Uronema
(protist: ciliate)

Overview

Protists are single-celled organisms with membrane-bound nuclei (eukaryotes). One protistan supergroup known as SAR comprises the Stramenopiles (with heterokont flagella), Alveolata (with cortical alveoli) and Rhizaria (with fine pseudopodia). Three major alveolate groups are recognized: ciliates, apicomplexans and dinoflagellates. Ciliated protozoa are unique eukaryotes as they exhibit nuclear dualism (vegetative macronucleus and reproductive micronucleus), the process of conjugation (exchange of micronuclei between pairs), have membrane-bound sacs (subpellicular alveoli) supporting the plasma membrane, and move using cilia (2+9 undulipodia with compound subpellicular infraciliature). Most ciliates are free-living in aquatic and terrestrial habitats, but some are symbiotic in vertebrate and invertebrate hosts. Ten major monophyletic lineages are recognized on the basis of their infraciliature, i.e. the ultrastructural organization of their kinetids (comprising basal bodies (= kinetosomes) and associated microtubular ribbons and fibrils). Members of the subphylum Intramacronucleata are united by the presence of microtubules inside the macronuclear envelope during division; including the oligohymenophoreans ('few membrane-bearer') with an adoral zone of buccal (oral) ciliature. Scuticociliates have a small paroral membrane located on the right side of the buccal cavity and three membranelles on the left side, plus a characteristic postoral scuticum usually composed of barren kinetosomes. Philasterids have a short paroral dikinetid membrane and uronematids have small ovoid sparsely-ciliated bodies. *Uronema* spp. are mostly free-living aquatic organisms, but several species are facultative histophagous parasites of marine fishes, sometimes causing significant mortalities in farmed tuna.

Uronema
clickable life-cycle
one-page PDF: overview and life-cycle

Classification:

Domain:	Eukaryota (membrane-bound nucleus)
Supergroup:	SAR (Stramenopiles + Alveolata + Rhizaria)
Group:	Alveolata (with cortical alveoli)
Phylum:	Ciliophora (with cilia, nuclear dualism, pellicular alveoli, reproductive conjugation)
Subphylum:	Intramacronucleata (microtubules occur inside macronuclear envelope during division))
Class:	Oligohymenophorea (distinct oral ciliature, comprising right paroral membrane and 3 left membranelles)
Subclass:	Scuticociliatia (with scuticum or scuticovestige)
Order:	Philasterida (short paroral dikinetid membrane)
Family:	Uronematidae (membranelles aligned with long axis, anterior pole non-ciliated)
Genus:	*Uronema* (opportunistic parasites in fish)
Species:	various species cause encephalitis in fish

Parasite biodiversity and host range: Protists are unicellular eukaryotes that move using undulipodia (flagella or cilia), pseudopodia (false-feet) or a unique gliding motion. Cells with different modes of locomotion do not form separate monophyletic assemblages as previously thought, but rather are distributed across several disparate supergroups (as evidenced by recent molecular phylogenetic analyses). One protistan supergroup known as SAR comprises the Stramenopiles (with heterokont flagella), Alveolata (with cortical alveoli) and Rhizaria (with fine pseudopodia). Three diverse alveolate groups are recognized: Ciliophora (with cilia), Dinoflagellata (with flagella) and Apicomplexa (with gliding motion, some also with flagellated microgametes). Ciliated protozoa are unique amongst the unicellular eukaryotes because they are the only group to exhibit nuclear dualism. Individual cells possess two different types of nuclei; vegetative macronuclei and reproductive micronuclei. Asexual reproduction occurs by transverse binary fission across rows of cilia (homothetogenic fission) whereas some species exhibit sexual reproduction by the phenomenon of conjugation (temporary fusion of two conjugates which exchange micronuclei). As their common name implies, ciliates are also characterized by the possession of simple cilia, or compound ciliary organelles, in at least one stage of their life cycles (compound subpellicular infraciliature is universally present even when cilia are absent). Cilia are elongate hair-like extensions of the cell membrane with an internal microtubular core (universal 2+9 configuration = 2 single central microtubules surrounded by 9 peripheral doublets). They are organelles of motility used for locomotion and/or feeding. Cilia (singular, cilium) are similar in ultrastructure to flagella (singular, flagellum), and they are collectively often called undulipodia (singular, undulipodium) because both use cross-linked proteins (dynein-walking mechanism) to undulate about their basal kinetosome (unlike the rotary motion unique to flagella in bacteria). Ciliates, together with dinoflagellates and apicomplexans, possess subpellicular alveoli which are membrane-bound sacs beneath the plasma membrane. Alveoli are thought to serve many varied functions: ranging from support (helping maintain body shape, act as fulcrum for undulipodia); metabolism (storage); osmoregulation (mucocysts); excretion (extrusomes); protection (toxicysts, trichocysts); and even hunting (haptocysts).

