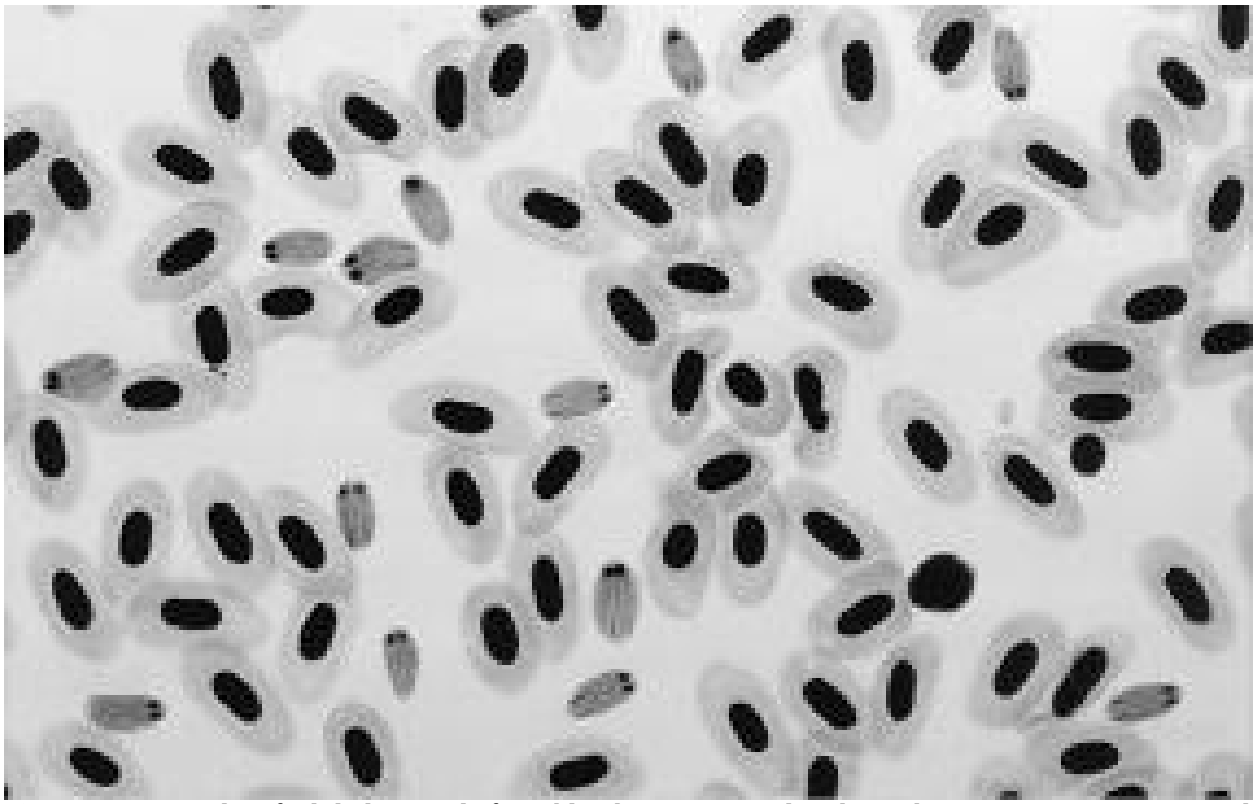


Figure 6. Unidentified diplomonads from blood smear stained with Leishman's Giemsa; note paired nuclei and passage of recurrent flagella through the cell. Originally published in Diseases of Aquatic Organisms 1992 (**14,** 81–89) and reproduced with permission of Inter-Research.

