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Histophagous scuticociliatids (Ciliophora) parasitizing turbot *Scophthalmus maximus*: morphology, *in vitro* culture and virulence

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Abstract. Systemic ciliatosis caused by histophagous ciliates constitutes a serious disease of cultured turbot. Six ciliate isolates were obtained from parasitized turbot during six epizootics at four different farms located in Spain, France and Portugal. Axenic cultures of the six isolates were obtained by periodical subculturing in ATCC 1651MA or supplemented L-15 media. In basal media or seawater, the parasites could survive starving for long periods with no apparent proliferation. In adequate media, growth kinetics was found to be very similar for isolates A and B, with a clear influence of temperature. Morphological studies demonstrated that all isolates share common features that allows their assignment to either *Philasterides* Kahl, 1931 or *Miamiensis* Thompson et Moewus, 1964. However, statistically significant differences were evident in pairwise comparisons of the isolates from the four farm sites in 16 taxonomically relevant morphometric features. This could allow the discrimination of different species or strains. Virulence of isolates A and B for healthy turbot was tested in several experiments. Differences in the virulence were especially evident after long-term *in vitro* culturing, isolate A being clearly attenuated after 35–42 passages, whereas isolate B became more virulent after 20–42 passages. The need of further studies to confirm such virulence variability and its implications in pathogenesis and prevention of turbot scuticociliatoses is stressed.

Scuticociliates are ciliate protozoans (Ciliophora) occurring abundantly in eutrophic coastal and saprobic maricultural waters. Some of them can behave as opportunistic histophagous parasites causing severe infections in crustaceans and fish. Small (1967) proposed the new order Scuticociliatida including the suborders Pleuronematina, Pseudocohnilembina and Philasterina. Further revisions of the Ciliophora (Lynn and Small 2000) granted scuticociliates the level of subclass (Scuticociliatia), and the suborder Philasterina was ranked as order (Philasterida). Since the criteria used hitherto to classify the families, genera and species of scuticociliates have been variable, the taxonomy of these organisms is currently rather confusing.

Recently, different scuticociliates have become an important threat for cultured marine fish such as tuna and different flatfish. Fatal encephalitis of bluefin tuna, attributed to *Uronema nigricans*, was reported by Munday et al. (1997). Among flatfish, Jee et al. (2001) described scuticociliatosis of Japanese flounder, *Paralichthys olivaceus*. In turbot, different episodes of scuticociliatosis have been reported in Spain (Dyková and Figueras 1994, Iglesias et al. 2001, authors' unpublished data) and Norway (Sterud et al. 2000). Only in one of these turbot epizootics the causative organism was identified to specific level as *Philasterides dicentrarchi* Dragesco, Dragesco, Coste, Gasc, Romestand, Raymond et Bouix, 1995 by Iglesias et al. (2001). This species was originally described from an episode of Mediterranean seabass (*Dicentrarchus labrax*) mortality by Dragesco et al. (1995). Since the taxonomy of scuticociliates is complicated, in several cases of scuticociliatosis the involved parasite has not been identified to species level (Yoshinaga and Nakazoe 1993, Dyková and Figueras 1994, Umehara et al. 2003).

The frequency and severity of scuticocilatoses have increased in recent years in turbot cultures in different countries. Main clinical signs and general histopathology are similar in the different outbreaks, but some differences have been pointed out (Iglesias et al. 2001). Apart from the description of the disease, the most complete information available deals with the parasite reported as *Philasterides dicentrarchi* by Iglesias et al. (2001) and includes data on cultivation (Iglesias et al. 2003a), antigenicity (Iglesias et al. 2003b) and invasion routes of the ciliate (Paramá et al. 2003).

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In recent years, we have studied different outbreaks of scuticociliatosis in several turbot farms from four different geographic locations. In the present paper, preliminary morphological studies suggesting the existence of different species or strains are presented. In addition, data on *in vitro* cultivation, growth kinetics and virulence of some isolates are included.

MATERIALS AND METHODS

Outbreaks studied and obtainment of ciliates

Ciliates were isolated during outbreaks in four different turbot farms located in Spain, France and Portugal, as detailed in Table 1. All the farms had a flow-through water supply of seawater (pump-ashore sites) or well water.

During the outbreaks in the farms, samples of ascitic fluid and brain were taken from affected fish and sent refrigerated to the Institute of Aquaculture Torre de la Sal (IATS) facilities for further processing. Upon arrival to the laboratory, fresh smears of the ascitic fluid or squashes of brain were examined for the presence of motile ciliates. This material was used for morphological studies and as inoculum for *in vitro* culture.

In vitro culture

Ascitic fluid containing active ciliates was directly inoculated in different media. In some cases, ciliates were previously washed and concentrated by dilution of the samples with Hanks' Balanced Salt Solution (HBSS) containing an antibiotic/antimycotic mixture (PSA) at 1× to 3× concentration (1× PSA = 100 $U \cdot ml^{-1}$ penicillin, 100 $\mu g \cdot ml^{-1}$ streptomycin, and 0.25 $\mu g \cdot ml^{-1}$ amphotericin B), followed by centrifugation at 500 × *g* for 15 min. This process was repeated as needed to obtain inocula with clean and viable parasites.

Culture media. A sterile, artificial saltwater (20‰ salinity), prepared using Instant Ocean mixture (Aquarium Systems, Sarreborg, France), was used as a basal medium for the initial primary cultures and for starvation experiments. Other media tested include American Type Culture Collection (ATCC) 1651MA medium and modified Leibovitz's L-15 medium (Gibco-Invitrogen, Paisley, UK) containing 1.28% artificial marine salts (for a final salinity equivalent to approximately 20‰).

Using L-15 and artificial seawater media, several supplements were tested, alone or combined, as additional food sources to support ciliate growth: 10% heat-inactivated Foetal Bovine Serum (FBS), HBSS-washed turbot blood cells (TBC), 1× Eagle's Basal Medium (BME) vitamin mixture, and ribonucleic acid from torula yeast.