Most ciliate species are free-living in aquatic or terrestrial habitats but many are commensals in vertebrate or invertebrate hosts and some are parasitic. Early classification systems recognized three main classes of ciliates mainly on the basis of their patterns of somatic (body) and buccal (oral) ciliation. The ‘lower holotrichs’ have simple body and oral ciliature; most are free-living species but some are highly specialized symbionts aiding cellulose digestion in herbivores. The ‘higher holotrichs’ have simple body ciliature but more specialized oral ciliature forming membranelles; most occur as free-living organisms but some live as commensals or parasites in a range of animals. The ‘spirotrichs’ have reduced body ciliation but well-developed oral ciliature forming an adoral zone of membranelles; most are bactivores living in aquatic and terrestrial habitats. More recently, ten major monophyletic lineages have been recognized on the basis of their infraciliature; i.e. the ultrastructural organization of their kinetids (comprising basal bodies (= kinetosomes) and associated microtubular ribbons and fibrils). These lineages (ranked as classes) have been well supported by modern molecular biological studies using several gene sequences.

Class	**Etymology**	**Defining characters**	**Lifestyles***	**Genera covered**
Subphylum: **Postciliodesmatophora** [somatic dikinetids with postciliodesmata (overlapping microtubular ribbons)]				
Karyorelictea	‘primitive-nucleus’	macronuclei not dividing but replaced by division of micronuclei	free-living (aquatic benthic/planktonic)	
Heterotrichea	‘different-hair’	compound ciliary organelles around mouth, macronuclei divided by external microtubules	free-living (aquatic planktonic/benthic)	
Subphylum: **Intramacronucleata** [macronuclei divided by internal microtubules]				
Spirotrichea	‘coiled-hair’	conspicuous right and left oral ciliature, left polykinetids leading into oral cavity	free-living (aquatic, terrestrial)	
Litostomatea	‘simple-mouths’	cytostome with noncurved tubular cytopharyngeal apparatus (rhabdos)	free-living (often predatory), symbiotic	*Balantidium*
Phyllopharyngea	‘leaf-throated’	mouth with radial microtubular ribbons (phyllae), some with sticky feeding tentacles	free-living (aquatic), epizoic, symbiotic	*Chilodonella*
Colpodea	‘breast-shaped’	reniform bodies, somatic cilia with transverso-desmata (overlapping ribbons)	terrestrial, some aquatic (bactivores)	
Nassophorea	‘pot-bearer’	oral nematodesmata well-developed (basket-like nasse or cyrtos supporting cytopharynx)	free-living (aquatic, terrestrial)	
Prostomatea	‘before-mouth’	simple apical mouths, some with oral microtubular band, some with oral brush	free-living (often predatory)	
Plagiopylea	‘misshapen-marker’	with twisted oral tubes, most with hydrogenosomes	free-living (anoxic habitats)	
Oligo-hymenophorea	‘few membrane-bearer’	typically with ventral groove containing mouth and compound ciliary organelles (usually adoral zone of three membranelles)	free-living, epizoic, symbiotic (micro-phagous)	*Uronema, Ichthyophthirus, Tetrahymena, Trichodina, Vorticella*

*Symbiosis *sensu latu* ranges from commensalism, mutualism and parasitism (depending on the benefit/detriment to the host)