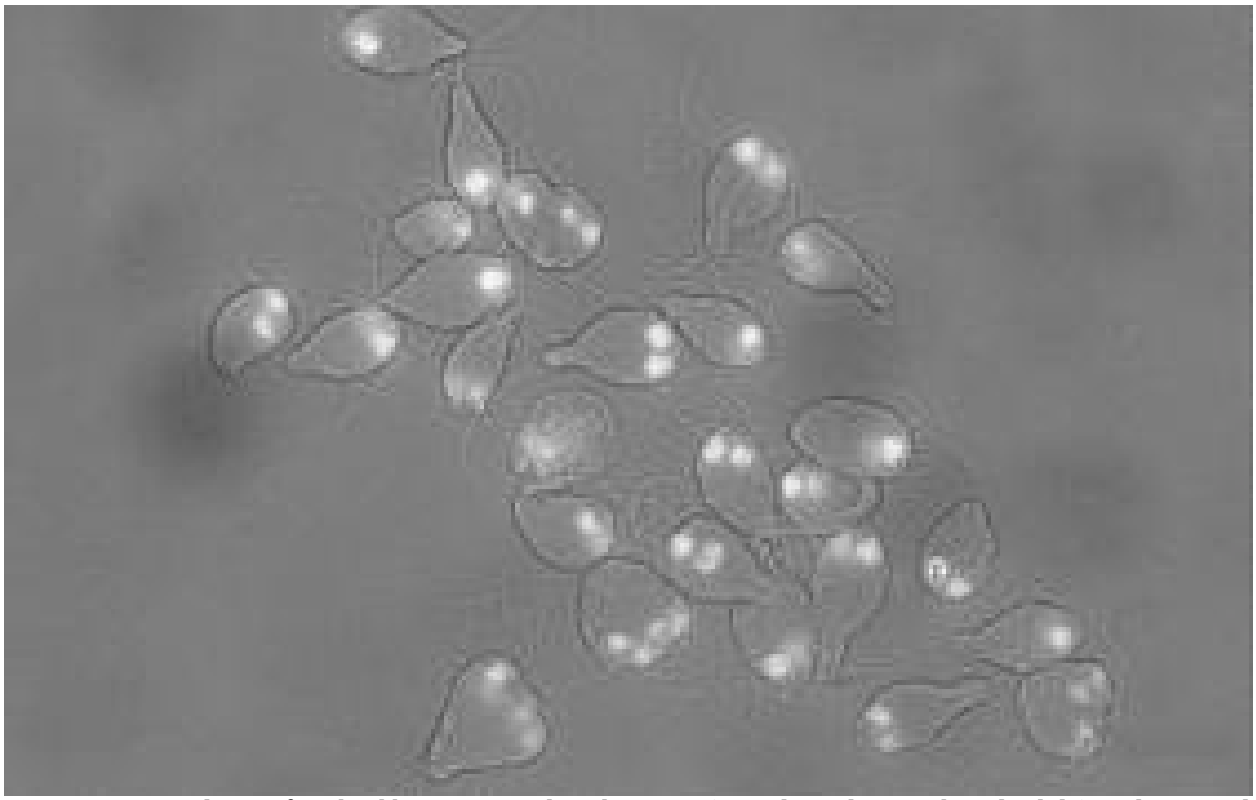


Figure 7. Culture of *S. barkhanus* stained with DAPI (4'-6-diamidino-2-phenylindole) and exposed to bright field and UV illumination, nuclei appear blue/violet (white in this photograph).