Culture conditions. Ciliates were maintained in 25-cm^2 T-flasks, with 7 ml media at 15 or 20°C. They were routinely observed and photographed using an inverted microscope. Subcultures were initiated by harvesting cultures in stationary phase, by centrifugation (500–650 × *g*, 15 min) and washing with HBSS containing 2× PSA. Parasites were counted in Neubauer chambers and inoculated into fresh media. Primary cultures included high doses of antibiotic/antimycotic mixture (3× PSA) but these were progressively lowered to a routine 1× PSA in subsequent passes, when no evidence of bacterial contamination was apparent. Axenic tests were performed by inoculating ciliates in basal media devoid of antibiotics that did not support ciliate multiplication but did allow bacterial growth if cultures were contaminated.

Growth kinetics. Ciliates from an early stationary phase culture of isolates A and B were seeded in 12-well plates containing 1.5 ml supplemented L-15 medium per well and incubated at two different temperatures (15 and 20ºC). For both isolates and temperatures, wells were inoculated 375 or 750 ciliates per well. The cultures were monitored daily with an inverted microscope and the number of ciliates was counted in triplicate wells at 0, 6, 24, 48, 72, 96, 120, 144 and 168 h post-seeding at 20ºC and at 0, 24, 72, 96, 120, 144, 168, 216, 264 and 366 h at 15ºC. The presence of dividing or conjugating ciliates was also assessed.

Morphological studies

The morphological study of ciliates freshly obtained from diseased fish was performed using conventional and inverted light microscopes. The observation under the light microscope was facilitated by adding 2% $MgCl_2$ in basal artificial seawater (Repak 1992). Measurements of the observable structures were taken. In addition, ciliates from the stationary phase of axenic cultures of the different isolates were used for morphological studies using two techniques:

Silver impregnation. Ciliates were stained using the wet silver nitrate method described by Foissner (1992) with some modifications. Briefly, ciliates were fixed in Champy's fluid, washed, and stored in Da Fano's fluid at 4ºC until further processing. Between the following steps, ciliates were pelleted by centrifugation at 14,000 × *g* for 2 min. After removal of the supernatant and washing with double-distilled water, ciliates were silver-impregnated in suspension with a 1% silver nitrate solution for 2 min, washed again and layered on Superfrost-plus slides (Menzel-Glaser, Germany), without additives. The aqueous suspension of silver-impregnated ciliates was irradiated under a coverslip with a 60 W incandescent lamp at 6 cm for 15–30 min. Slides were air-dried and dehydrated through a graded ethanol series, cleared with xylene and mounted in DPX.

Scanning electron microscopy (SEM). Ciliate suspensions were fixed with an equal volume of fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) at 4ºC for 30 min, pelleted by centrifugation (430 × *g* for 5 min), suspended again in fixative for 1.5 h, and washed three times with the same buffer. They were postfixed in 1% (v/v) cacodylic osmium tetroxide for 2 h at 4ºC, washed as above and dehydrated in a graded ethanol series up to 70%. Ciliates were then layered on poly-L-lysine-coated coverslips for 1 h in a humid chamber. The coverslips were passed through a graded ethanol series up to absolute, critical point dried and coated with a gold-palladium mixture in a Bio-Rad E5600 sputter coater. Samples were examined with a Hitachi S-4100 scanning electron microscope.

A detailed morphological description is beyond the scope of this paper. However, a comparison of four isolates was made in order to assess the similarities and differences. Sixteen features considered relevant for scuticociliate taxonomy were selected for statistical comparison: length/width ratio; number and arrangement of somatic kineties; distance from the posterior edge of the oral area to the apical end; oral area length to total length ratio; length of oral polykinetids OPK1,

OPK2 and paroral membranelles PM1 and PM2, and their distance to the apical end; distance of OPK3 to the apical end; distance of scutica to the apical end; and length of the cytoproct and its distance to the apical end. In addition, other characters such as the number and arrangement of kineties in the oral structures, the arrangement of the circular pole fibre and the posterior vacuole, and the presence and length of caudal cilium, were considered.

Statistics. Differences in values of the above mentioned 16 characters among the ciliates from the four sites were analysed using one-way ANOVA or ANOVA on ranks, followed by Student-Newman-Keuls or Dunn's methods, respectively ($P<0.05$). All the statistical analyses were performed with Sigma Stat software (SPSS Inc., IL).

Virulence assays

As new outbreaks occurred in farms 1 and 2, isolates A and B were selected for additional studies on their virulence. Experiments were carried out in two different facilities:

Facility 1: Small-scale experimental unit at Universitat Autònoma de Barcelona (UAB) facilities, consisting of eight square, 0.50m^2 tanks arranged as independent recirculation systems with individual biological filters. Daily controls of pH, temperature, salinity, ammonium and nitrite were carried out and partial water changes were performed as required. Fish were kept at 19 ± 1°C.

Facility 2: The pathology unit of an experimental facility in Galicia (north-west Spain) including 18 square, 1m^2 tanks with a flow-through supply of cartridge-filtered, UV-irradiated seawater. The temperature ranged from 15 to 19.5ºC during the experimental period.

Fish weighting about 70–90 g, with no previous contact with the ciliate, were used for the different experiments. After an acclimatisation period at the experimental units, fish were injected intracoelomically with the appropriate dose of ciliates in 0.2 ml of HBSS containing 0.35% NaCl. Control fish received the same volume of saline without ciliates.

Inocula. Two types of inocula were used: i) ciliates from primary cultures recently obtained from diseased fish, kept for short periods in basal artificial seawater and fed pieces of fish brain while their numbers multiplied until inoculation; and ii) ciliates from axenic cultures, harvested at different passages (Table 2). In both cases, ciliates were harvested, washed, counted and transferred to the adequate volume of HBSS plus 0.35% NaCl as a mother, concentrated stock. Within 20–24 hours, this stock was diluted to the inoculation doses that were injected to the fish, as described above. The active condition of the ciliates in all the treatment doses was verified by observation of a subsample under the microscope.

Experimental design. In Table 2, the different virulence assays carried out are summarised, with indication of the experimental unit, number of fish, and doses inoculated. Experiments in facility 2 were designed to verify at larger scale some previous results, with the final aim of assessing the apparent variation of virulence of cultured isolates for their potential use in immunoprophylaxis or challenge experiments.