The class Oligohymenophorea contains ciliates whose somatic (body) kinetosomes (basal bodies) are associated with unique ultrastructural elements (collectively referred to as infraciliature) comprising anteriorly-directed overlapping kinetodesmal fibrils, divergent postciliary microtubular ribbons and radial transverse ribbons. Members are considered to be ‘higher’ holotrichs as there is clear distinction between the oral and somatic ciliature. They possess distinctive oral kineties consisting of a right paroral kinety (membrane) and typically 3 left oral polykinetids (membranelles) located in a buccal or infundibular cavity with an inconspicuous cytopharyngeal apparatus. They are widely distributed as free-living or symbiotic forms, although a few are parasitic mainly in fish. The class contains 6 subclasses (Apostomatia, Astomatia, Hymenostomatia, Peniculia, Peritrichia, Scuticociliatia) differentiated predominantly on the basis of their patterns of somatic and oral ciliation. Scuticociliates have a small paroral membrane located on the right side of the buccal cavity and three membranelles on the left side (paroral files (membranoids) of dikinetids with a, b and c segments), plus a characteristic postoral scuticum (or scutico-vestige) usually composed of barren kinetosomes. Three scuticociliate orders are recognized: Philasterida; Pleuronematida; and Thigmotrichida. Philasterids have elongate bodies with uniform somatic ciliation and a long caudal cilium as well as oral ciliature with a short paroral dikinetid membrane (typically by reduction of a and c segments). A total of 15 philasterid families are recognized, including the Uronematidae comprising 7 genera (*Homalogastra, Pseudouronema, Urocyclon, Uronema* (syn. *Uronemita*), *Uronemella, Uronemopsis,* and *Uropedalium*) whose members have small ovoid sparsely-ciliated bodies with a long caudal cilium, a flat non-ciliated anterior pole with a long buccal area and oral membranelles aligned longitudinally. The genus *Uronema* contains both freshwater and marine species which are mostly free-living filter-feeding scavengers, but several species may become histophagous and opportunistically infect fish causing skin and/or gill lesions, sometimes invading internal organs (viscera and brain) with fatal consequences.

*Uronema** species	Length (µm) [no. somatic kineties]	Life-style (Hosts) [Clinical signs]	Distribution
U. acutum	24-70 [9-14]	marine, free-living	North Sea
U. apomarinum	25-35 [12-13]	brackish water, free-living	China
U. castellonesis	28-38 [13]	freshwater, free-living	Europe
U. elegans (syn. *Cryptochilum*)	33-90 [21-27]	marine, free-living	Mediterranean
U. gallicum	20-30 [13-15]	marine, free-living	Europe
U. granulatum	25	freshwater, free-living	Europe
U. heteromarinum	25-50 [15-16]	marine, free-living	China
U. marinum [type species] (syn. *U. marina, schewiakoffi, Cohnilembus pusillus, Loxocephalus putrinus*)	19-60 x 7-27 [10-16]	marine, free-living (esp. amongst algae) and parasitic in skin, gills and viscera of fish (Acanthuriformes: acanthurid (yellow tang); Labriformes: labrid (California sheephead wrasse, red-lined wrasse, cunner); Moroniformes: moronid (European seabass); Perciformes: chaetodontid (pennant coralfish, teardrop butterflyfish, diagonal butterflyfish, threadfin butterflyfish, bluecheek butterflyfish, vagabond butterflyfish, copperband butterflyfish, longnose butterflyfish), opistognathid (yellowhead jawfish), pomacanthid (threespot angelfish, goldspotted angelfish, pygmy angelfish), pomacentrid (garibaldi, Vanderbilt's chromis, bluegreen damselfish, maroon clownfish, tomato clownfish), pseudochromid (orchid dottyback), serranid (sea goldie); Pleuronectiformes: paralichthyid (olive flounder), scophthalmid (turbot); Salmoniformes: salmonid (Atlantic salmon); Scombriformes: stromateid (silver pomfret); Scorpaeniformes: scorpaenid (lionfish); Syngnathiformes: syngnathid (lined seahorse, yellow seahorse); Tetraodontiformes: tetraodontid (Japanese puffer)] [lesions, respiratory distress, death]	worldwide (patchy)
U. nigricans (syn. *Cyclidium, U. parduczi*)	21-29 [11-16]	freshwater/marine, free-living and parasitic in gills and brain of fish (Cyprinodontiformes: poeciliid (guppy); Carangiformes: carangid (yellowtail amberjack); Scombriformes: scombrid (southern bluefin tuna)) [lesions, respiratory distress, swimmer syndrome, death]	worldwide (patchy)
U. orientalis	25-58 [15-20]	marine, free-living	China
U. pluricaudatum		marine, free-living	Europe
U. rabaudi	40	marine, epizoic on carapace of invertebrates (Copepoda: acartid (*Acartia clausi*), clausiid (*Clausia elongata*))	Europe
U. tortum (syn. *Cryptochilum*)	36-58 [6-8]	marine, free-living	Mediterranean