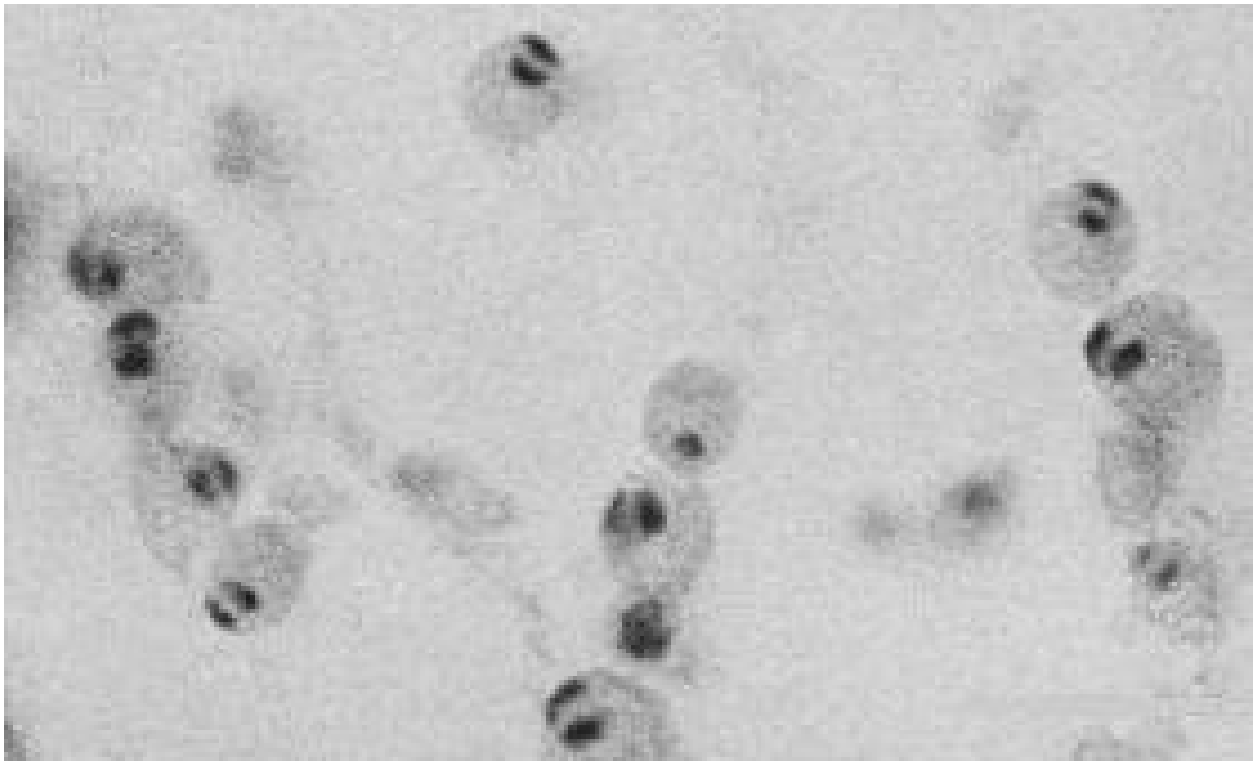


Figure 8. *S. torosa* in tissue section (lumen of rectum) stained by the Feulgen reaction. Originally published in the Journal of Protozoology 1990 (**37,** 369-383) and reproduced with permission of the Society of Protozoologists.