After inoculation, fish were monitored daily regarding behaviour, ingested food, and mortality. Dead fish were weighed and the presence of ciliates was assessed by microscopic examination of a sample of ascitic fluid. The experiment was considered concluded several days after all mortality appeared to cease, when surviving fish returned to normal behaviour and feeding (20–30 days after inoculation). Cumulative mortality was calculated for each dose and the lethal dose 50 (LD_{50}) was obtained using the formula of Reed and Muench (1938).

RESULTS

Morphological studies

The morphology of freshly isolated ciliates from the four sites was very similar under both conventional and inverted light microscopes. However, for all isolates, ciliate shape and size were very variable according to their age, tissue from which they were obtained, and feeding status. The presence of very large individuals, often with several host cells engulfed within their body and deforming their normal shape was common in this material. Therefore, we used ciliates from axenic cultures, kept in the same conditions and harvested at standardised times after inoculation, for an accurate comparison of morphometric features.

Silver nitrate staining revealed information on the arrangement of somatic kineties, oral polykinetids (OPK1, OPK2, OPK3) and paroral membranelles (PM1, PM2) (Figs. 1–3). With this technique the number of cilia present in some of these structures was also counted. SEM provided some additional information on the visible structures, depending on the ciliate position (Figs. 4–6). Thus, the number of cilia of OPK1, PM1 and PM2 could be easily counted, and sometimes OPK3

Table 1. Details on the turbot farms, outbreaks and scuticociliate isolates studied.

Farm no.	Geographic location	Water supply (salinity)	Isolate	Date of obtention	Studies	
					In vitro and morphology	Virulence
1	Cantabric, France	Well water (28‰)	A-1	Jan 2002	yes	yes
			A-2	Aug 2002	yes	yes
2	Cantabric, NW Spain	Seawater (35‰)	B-1	Apr 2002	yes	yes
			B-2	Oct 2002	yes	yes
3	Atlantic, NW Spain	Seawater (32‰)	C	Aug 2001	yes	no
4	Atlantic, Portugal	Well water (24‰)	D	Oct 2002	yes	no

Table 2. Details on the virulence tests performed using different isolates of scuticociliates from turbot. In trials 2 and 4 the two isolates were tested simultaneously. No mortality was registered in control groups.

Trial no.	Type of culture	Isolate and passage (p)	Facility	Ciliates/fish ($\times 10^3$)	No. fish	Mortality (%)	Mortality interval (days p.i.)	LD_{50} ($\times 10^3$)
1	Primary cultures	A-2	1	5 15 45 135	10 10 10 10	20 30 0* 40	11–17 11–12 – 8–22	72.4
		B-2	1	5 15 45 135	10 10 10 10	0 20 40 70	– 9–10 7–11 8–11	81.2
2	Axenic cultures	A-1 p-35	1	20 60 180	10 10 10	0 40 70	– 8–15 7–18	210
		B-1 p-27	1	20 60 180	10 10 10	50 90 100	6–10 3–8 3–12	25
3	Axenic cultures	A-1 p-38	1	10 20 40	20 20 12	0 0 8.3	– – 13	>40
4	Axenic cultures	B-1 p-34	2	20 40 80	20 20 20	50 90 95	5–13 6–16 5–12	27.5
		B-2 p-20	2	20 40 80	20 20 20	65 90 95	8–18 7–15 7–13	<20
5**	Axenic cultures	A-1 p-42	2	5 10 20	100 300 300	4 26 34.3	9–13 4–22 7–19	>20

*LD_{50} was calculated omitting this dose; **Fish suffered from flexibacteriosis.

and part of OPK2 were also visible. The disposition of somatic dikinetids could also be observed.

The four geographical isolates shared several characteristics such as the presence of three oral polykinetids anterior to the cytostome, and paroral dikinetid with two different segments (PM1 and PM2), as well as the general disposition of these elements. They were also very similar in the length of the caudal cilium and the position of the posterior vacuole. However, they differed in some other features, mainly in the length/width ratio, the number of somatic kineties, the number of cilia in each oral polykinetid and paroral membranes, and the relative disposition of these structures in the body.

The pairwise statistical comparison of the 16 morphological features studied detected at least 6 significant differences among some isolates (A versus B and A versus D), although significant differences reached up to 10 characters in B versus D (Table 3).

In vitro culture

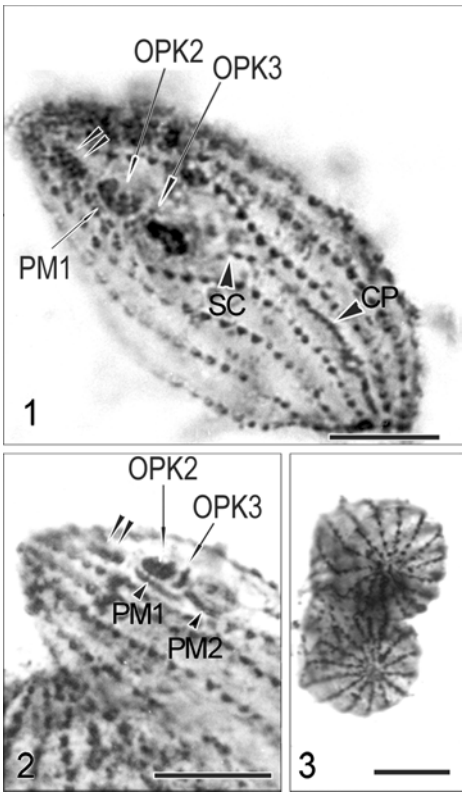
The initial attempts to maintain the parasite in marine salts or basal media without supplements allowed parasite survival for long periods. Proliferation of ciliates, however, was not attained under these conditions.

Table 3. Number of statistically significant differences ($P<0.05$) among the four geographical ciliate isolates in a pairwise comparison of 16 selected morphometrical characters. See text for details on the features compared.

