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The dinoflagellates *Pfiesteria shumwayae* and *Luciella masanensis* cause fish kills in recirculation fish farms in Denmark

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ABSTRACT

Fish kills in two geographically separate fish farms in northern Denmark in 2012, one using marine, the other brackish water 'Recirculation Aquaculture Systems' (RAS), were found to be caused by *Pfiesteria shumwayae* and *Luciella masanensis*, two species of dinoflagellates belonging to the family Pfiesteriaceae. There were no other harmful algae present in either of the aquaculture plants. Serious fish kills in the US have been attributed to Pfiesteria during the past 20 years, but this type of mortality has not been documented elsewhere. *L. masanensis*, described recently from Korea and USA, has not been previously reported to be the source of fish kills. In the marine farm, the affected fish was rainbow trout, in the brackish water farm pikeperch. Light microscopy is presently insufficient to discriminate between the approx. 20 species of the family Pfiesteriaceae described. Identification of the two algal species was therefore based on molecular sequencing of nuclear-encoded LSU rDNA, confirmed by scanning electron microscopy and, eventually, also by examination of the very thin amphiesmal plates of the flagellates by calcofluor-stained cells in a fluorescence microscope.

Although the two fish farms differed in light and salinity conditions, both farms used re-circulating water in closed circuit systems. The dinoflagellates were examined in detail and shown to feed on organic material such as live, damaged nematodes, as described for the single pfiesteriacean flagellate known from freshwater, *Tyrannodinium edax*. Algal cells were observed to attach to their prey by an attachment filament and subsequently used a peduncle to suck up the food. Fish farms utilizing water recirculation technology are gaining popularity due to their reduced effect on the environment. The two cases from Denmark are apparently the first RAS farms in which serious fish kills have been reported. In the marine farm (*Luciella*) fish mortality increased dramatically despite treatment of the water with peracetic acid and chloramine-T. The plant was temporarily closed down pending investigation into the cause of mortality and subsequently to determine a method of management to control the dinoflagellate and avoid future fish kills. In the brackish water farm (*Pfiesteria*), water was treated with chloramine-T, which caused the dinoflagellates to disappear temporarily from the water column, apparently forming temporary cysts. The treatment was repeated after a few days to a week, when the temporary cysts appeared to germinate and the dinoflagellates reappeared in the water column.

1. Introduction

Pfiesteria shumwayae Glasgow & Burkholder is a very small heterotrophic dinoflagellate first described from the southeastern USA as the cause of extensive fish kills during the years 1991–1996 (Glasgow et al., 2001). It was transferred to a separate genus, *Pseudopfiesteria*, by Litaker et al. (2005), based mainly on its possession of an extra singular plate, compared to the type and only other species of *Pfiesteria* known, *Pfiesteria piscicida*. The transfer was disputed by Marshall et al. (2006), and we have followed this recommendation, as molecular evidence indicates that the two species are closely related. *Pfiesteria* belongs in the family Pfiesteriaceae of the order Peridiniales, and the family presently comprises eight genera, most of which are small,

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morphologically alike and difficult to identify (Calado et al., 2009). They differ morphologically mainly in the number of amphiesmal plates on the epicone, as illustrated graphically by Calado et al. (2009). The upsurge in the interest in these dinoflagellates was caused by the massive fish kills attributed to *Pfiesteria*, particularly in Maryland and North Carolina (Glasgow et al., 2001), which resulted in huge economic losses. Effects on humans were also reported (Glasgow et al., 1995; Grattan et al., 1998). However, a potential toxin was difficult to identify. Fish kills in the laboratory were found by Gordon et al. (2002) and Gordon and Dyer (2005) found the most consistent fish mortality to require physical contact between fish and *Pfiesteria* cells. A toxin was identified in the two *Pfiesteria* species by Moeller et al. (2007). However, some researchers were unable to confirm the toxicity data and questioned their validity, resulting in a controversy from the mid-1990s (see also Rabinsky and Flemming, 2004). Foremost among the papers questioning the data was Place et al. (2008), while a recent paper by Burkholder and Marshall (2012) summarized the data in favor of *Pfiesteria* species causing the fish kills.