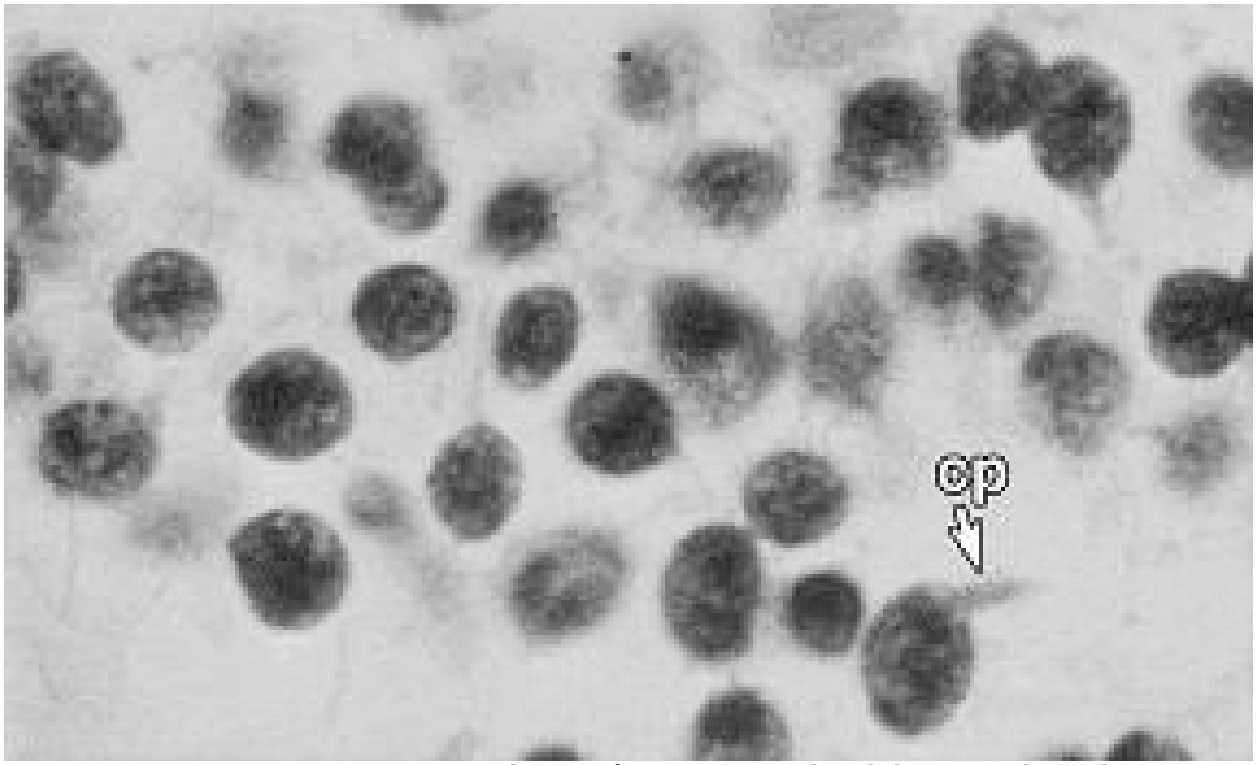


Figure 9. *S. torosa* in tissue section (lumen of rectum) stained with haematoxylin and eosin; (cp) caudal projection. Originally published in the Journal of Protozoology 1990 (**37,** 369-383) and reproduced with permission of the Society of Protozoologists.