	D	C	B	A
A	6	8	6	0
B	10	7	0	
C	8	0		
D	0			

Ciliate division and proliferation was observed when fish tissues (brain) or blood cells were added to the cultures or when bacterial contamination occurred (situation observed in several attempts). In such conditions, proliferation lasted as long as the external source of food, and ciliates acquired the globe shape frequently observed in those recently obtained from fish (Fig. 7). In the old cultures, aberrant forms, narrow or of unusual shapes, were common (Fig. 8).

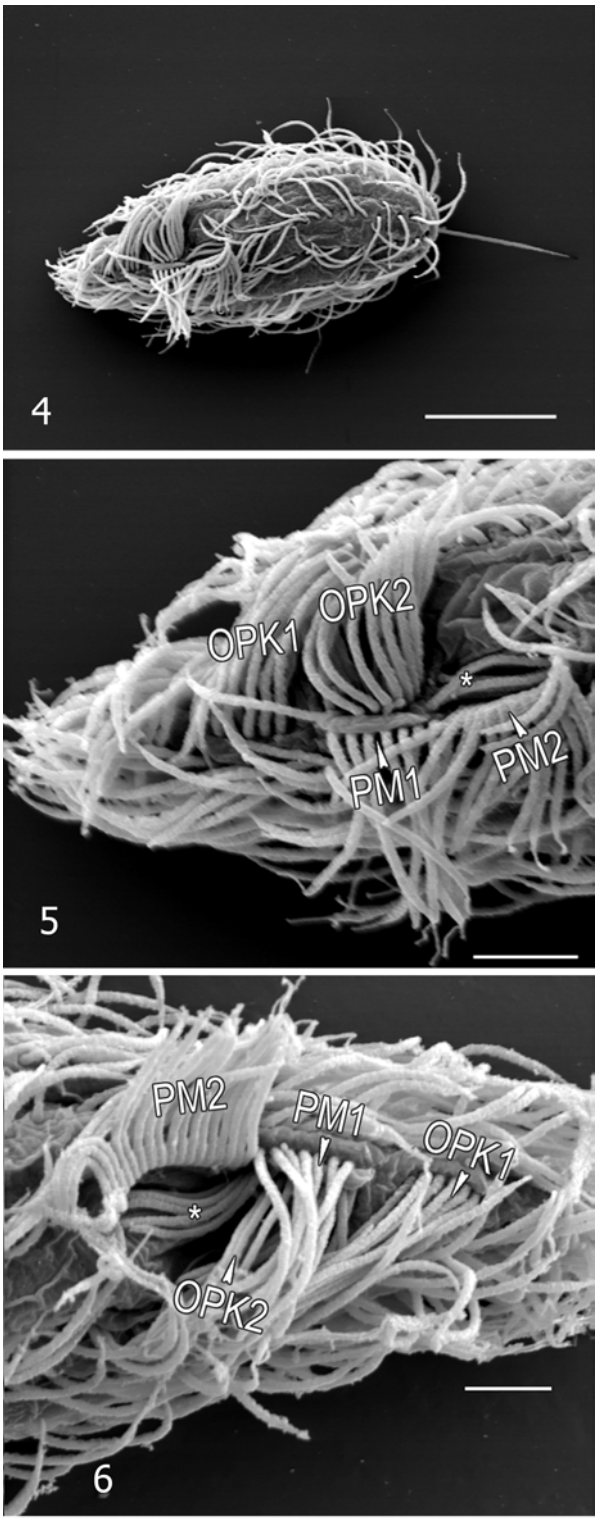
Proliferation of ciliates in axenic culture was achieved in ATCC 1651MA medium and in L-15 based media when adequately supplemented with FBS, vitamins and RNA. Axenic cultures were obtained of ciliates from the four sites (two isolates from both A and B sites, and one isolate each from C and D sites).



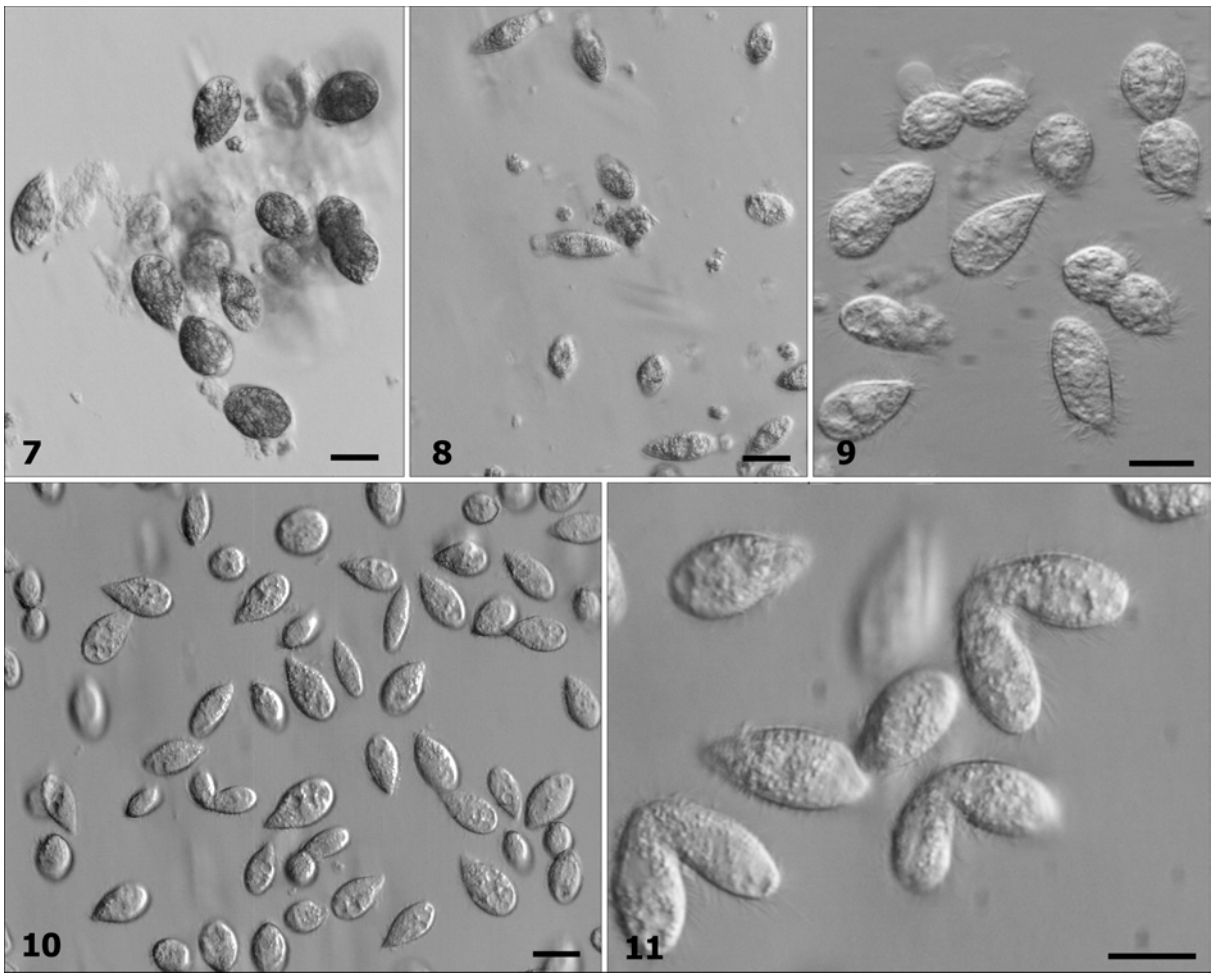
Figs. 1–3. Silver nitrate-impregnated scuticociliates from turbot *Scophthalmus maximus*. **Fig. 1.** Details of the oral infraciliature and other structures in a ciliate C. **Fig. 2.** Anterior end of a ciliate A, showing details of the oral infraciliature. **Fig. 3.** Posterior view of two A ciliates. OPK2, OPK3 – oral polykinetids; PM1, PM2 – paroral membranes; SC – scutica; CP – cytoproct. Double arrowheads point to OPK1. Scale bars = 10 μm.