Like many other marine dinoflagellates, the two species are geographically widespread. Using a PCR assay, *Pfiesteria shumwayae* was reported from Asia, Australia, New Zealand, Europe and both Americas (Rublee et al., 2004). In Europe the closest known occurrence is in Norway (Jakobsen et al., 2002). The potentially lethal effects of these algae on fish have caused concerns, particularly in fish farms. However, up until now, algal-related fish kills and other negative effects in wild fish stocks or in fish farms have not been reported outside the US.

The present article describes two fish kills within re-circulated water aquaculture plants in Denmark. The first event took place in December 2011 in a brackish water farm in northern Jutland, where the toxic alga responsible was identified to be *Pfiesteria shumwayae*. A second fish kill occurred 6 months later on a land-based marine farm in Hirtshals, northern Jutland. It was identified as *Luciella masanensis*, a species known from North America and Japan (Mason et al., 2007), but not previously associated with fish kills. From studies in Korea, it is known to feed on several other algae and fish blood cells (Jeong et al., 2007a; Baek et al., 2010). The water utilized in the aquaculture plants was re-circulated, and such plants are considered to be a positive development in mass culture of fish, due to their reduced impact on the environment. In the second farm, the harmful effect of the algae eventually resulted in the closure of the unit. Both these cases will be described and discussed below.

2. Materials and methods

2.1. Material

The material derived (1) from Ejsing Seafarm, Venø Bugt and (2) from Section for Aquaculture, National Institute of Aquatic Resources, the North Sea Science Park, Hirtshals (Technical University of Denmark) (see Fig. 1 for locations in the northern part of Jutland, Denmark). Water samples containing live dinoflagellates were posted to Dept of Biology, University of Copenhagen and identified using molecular, light- and scanning electron microscopical analyses. The Ejsing material (Fig. 1S, Figs. 2 and 3) was maintained at 15 °C either as enrichment cultures grown in L16 freshwater medium (Lindström, 1991) and fed the blue-green cryptomonad *Komma* sp. (strain SCCAP K-1622), or in L1 medium (Guillard and Hargraves, 1993) with a salinity of 30 psu and fed the red cryptomonad *Rhodomonas*/*Storeatula* sp. (strain SCCAP K-1488). The material from Hirtshals (Fig. 2S, Figs. 4 and 5) was single-cell isolated and maintained at 15 °C in L1 medium (PSU 30), and fed *Rhodomonas*/*Storeatula* sp. (SCCAP strain K-1488).

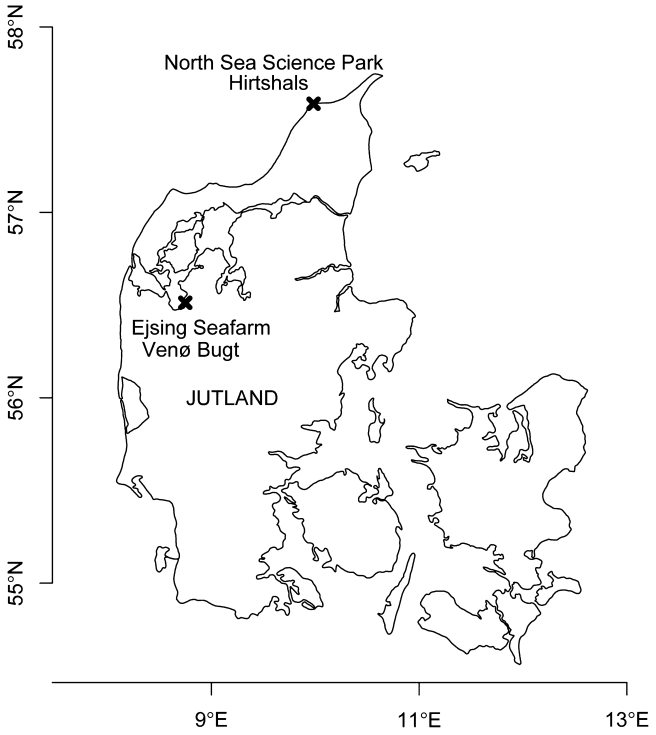

Fig. 1. Map showing locations of the two Danish fish farms that experienced fish kills from December 2011 onwards at Ejsing Seafarm, Vinderup, and in July 2012 at the North Sea Science Park, Hirtshals.