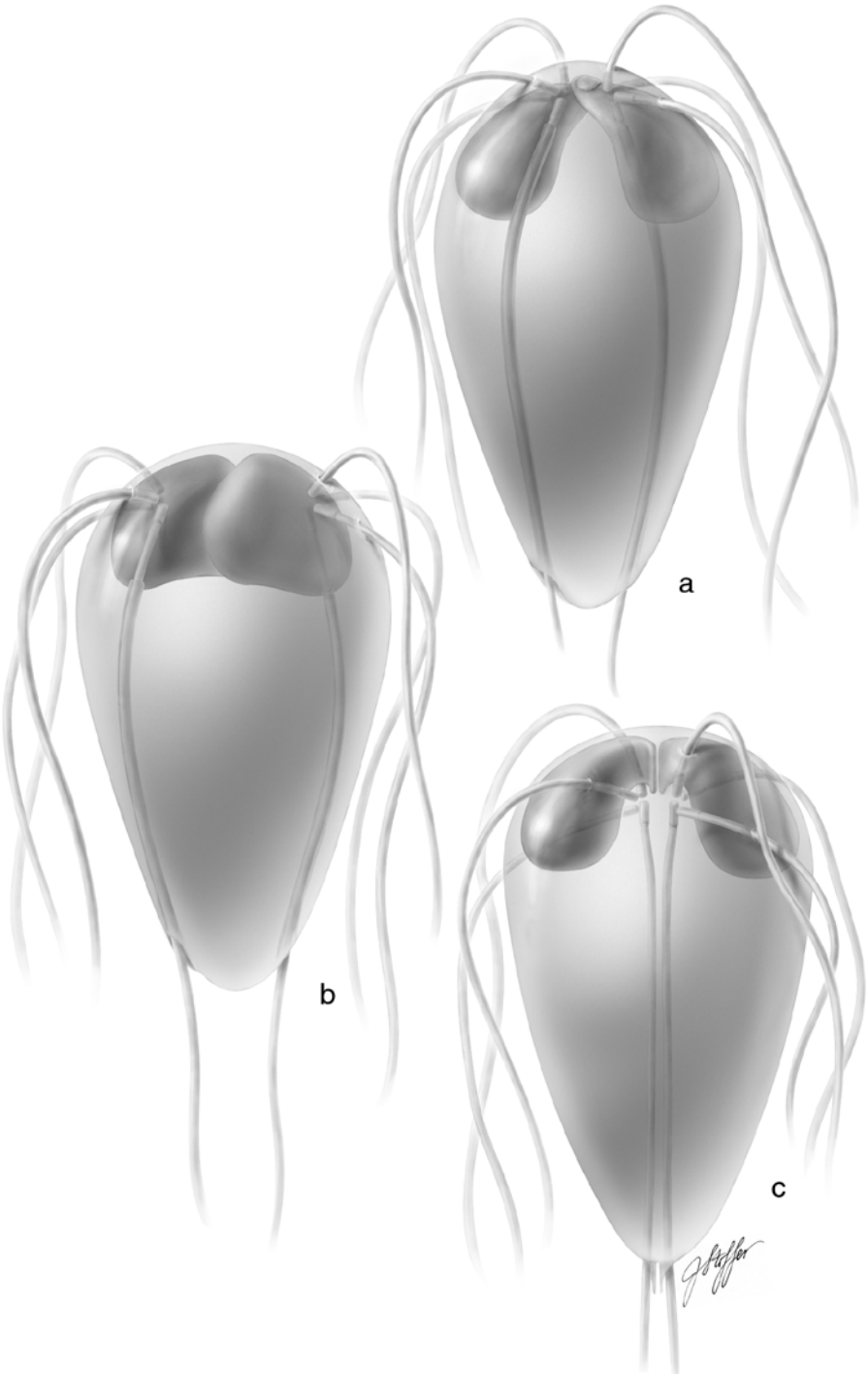


Figure 10. Principal distinguishing features of the three genera of diplomonads within the suborder Diplomonadina that have been reported from fish, (a) *Spironucleus*; (b) *Hexamita*; (c) *Octomitus*. Organisms shown in ventral view. Note especially the presence or absence of flagellar pockets (cytostomal canals) (evident as sheaths around the recurrent flagella of *Spironucleus* and *Hexamita,* and absent from *Octomitus*), shape of the nuclei, and locations of kinetosomes and tract of the recurrent flagella passing posteriorly. Surface ornamentation, microtubular bands, and endoplasmic reticulum are excluded for simplicity, and flagella are shortened in this illustration. Original by Judith A. Stoffer after Brugerolle (1974), Brugerolle *et al*. (1973b, 1974) and Kulda & Nohýnková (1978). © 2001 Judith A. Stoffer.

References

Brugerolle, G. 1974. Contribution l' étude cytologique et phylétique des diplozoaires (Zoomastigophores, Diplozoa, Dangeard 1910). III. Étude ultrastructurale de genre *Hexamita* (Dujardin 1836). Protistologica 10:83-90.

Brugerolle, G. 1975. Contribution l' étude cytologique et phylétique des diplozoaires (Zoomastigophores, Diplozoa, Dangeard 1910). VI. Caractères généraux des diplozoaires. Protistologica 11:111-118.

Brugerolle, G., L. Joyon, and N. Oktem. 1973. Contribution a l'étude cytologique et phylétique des diplozoaires (Zoomastigophorea, Diplozoa, Dangeard 1910). II. Étude ultrastructurale du genre *Spironucleus* (Lavier 1936). Protistologica 9:495-502.

Brugerolle, G., L. Joyon, and N. Oktem. 1974. Contribution a l'étude cytologique et phylétique des diplozoaires (Zoomastigophorea, Diplozoa, Dangeard 1910). IV. Étude ultrastructurale du genre *Octomitus* (Prowazek 1904). Protistologica 10:457-463.

Brugerolle, G., and J. J. Lee. 2000. Order Diplomonadida. Pages 1125-1135 *in* J. J. Lee, G. F. Leedale, and P. Bradbury, editors. An Illustrated Guide to the Protozoa, Second Edition. Society of Protozoologists. Lawrence, Kansas.

Buchmann, K., and A. Uldal. 1996. Temperature, pH, and bile dependent *in vitro* cultivation of *Hexamita salmonis* from rainbow trout *Oncorhynchus mykiss* intestine. Diseases of Aquatic Organisms 24:169-172.