Several free-living species previously assigned to the genus *Chilodonella* have since been reassigned to other genera: including *U. biceps* [= *Balanonema*]; *U. digitiformis* [= *Philasterides*]; *U. filificum* [= *Uronemella*]; *U. microcrepis* [= *Dexiotrichides*]; and *U. sociale* [= *Platynematum*]. *Confusingly, the genus name *Uronema* (and some species names, e.g. *U. marinum*) are shared in the botanical nomenclature with some epiphytic microfilamentous green algae (Chlorophyceae: Chaetophorales: Uronemataceae) living in freshwater and marine environments (viz: *U. acuminatum, africanum, belkae, brasiliense, confervicola, curvatum, elongatum, falcatum, indicum, intermedium, marinum, minutum, simplicissimum, subelongatum, terrestre* and *trentonense*).

Parasite morphology: *Uronema* spp. form motile trophic stages (trophozoites) that swim continuously in the water column using holotrichous (whole body) ciliation. Cyst formation has not been reported. Trophozoites are typically ovoid-ellipsoid (sometimes pyriform) in shape with a rounded posterior and a truncated anterior end (flattened and nonciliated). Free-swimming ciliates are more slender and transparent than those recovered from fish which are robust and opaque. Trophozoites vary in size from 20-50 µm in length depending on their nutritional state (well fed individuals being more rotund) and species (*U. marinum* 30-50 x 10-20 µm;

U. nigricans 20-34 x 12-20 µm). The ciliates are clearly hymenostomes with well-defined oral and somatic ciliature. Members of the genus *Uronema* have a large shallow buccal cavity located in the upper half of the body, surrounded by distinctive rows of cilia forming a paroral undulating membrane and 3 membranelles aligned with the long axis of the body. Like other members of the suborder Philasterina, the undulating membranes were shorter than the other oral structures and they were not reinforced by ribbed walls. The first membranelle consisted of one row of basal bodies (kinetosomes), the second and third membranelles had 2 or more longitudinal rows, and the paroral membrane extended forward to the anterior end of the second membranelle. A distinctive scuticum (consisting of several basal body pairs) was located immediately below the oral structures (characteristic for all scutico-ciliates). The cell body was uniformly covered with longitudinal rows (kineties) of short somatic cilia, except for the nonciliated anterior pole. The number of kineties ranged from 6-27 depending on the species, with *U. marinum* and *U. nigricans* typically having 10-16. Each row of cilia had 12-32 kinetids, with the anterior half being dikinetids (paired basal bodies) and the posterior half being monokinetids (single basal bodies). Trophozoites also possessed a long prominent caudal cilium, being 1-2 times the length of the body. Cells contained a single large ovoid macronucleus and a small round adjacent micronucleus, both located just anterior to the midbody. Trophozoites contained a single translucent contractile vacuole located near the posterior pole, and their cytoplasm appeared granular due to the presence of food vacuoles in the posterior half of the body. While cyst formation has not been observed, experimental studies on *U. marinum* cultures have shown that starved trophozoites undergo 2-3 successive cell divisions forming 4-8 small slender 'swarmer' cells to aid dispersion.