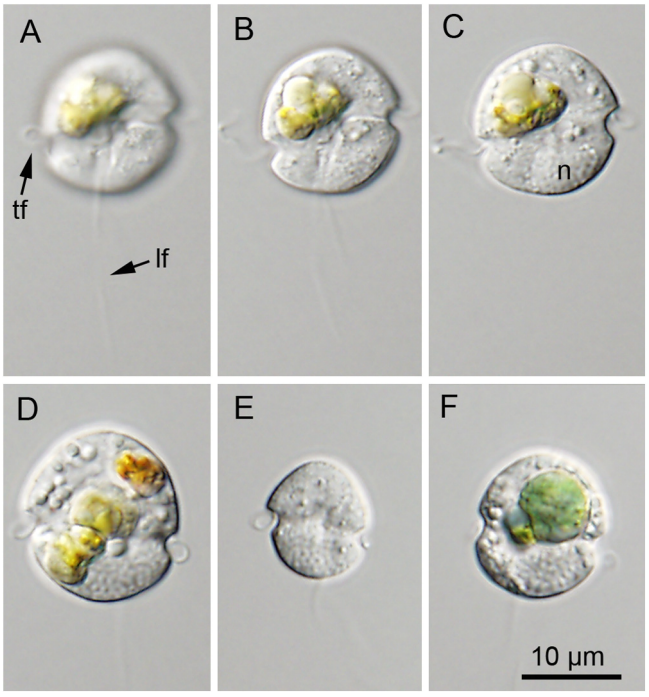

Fig. 2. Light microscopy of live cells of *Pfiesteria shumwayae* (differential interference contrast). (A–C) Same cell in different focal planes (ventral to dorsal). The cell has been fed the reddish cryptophyte *Storeatula*/*Rhodomonas* sp., and remnants are clearly visible in the food vacuole; n, nucleus; lf, longitudinal flagellum; tf, transverse flagellum. (D) Large cell containing several food vacuoles. (E) Small cell with no visible food vacuoles. (F) Cell that has been fed with the blue green cryptophyte *Komma* sp.

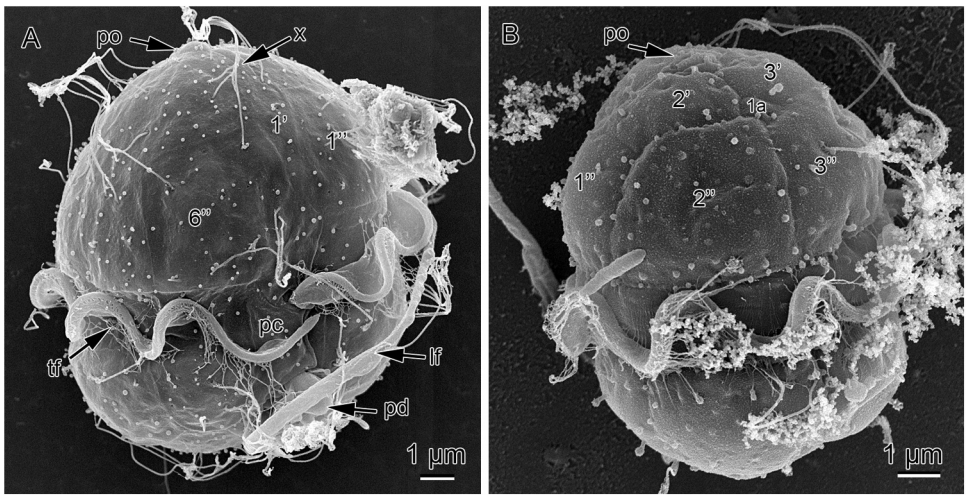

Fig. 3. Scanning microscopy of *Pfiesteria shumwayae*. (A) Cell seen in ventral view; lf, longitudinal flagellum; tf, transverse flagellum; pd, peduncle; pc, peduncle cover plate. (B) Cell seen in dorsal view. Notice small 4-sided 2a plate.