When observed under the inverted microscope, ciliate division was evident shortly after seeding in medium (Fig. 9) and the number of dividing ciliates increased progressively until stationary phase (Fig. 10), when conjugating ciliates usually started to appear (Fig. 11). The four isolates studied presented a similar development cycle under culture, though conjugating pairs appeared consistently earlier and in larger numbers for the B isolates.

In growth experiments, the pattern was very similar for isolates A and B (Fig. 12). After an exponential growth phase, organisms entered the stationary phase, in which the number of conjugating ciliates increased progressively. There was a clear influence of temperature on growth. Thus, the exponential phase started earlier and was shorter at 20ºC than at 15ºC (Fig. 12). At 20ºC, the exponential phase occurred at 72–96 h. The stationary phase started at about 120 h, and in the last count (168 h), the number of ciliates slightly decreased. No significant differences were observed when seeding 750 (Fig. 12) or 375 (data not shown) ciliates per well.



Figs. 4–6. Scanning electron micrographs of scuticociliates from turbot *Scophthalmus maximus*. **Figs. 4, 5.** Ciliate D in ventral view. **Fig. 4.** General view. **Fig. 5.** Details of the oral infraciliature. **Fig. 6.** Details of the oral infraciliature in a ciliate B (ventral view). OPK1, OPK2 – oral polykinetids; PM1, PM2 – paroral membranes. OPK3 (*) is deep in the oral cavity. Scale bars: Fig. 4 = 10 μm; Figs. 5, 6 = 2.5 μm.



Figs. 7–11. *In vitro*-cultured scuticociliates from turbot *Scophthalmus maximus*. **Fig. 7.** Primary culture. Note the bloated body shape due to ingested host materials. **Fig. 8.** Starved ciliates in artificial saltwater, of elongate and abnormal shape. **Figs. 9–11.** Axenic cultures in different growth phases. **Fig. 9.** Details of binary fission during proliferative phase. **Fig. 10.** Aspect of a culture in early stationary phase. **Fig. 11.** Detail of conjugating pairs in late stationary phase. Ciliates A (Figs. 7, 8, 10) and B (Figs. 9, 11). Scale bars = 20 μm.

Growth was delayed and slightly different at 15ºC. The exponential phase occurred at 144–216 h with 375 ciliates per well, and at 120–168 h with 750 ciliates per well. The stationary phase was prolonged and in the last observation (336 h) the number of ciliates had not started to decrease.

Virulence tests

Results of virulence assays are summarised in Table 2 and Figs. 13–14. Primary cultured isolates A-2 and B-2 appeared to produce different results. As shown in Fig. 13 and Table 2 (trial no. 1), the second highest A-2 dose (45×10^3 ciliates per fish) did not cause any mortality, although cumulative mortality was higher for the two lowest doses when compared to isolate B-2. As a result, if dose 45×10^3 is not considered, LD_{50} values are very similar for both isolates, whereas if this anomalous result is accepted, the LD_{50} is 237×10^3 for the A-2 isolate. Mortality occurred earlier and lasted a shorter period for B-2 (7–12 days) than for A-2 (8–18 days).

When virulence of axenically cultured isolates from these same farms was tested, the situation was completely different, as the LD_{50} was clearly higher for isolate A-1 compared to isolate B-1 (210×10^3 versus 25×10^3). Again, most fish injected with B-1 died earlier and in a shorter period than those inoculated with A-1 (Table 2, trial no. 2 and Fig. 13.).