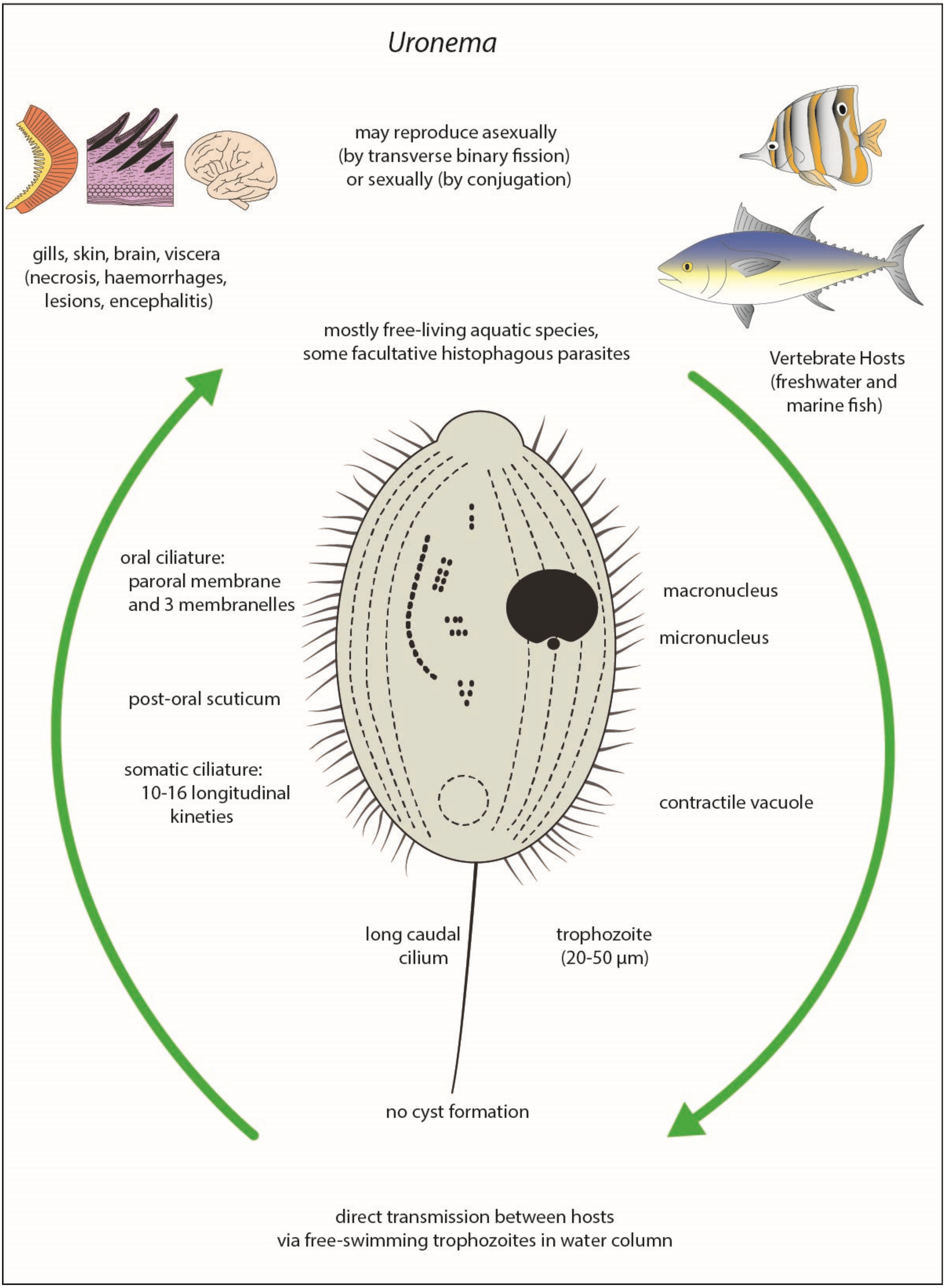
Site of infection: All *Uronema* spp. are free-living filter-feeding scavengers in aquatic environments, 9 species occurring in marine waters (including *U. marinum*), 2 in freshwater, one in brackish water, and one (*U. nigricans*) in marine and freshwater. Both *U. marinum* and *U. nigricans* may also occur as opportunistic histophagous parasites in the tissues of fish, particularly ornamental and farmed species. Infections have been recorded in some 35 fish species belonging to 19 families in 12 families (mainly in Perciformes, but also in Acanthuriformes, Carangiformes, Cyprinodontiformes, Labriformes, Moroniformes, Pleuronectiformes, Salmoniformes, Scombriformes, Scorpaeniformes, Syngnathiformes and Tetraodontiformes). Parasitic ciliates may be found in fish in the gills, skin, musculature and internal organs (viscera and brain).