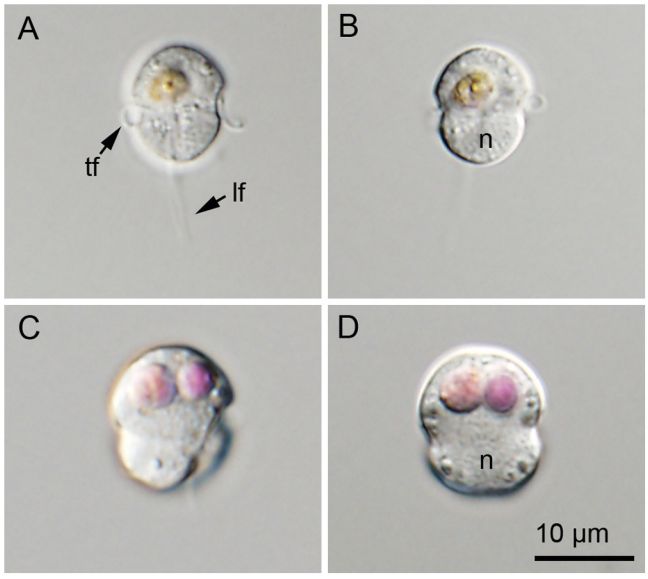

Fig. 4. Light microscopy of live cells of *Luciella masanensis* (differential interference contrast) fed *Storeatula/Rhodomonas* sp. (A and B) Same cell in different focal planes. One food vacuole is present. (C and D) Large cell containing two food vacuoles.

2.2. Light microscopy

The light micrographs included (Figs. 2 and 4) were taken on an Olympus BX51 microscope equipped with differential interference contrast and an Olympus DP-72 digital camera (Olympus, Japan). For epifluorescence of calcofluor-white-stained material approx. 5 ml of a culture of *Luciella masanensis* was fixed in glutaraldehyde (final concentration 2%). Calcofluor White M2R (Polysciences; 0.2 ml of 10 mg l^{-1} solution in distilled water) was added to the fixed cells, and the sample was filtered onto a MontaMil® polycarbonate membrane filter (pore size 0.2 µm) under a slight vacuum. The filter was placed on an objective slide containing a drop of immersion oil. Before placing a cover slip on the filter, one additional drop of immersion oil was added to the filter. We used a Zeiss Axio Imager.M2 equipped with epifluorescence and filter set 49 from Zeiss (excitation 365 nm, emission 445 nm) to view the arrangement of thecal plates (Fig. 5A and B). The same method was used for *Pfiesteria*, but the results were not satisfactory.

2.3. Scanning electron microscopy (SEM)

Among several fixation schedules tried, the following revealed, to some extent, the plate pattern: The material from Ejsing grown in L16 was fixed for 15 min in a mixture of 0.5% OsO_4 and 8.7%

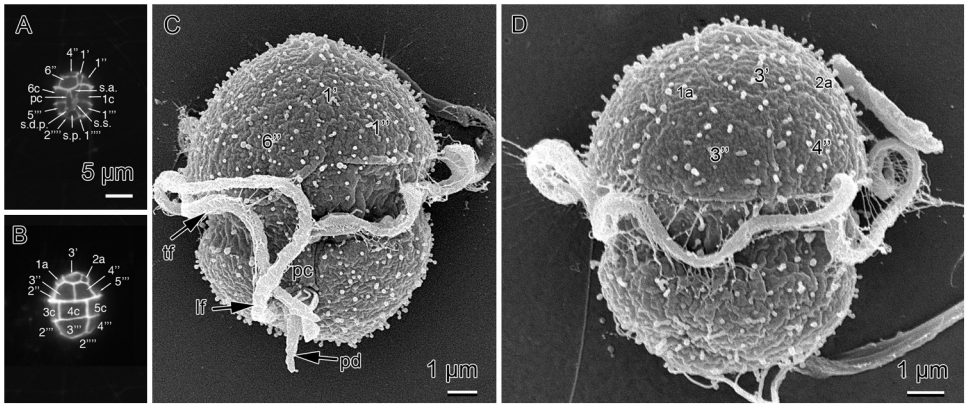

Fig. 5. Epifluorescence (calcofluor stained cells: A and B) and scanning electron microscopy (C and D) of *Luciella masanensis*. (A) Ventral view. (B) Dorsal view. (C) Ventral view. (D) Dorsal view. Notice the presence of the two small intercalary plates (1a and 2a), other plates are also labeled.