Ferguson, H. W. 1979. Scanning and transmission electron microscope observations on *Hexamita salmonis* (Moore, 1922) related to mortalities in rainbow trout fry *Salmo gairdneri* Richardson. Journal of Fish Diseases 2:57-67.

Ferguson, H. W., and R. D. Moccia. 1980. Disseminated hexamitiasis in Siamese fighting fish. Journal of the American Veterinary Medical Association 177:854-857.

Kent, M. L., J. Ellis, J. W. Fournie, S. C. Dawe, J. W. Bagshaw, and D. J. Whitaker. 1992. Systemic hexamitid (Protozoa: Diplomonadida) infection in seawater pen-reared Chinook salmon *Oncorhynchus tshawytscha*. Diseases of Aquatic Organisms 14:81-89.

Kulda, J., and J. Lom. 1964a. Remarks on the diplomastigine flagellates from the intestine of fishes. Parasitology 54:753-762.

Kulda, J., and J. Lom. 1964b. *Spironucleus elegans* Lavier, parasite of fish. Cěskoslovenská Parasitologie XI:187-192.

Kulda, J., and E. Nohýnková. 1978. Flagellates of the Human Intestine and of Intestines of Other Species. Pages 1-138 *in* J. P. Kreier, editor. Parasitic Protozoa, Volume II. Academic Press, New York.

Paull, G. C., and R. A. Matthews. 2001. *Spironucleus vortens*, a possible cause of hole-in-the-head disease in cichlids. Diseases of Aquatic Organisms 45:197-202.

Poppe, T. T., T. A. Mo, and L. Iversen. 1992. Disseminated hexamitosis in sea-caged Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms 14:91-97.

Poynton, S. L., and C. M. Morrison. 1990. Morphology of diplomonad flagellates: *Spironucleus torosa* N.Sp. from Atlantic Cod *Gadus morhua* L., and haddock *Melanogrammus aeglefinus* (L.) and *Hexamita salmonis* Moore from brook trout *Salvelinus fontinalis* (Mitchill). Journal of Protozoology 37:369-383.

Poynton, S. L., and E. Sterud. 2002. Guidelines for species descriptions of diplomonad flagellates from fish. Journal of Fish Diseases 25:15-31.

Poynton, S. L., W. Fraser, R. Francis-Floyd, P. Rutledge, P. Reed, and T. A. Nerad. 1995. *Spironucleus vortens* n.sp., from freshwater angel fish *Pterophyllum scalare*: morphology and culture. Journal of Eukaryotic Microbiology 42:731-742.

Sangmaneedet, S., and S. Smith. 2000. *In vitro* studies on optimal requirements for the growth of *Spironucleus vortens*, an intestinal parasite of freshwater angelfish. Diseases of Aquatic Organisms 39:135-141.

Sterud, E. 1998. *In vitro* cultivation and temperature dependent growth of two strains of *Spironucleus barkhanus* (Diplomonadida: Hexamitidae) from Atlantic salmon *Salmo salar* and grayling *Thymallus thymallus*. Diseases of Aquatic Organisms 33:57-61.

Sterud, E., T. A. Mo, and T. T. Poppe. 1997. Ultrastructure of *Spironucleus barkhanus* n. sp., (Diplomonadida: Hexamitidae) from grayling *Thymallus thymallus* (L.) (Salmonidae). Journal of Eukaryotic Microbiology 44:399-407.

Sterud, E., T. A. Mo, and T. T. Poppe. 1998. Systemic spironucleosis in sea-farmed Atlantic salmon *Salmo salar*, caused by *Spironucleus barkhanus* transmitted from feral Arctic char *Salvelinus alpinus*? Diseases of Aquatic Organisms 33:63-66.

Woo, P. T. K., and S. L. Poynton. 1995. Diplomonadida, Kinetoplastida, and Amoebida (Phylum Sarcomastigophora). Pages 27-96 *in* P. T. K. Woo, editor. Fish Diseases and Disorders, Volume 1. Protozoan and Metazoan Infections. CAB International, Wallingford, London.