Pathogenesis: Scuticociliates may cause clinical disease (known generally as scuticociliatosis or scutica infection) in a range of fish, crustaceans and molluscs. Two *Uronema* spp. opportunistically infect fish causing superficial and/or systemic disease (generically known as uronemosis, with specific regional variants such as red band disease in ornamental fish, red sores in damselfish, or blue swimmer syndrome in tuna). When free-living bactivorous trophozoites come into contact with fish, they may attach to superficial surfaces (skin and gills) but may sometimes invade to deeper tissues (muscles, viscera, brain) causing significant disease and mortality. Trophozoites break down host tissues using proteolytic enzymes and they feed on the resultant cellular debris as well as on host erythrocytes. Ciliate secretions also appear to impair host cellular responses by inhibiting or neutralizing the respiratory burst activity used by phagocytes to kill pathogens. Feeding trophozoites may cause white slimy necrotic skin patches which progress to areas of depigmentation, scale loss, haemorrhagic lesions, or open ulcerated wounds (red sores). Infections in ornamental aquarium fish may also involve behavioural abnormalities (rubbing skin on substrates, hiding, listlessness, disorientation) and breathing difficulties (gasping, gaping, gulping) resulting in some mortalities. Parasite may also enter the bloodstream through lesions and invade the musculature, stomach, intestines, liver, spleen, brain, spinal canal, kidneys or urinary bladder, with infections often becoming systemic and causing extensive degeneration, necrosis, haemorrhages, cellular vacuolization, inflammation, granuloma formation, epithelial hyperplasia, abdominal swelling, reddened ascites, muscular oedema, necrotizing myositis, meningitis, olfactory neuritis, perineuritis and brain liquefaction. Trophozoites are thought to first invade olfactory rosettes and then track along branches of the olfactory nerve to enter brain. Intraocular infections can lead to exophthalmos (bulging) with the eyes becoming cloudy and possibly leading to blindness. Infected fish may exhibit locomotory dysfunction, respiratory distress, compulsive vigorous or erratic swimming, discolouration (blue swimmer syndrome in tuna), rapid loss of condition and death may occur within days of infection (due to cumulative respiratory, excretory and neural dysfunction). Risk factors for disease include stressed and immunocompromised fish (due to captivity, poor nutrition, poor water quality), particularly cultured or aquarium fish which are crowded in unhygienic conditions (often due to bacterial blooms due to overfeeding).

Developmental cycle and mode of transmission: *Uronema* spp. are free-living ciliates aquatic environments where they filter feed on bacterial flora, being commonly found among decaying algae. Trophozoites are able to survive in water over a wide range of temperatures (6-30°C) and salinities (10-35 ppt), with optimal growth occurring when bacteria are plentiful in waters ranging from 10-25°C. When food is abundant, they are able to multiply rapidly (within several hours) by asexual transverse binary fission. When food becomes limited, they may initiate sexual reproduction by conjugation involving pairs fusing anteriorly and exchanging genetic material (micronuclei). Some *Uronema* spp. also exhibit a starvation response whereby they rapidly divide to form small slender cells that begin rapid erratic 'swarming' behaviour and may survive starvation for 100-170 hours (i.e. the equivalent of ~ 45 generations of well-fed cells). It is thought swarming increases the likelihood of ciliates encountering hosts with higher body temperatures more suitable for growth and development. Only 2 species (*U. marinum* and *U. nigricans*) have been found to opportunistically infect fish, especially stressed and immunocompromised fish due to recent capture, transport, poor diet, crowded culture, or concomitant infections. Transmission between fish is direct via free-swimming stages in the water column. Ciliates are released from live and dead fish back into aquatic environments where they can survive for long periods as free-living individuals or they can opportunistically infect new susceptible fish. Some infected fish may act as asymptomatic carriers, with disease only developing in highly susceptible species or compromised individuals (especially in ornamental aquarium fish). Many infections in cultured fish species (particularly southern bluefin tuna) occur during the winter when water temperatures drop below 15-18°C.