In a further trial in facility 1, a higher number of fish was used in order to assess the virulence of low doses of axenically cultured isolate A-1, as its low virulence suggested its good potential for immunoprophylactic purposes. Only one fish died with the highest dose used (8.3% mortality) (Table 2, trial no. 3). Thus, a subsequent experiment was carried out with low doses of this axenically cultured A-1 isolate, at a larger scale in

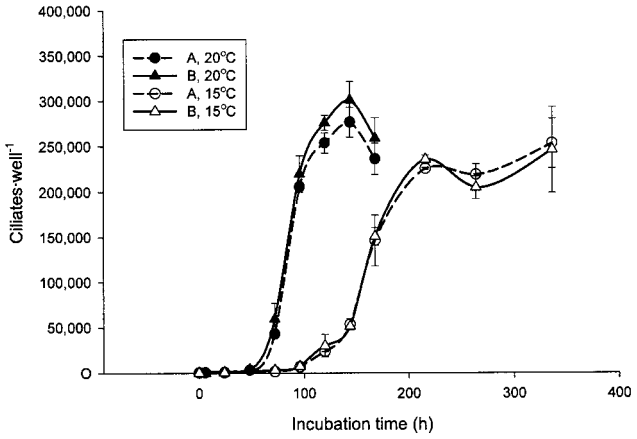


Fig. 12. Growth kinetics of ciliates A and B in axenic culture (L-15 medium supplemented with 10% FBS, 1% RNA, 1× BME vitamins and 1.28% marine salts). Ciliates (750 per well) were seeded in triplicate wells, cultured at two temperatures and counted at different incubation times.

facility 2. Lethal dose 50 could not be calculated, but mortality was higher than in the previous experiments with the same isolate using equivalent doses (Table 2, trial no. 5, Fig. 14). Also tested in facility 2, axenically cultured isolates B-1 and B-2 were inoculated to fish from the same stock, resulting in a LD_{50} clearly lower than that of A-1, tested under equivalent conditions (Table 2, trial no. 4, Fig. 14). The mortality patterns were similar to those obtained in previous experiments, as mortality occurred earlier and lasted a shorter period with isolates B compared to isolates A.

No mortality was registered in control fish in any of the virulence tests carried out.

DISCUSSION

Morphology and taxonomy

A combination of different techniques is necessary in order to obtain complete information on the morphology of these ciliates, as some methods do not allow the observation of certain structures.

Although a detailed morphological study of the different isolates is beyond the scope of this paper, some interesting information has been obtained. Our results demonstrate that the four isolates studied share several characteristics, such as the presence of three oral polykinetids anterior to the cytostome, paroral dikinetid with two different segments, and relatively shallow oral cavity. Such features allow their ascription to the same group, in the order Scuticociliatida Small, 1967 (subclass Scuticociliatia, according to the classification of Lynn and Small, 2000). They also have the characters of the order Philasterida, characterised by the paroral dikinetid shorter than other oral structures, and the lack of a ribbed wall from the paroral dikinetid towards the cytostome. According to the system of Lynn and Small (2000), there are 15 families in this order, including Uronematidae and Philasteridae, differentiated by the anterior end (a non-ciliated, flat disc in Uronematidae versus a non-flattened disc in Philasteridae). In addition, the Uronematidae present reduced oral polykinetids with OPK1 unciliated and a long oral area; in contrast, the Philasteridae present triangular OPK1, equal to or smaller than OPK2, and a rather shallow and anterior oral cavity. According to these criteria, our ciliates belong to the family Philasteridae.

Genus and species assignment is more difficult, as the taxonomy of many members of Scuticociliatida is unclear. The four isolates have two separated paroral segments, so they could belong to either *Philasterides* Kahl, 1931 or *Miamiensis* Thompson et Moewus, 1964. A different isolate from turbot was identified as *Philasterides dicentrarchi* by Iglesias et al. (2001). However, the authors did not provide information on the number of cilia in each of the oral polykinetids and paroral segments, nor was this information included in the original description of this species from seabass (Dragesco et al. 1995). For further complication, Song and Wilbert (2000) redefined some scuticociliate genera, gave improved diagnosis for *Paranophrys* Thompson et Berger, 1965 and *Miamiensis*, revised their family assignment, and proposed that *Philasterides dicentrarchi* was synonymous to *Miamiensis avidus*. They included *Miamiensis* in the Uronematidae, in the light of general features of ciliature and despite its pointed anterior end, thus contradicting the above-cited classification of Lynn and Small (2000).

Thus, both the species assignment and the discrimination between isolates are not possible with the available information. Further morphological studies, including stomatogenesis and the possible existence of polymorphic life cycles (Grolière 1974, 1980, Fenchel 1990, Pérez-Uz and Guinea 2001), could generate additional insights for identification. Therefore, in our view, considering the confusing taxonomy of this group of ciliates, life-cycle and molecular studies are necessary for an accurate comparison allowing a definitive taxonomic placement, as it has been stressed by some authors (Diggles and Adlard 1995, Wiackowski et al. 1999). Thus, DNA-based molecular studies of our isolates are underway, and will be presented elsewhere in parallel with a complete morphometric study.

In vitro studies

There are many reports on *in vitro* culture of different scuticociliates. Several authors use bacteria as a food source for different species from marine waters (Parker 1976, Nerad and Daggett 1992, Pérez-Uz 1995, Crosbie and Munday 1999). Some authors describe the maintenance of ciliates in saltwater, at least for short terms, by feeding fish brain tissue (Yoshinaga and Nakazoe 1993, Kwon et al. 2003). However, culture of marine scuticociliates in axenic conditions has also been described, using relatively simple media and even chemically defined media (see Nerad and Daggett 1992).

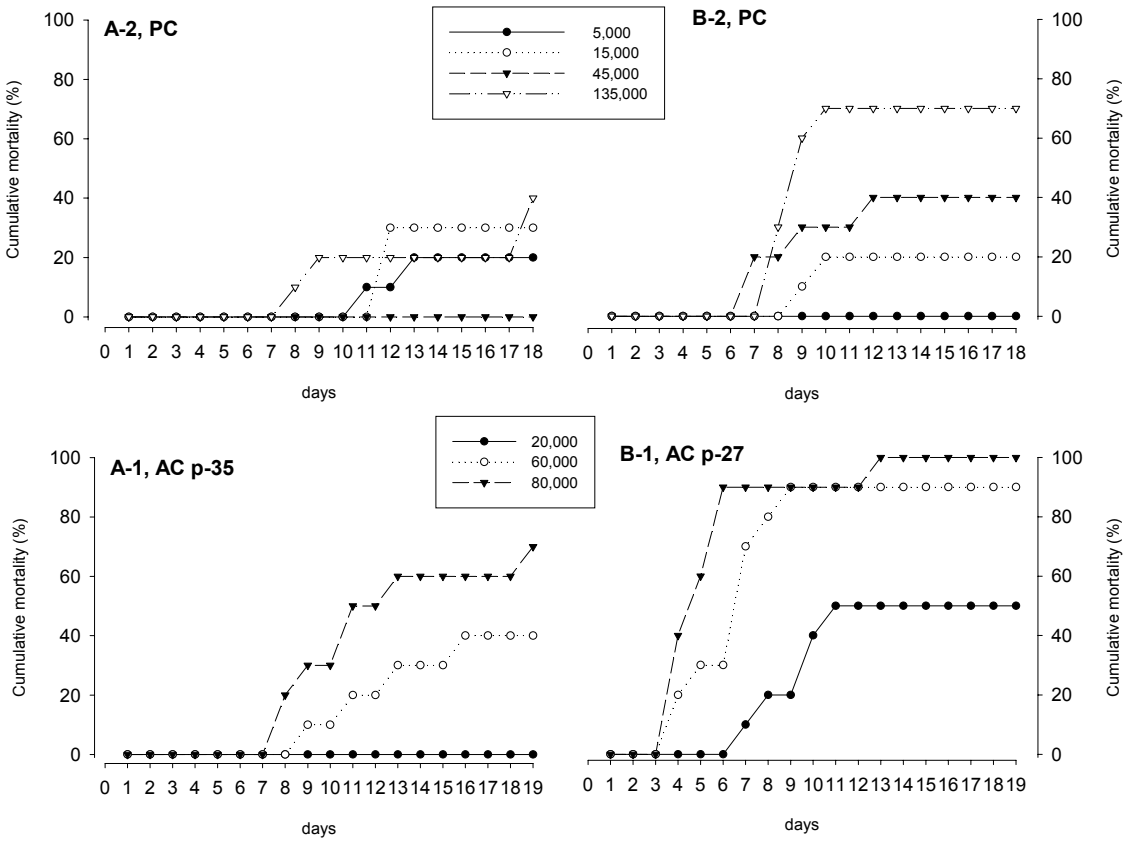


Fig. 13. Daily cumulative mortality of turbot in virulence tests (trials no. 1 and 2) using different doses of ciliates A and B from primary cultures (A-2, PC and B-2, PC; top) and from axenic cultures at different passages (A-1, AC, passage 35 and B-1, AC, passage 27; bottom). Doses used (ciliates per fish) are indicated in the legend boxes. No mortality was registered in control groups of fish (not shown).

We could also easily grow the ciliates in the presence of bacteria or by adding blood or pieces of brain as food source. However, our purpose was to obtain cultures in standardised conditions to be used for further experiments on morphology, behaviour, pathogenesis and virulence. Thus, we obtained axenic cultures of the six isolates studied (two different isolates from both sites A and B and one isolate from each site C and D). Cultures were initially developed using modified ATCC 1651 MA medium. This medium is based on formulations by Soldo and Merlin (1972) and consists of artificial brackish water (approximately 20‰ salinity) containing peptone, trypticase, L-alpha-lecithin and RNA as food sources, and including a mixture of vitamins. Our modification of this medium did not include L-alpha-lecithin and used a commercially available vitamin mixture with a somewhat different composition (BME vitamin solution 100×). This medium supported ciliate growth, but we formulated a more defined medium based on standard, commercially available tissue culture media and supplements. We found L-15 medium, supplemented with 1.28% artificial marine salts, 10% FBS, 1% RNA from torula yeast and 1× BME vitamin solution, a very convenient formulation which fully supported ciliate growth. The different isolates of this study have been maintained in axenic culture in this medium for up to 24 months. Iglesias et al. (2003a) reported the use of L-15 with 10% FBS for the culture of the ciliate obtained from turbot and identified as *Philasterides dicentrarchi*. However, they supplemented the medium with glucose, nucleosides, lecithin and Tween 80. The adaptability of histophagous scuticociliates to different media formulations, given that essential nutrients and some growth factors are present, is remarkable.

We could assess the high capability of these ciliates to starve for several months, using basal medium or medium with exhausted nutrients. In such conditions, the ciliates become slender and acquire unusual shapes. The significance of such changes for the ciliates' ecology, if any, needs further investigation. However, ciliates recovered and initiated normal growth and division when food (pieces of brain or TBC) was added to starving cultures, or when they were changed to complete medium.

The influence of temperature was demonstrated by Iglesias et al. (2003a) for cultured *Philasterides dicentrarchi*, with optimum growth at 18–23ºC and negligible growth at 13ºC. All the isolates studied in our work

grow well at 15ºC (the temperature of routine maintenance), although growth is much faster at 20ºC (Fig. 12). This temperature range could be relevant for the onset of infections, as 15ºC is close to the optimum for farmed turbot. Although outbreaks occur mainly at higher temperatures (18–20ºC), ciliates can be present in asymptomatic infections at lower temperature.

The growth of isolates A and B in axenic cultures was similar except for conjugation. In each culture, conjugating pairs usually occurred synchronically in the stationary phase or when nutrients were exhausted, but consistently were both found earlier and were more frequent in isolate B than isolate A. Conjugation in ciliates can be induced by starvation of mature individuals (Miyake 1996, Sugiura and Harumoto 2001), genetic diversity of mating types being also an important factor (Doerder et al. 1996). Thus, the conjugation difference found between isolates A and B might be due to their different sensitivity to nutrient exhaustion or be related to a possibly higher genetic diversity of isolate B than the other isolates (which, however, should yet be studied by DNA fingerprinting methods).

Virulence assays

Some differences in virulence were observed between isolates A and B. Also, virulence was found to be different between isolates recently obtained from fish and those after several *in vitro* passages.

As evaluated from LD_{50} values, primary cultures of isolates A-2 and B-2 showed very similar virulence (if the anomalous result of A-2 dose 45×10^3 ciliates per fish is eliminated from the calculations). However, the behaviour of isolate A-2 was somewhat different in the mortality pattern, the lower repeatability of results and the anomalous results obtained with some relatively high doses that did not cause mortality whereas some fish died with lower doses. This situation was also observed in another previous experiment (data not shown).