Differential diagnosis: Infections may be indicated by the presence of suspicious lesions on fish surfaces with behavioural changes suggesting breathing difficulties. Diagnosis is reliant, however, on the direct microscopic detection of parasitic ciliates in host tissues, including skin scrapings, lesion biopsies, gill clippings, blood, cerebrospinal fluid, diced organs and tissue fragments. Samples may be examined as wet mounts, impression smears, squash preparations, or histological sections of fixed tissues. Live ciliates may be counter-stained with methyl-green pyronin to reveal nuclei, but they are best enrobed on albuminized slides and subject to silver staining (usually silver proteinate (protargol), but also silver carbonate and silver nitrate) to reveal oral and somatic ciliary patterns (basal bodies are strongly argentophilic). Clinical biochemical studies conducted on infected tuna revealed nonspecific elevations in serum protein, magnesium, sodium, chloride, cortisol and glucose, with reduced levels of potassium. Trophozoites readily adapt to cultivation in the laboratory in filtered water or tissue broth infusions so long as bacteria are added as a food source (usually *Vibrio* strains in marine systems, *Enterobacter* in freshwater systems). Several attempts have been made to develop specific antibody labels to help identify ciliates in clinical samples, but most exhibited problems with reagent cross-reactivity. Molecular biological techniques have been used to detect and characterize ciliates following the polymerase chain reaction (PCR) amplification of nuclear (small subunit ribosomal DNA) and mitochondrial (cytochrome c oxidase subunit 1) gene sequences.

Treatment and control: Infections in fish may be superficial and/or systemic, the former responding in some cases to chemotherapy, while treatment of the latter is ineffective due to the speed and severity of disease. Fish with topical infections have been treated by bathing in various chemicals (formalin, malachite green, methylene blue, copper, acriflavine, hydrogen peroxide), fed foods medicated with nitroimidazoles (metronidazole) or dosed internally (stomach tube or injections) with metronidazole, and even some anti-malarial drugs (chloroquine, quinacrine). Concurrent antibiotic therapy (e.g. nitrofurazone) was often used as secondary bacterial infections were common in skin and gill lesions. Many of the chemicals used, however, exhibited some toxicity to fish with the development of adverse side-effects, and treated fish often became rapidly re-infected when returned to their source waters. There is no fallow (fishless) period that can be used to combat *Uronema* infections, as the parasites can survive in tanks almost indefinitely once present. Control measures must therefore be adopted to decontaminate culture facilities and aquaria, prevent the introduction and growth of parasites in holdings, and help fish resist infections by avoiding stressful situations. Clean water (filtered and chlorinated where possible) should be used to rear and hold fish, with good flow and regular exchange. Ozonation and ultraviolet illumination have been used in aquaria to kill ciliates, although best results are obtained by draining and drying tanks before restocking. Sea cages should be regularly moved to avoid the accumulation of organic wastes, and they should be periodically treated for biofouling. Fish should be subject to regular health screening and new stock should be held in quarantine. In the event of disease outbreaks, sick fish should be promptly removed and/or culled and carcases removed for land disposal (burn or bury). Immunocompromised fish are more susceptible to infection and disease, so every effort should be made to minimize stressful events (capture, handling, sudden changes in environment) and eliminating predisposing factors (over-stocking, poor water quality, poor nutrition, organic enrichment through over-feeding leading to bacterial blooms). Experiments have also shown that some dietary immunostimulants (such as plants extracts from *Punica granatum*, *Chrysanthemum cinerariaefolium* or *Zanthoxylum shinifolium*) enhanced the innate immunity of olive flounder and improved their survival to challenge with *U. marinum*. Experimental vaccination studies have yielded some promising results when variable surface proteins on trophozoites known as immobilization antigens (i-antigens) were bound to PLGA nanoparticles (poly d,l-lactide-co-glycolic acid) and inoculated into kelp groupers where they significantly reduced mortalities when challenged with *U. marinum.*


Uronema
may reproduce asexually
(by transverse binary fission)
or sexually (by conjugation)
gills, skin, brain, viscera
(necrosis, haemorrhages,
lesions, encephalitis)
mostly free-living aquatic species,
some facultative histophagous parasites
Vertebrate Hosts
(freshwater and
marine fish)
oral ciliature:
paroral membrane
and 3 membranelles
post-oral scuticum
somatic ciliature:
10-16 longitudinal
kineties
macronucleus
micronucleus
contractile vacuole
long caudal
cilium
trophozoite
(20-50 µm)
no cyst formation
direct transmission between hosts
via free-swimming trophozoites in water column

Uronema trophozoite from salmon sea-cage

Uronema trophozoite from salmon skin (silver stain)