In addition, both isolates showed a completely different virulence when assayed after many passages in axenic culture. In initial trials with axenically cultured isolate A-1 (using high doses of ciliates) very high LD_{50} (low virulence) was observed, although in a subsequent trial, virulence was somewhat higher with comparable doses, such as 20×10^3 ciliates per fish. However, in this last trial at larger scale, some fish suffered from flexibacteriosis during the experiment, which undoubtedly amplified the pathogenic effect of the ciliate. On the other hand, axenically cultured isolates B-1 and B-2 increased their virulence (lower LD_{50}) after several culture passages, compared to primary cultured B-2 isolate freshly obtained from fish. The observed differences in the virulence of our isolates A and B (coming from farms both geographically distant and with different salinity) could have implications in the pathogenicity of these ciliates. The increased virulence of the attenuated, axenically cultured A-1 isolate, when tested in fish suffering from flexibacteriosis, also evidences the influence of stressful conditions or concomitant infections on the pathogenic effects of these ciliates, as already pointed out by different authors (Cheung et al. 1980, Dragesco et al. 1995, Sterud et al. 2000).

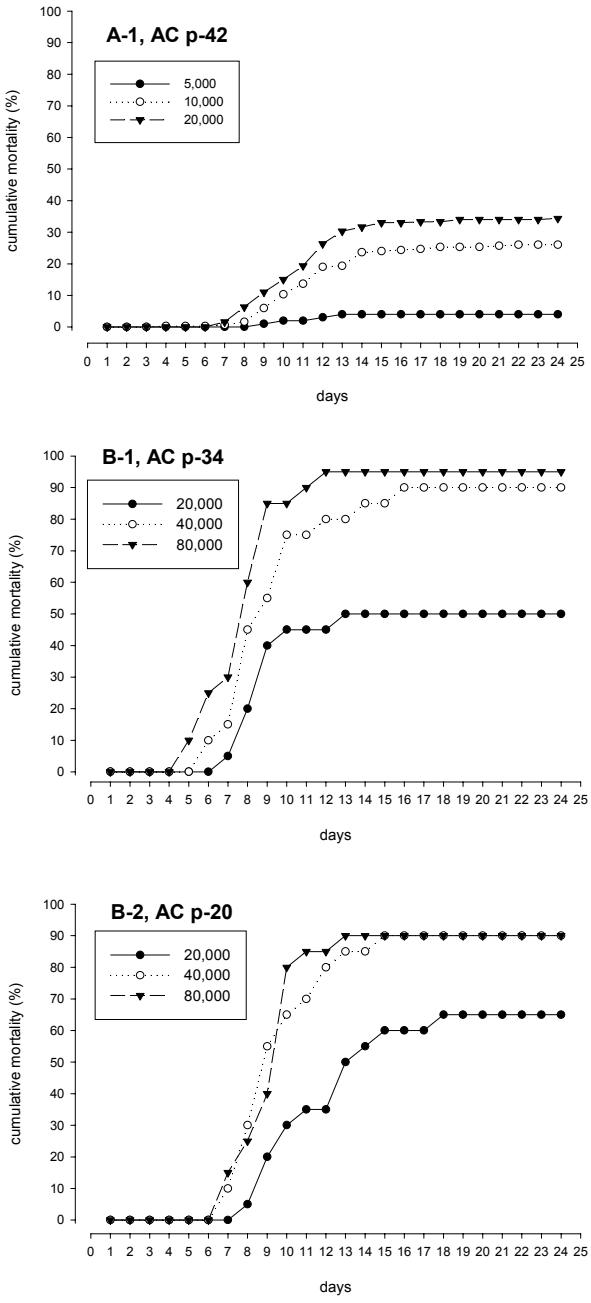


Fig. 14. Daily cumulative mortality of turbot in virulence tests (trials no. 4 and 5) using axenically cultured ciliates A and B at different passages: A-1, AC passage 42 (top), B-1, AC passage 34 (middle), and B-2, AC passage 20 (bottom). Doses used (ciliates per fish) are indicated in the legend boxes. No mortality was registered in control groups of turbot (not shown).

Some attempts of experimental infection of fish with scuticociliates have been reported previously, but we have not found precise references to virulence tests or LD_{50} calculations. Paramá et al. (2003) inoculated turbot intracoelomically with *Philasterides dicentrarchi* (from cultures bacterized with *Vibrio anguillarum*) causing higher mortality rate than similar doses of our isolate B. However, they used smaller fish. In different experiments by the same authors (Iglesias et al. 2003b), 5×10^5 ciliates from an axenic culture were used as a challenge, a dose much higher than the ones they had used previously (Paramá et al. 2003). However, the differences in the ciliates' culture conditions reported in both studies are significant and, furthermore, it is not clear if the same isolate was used in both series of experiments or for how long the ciliates had been maintained under culture. As evidenced from our results, both the origin of the isolates and their *in vitro* cultivation could have a significant influence on their behaviour and virulence. In the current study, axenically cultured ciliates had clearly different virulence than primary cultured ciliates of the same origin but, interestingly, virulence increased after axenic culture for ciliate B but decreased for ciliate A. Kwon et al. (2003) reported extracts from long-term cultures of *Uronema marinum* to have a lower protease activity, and less effect on olive flounder phagocyte respiratory burst than short-term cultures of that histophagous scuticociliate. A possible relation to a lower infection potential, although suggested by the authors, was not experimentally demonstrated.

Attenuation of virulence has been reported for other parasites cultured *in vitro*. Some of them are even used in different vaccine preparations (reviewed in Dalton and Mulcahy 2001). An experimental vaccine for the fish flagellate *Cryptobia salmositica*, using a strain attenuated through *in vitro* culture, is also available (Woo 1990). In the current study, the effect of axenic *in vitro* cultivation on the virulence expressed by different isolates was demonstrated, but the attenuation of isolate A was not complete with the doses and conditions tested. Further studies are needed to explore the possible attenuation of turbot scuticociliates, using prolonged *in vitro* culture or other modulating strategies contributing to a decrease in virulence.

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