$HgCl_2$ (final concentrations). Material grown in L1 was fixed for 15 min in a mixture of 2% OsO_4 and 0.125% glutaraldehyde (final concentrations). The material from Hirtshals was fixed 30 min in 2% OsO_4 (final concentrations). In all cases the fixed material was placed on poly-L-lysine coated circular coverslips. After washing in dH_2O for 30 min, samples were dehydrated in an ethanol series: 30%, 50%, 70%, 96%, 99.9% for 10 min in each change, and finally in two changes of 100% ethanol, 30 min in each change. Critical point drying was in a BAL-TEC CPD-030 (Balzers, Liechtenstein). The cover slips were mounted on stubs and coated with palladium-platinum and examined in a JEOL JSM-6335F field emission scanning electron microscope (Tokyo, Japan) (Figs. 3 and 5C and D).

2.4. Molecular identification

The molecular characterization used to identify the material to species level was based on single-cell PCR and determination of partial nuclear-encoded LSU rDNA. Single cells were isolated with a minimum volume of water and frozen until PCR amplification. To amplify approx. 1800 base pairs of this gene we used a eukaryotic primer (D1F) in combination with a primer designed specifically for dinoflagellates (Dino-ND; Hansen and Daugbjerg, 2004). Semi-nested and nested PCR amplifications were used to obtain visible fragments of expected length. For semi-nested amplifications we used D1F in combination with D3B, and for nested amplifications we used D3A and ND-1483R (for primer sequences, see Hansen et al., 2007). PCR included the EmeraldAmp GT PCR Mater Mix (TakaRa Bio Inc., Japan) following the instructions of the manufacturer, or as in Lundholm et al. (2002) with an initial 5-min heating at 94 °C. PCR products were purified using either the NucleoFast 96 PCR kit from Macherey-Nagel (GmbH & Co. KG, Düren, Germany) or the QIAquick PCR purification kit (Qiagen), following the manufacturer's recommendations. Air-dried PCR products (550 ng) were sent to Macrogen (Holland) for sequence determination in both directions or was cycle-sequenced using the ABI PRISM Dye Terminator Cycle Ready Reaction kit with AmpliTaq DNA polymerase. Subsequent sequencing was conducted on an ABI 3130XL automated sequencer (Applied Biosystems).

3. Results

3.1. The Ejsing Seafarm (AquaPri innovation A/S)

The fish farm comprises several indoor basins, divided into two units, the larger unit comprising two large raceways; each raceway has a volume of 1000 cubic meters (Fig. 1S). The fish cultured is pikeperch (*Stizostedion lucioperca*), which has been introduced to several Danish lakes for subsequent human consumption and/or for angling or biocontrol. It is grown to a market size of 800 g to 1.2 kg. The salinity of the water was maintained at 1–3 psu, the temperature at 16–23 °C. When the fish kill occurred, both raceways were in use, however mass mortality was observed in only one raceway, containing the oldest and largest fish. The total fish mortality was estimated to be 20 tons, but the number of skin lesions or other abnormalities observed did not exceed what occurred in unaffected basins. In addition to *Pfiesteria*, the water of the raceways contained only bacteria, no other eukaryotic organism was observed. In the second raceway, which had been stocked later, various treatments were attempted, which resulted in the fish being saved. Experimental treatment of the infected water using fish sampled from the farm was carried out to identify an effective treatment to control the algae. Various chemotherapeutants were tested, such as copper sulphate, peracetic acid and hydrogen peroxide. These compounds were either ineffective and/or had a negative impact on the fish at the concentrations needed to kill the dinoflagellates. Chloramine-T proved to be the only effective therapeutant that controlled the level of dinoflagellates without impairing the survival of the fish. The final effective concentration was determined at 4–8 mg l^{-1} of chloramine-T weekly. The general recommended concentration is 4 mg l^{-1}. A concentration of 10 mg l^{-1} proved lethal to the fish. After treatment with oxidizing agents such as peracetic acid and hydrogen peroxide, the dinoflagellates stopped moving and the fish improved quickly. However, the dinoflagellates soon resumed swimming, and the negative effect on the fish returned. When a concentration of 2–3 million dinoflagellate cells l^{-1} had been reached, the negative impact on the fish was observed and the highest concentration of dinoflagellates during the fish kill was 6.6 million cells l^{-1}. Treatment of water with ultraviolet light was also effective, resulting in constant minimum algal concentration (200 ml^{-1}) after 48 h and no fish kills. However, up scaling of this treatment to the entire volume of the plant was not economically feasible. During the recirculation process, the water was filtered for particulate matter. However, due to the farm design, the particles are difficult to remove, resulting in water with a high organic content. This is believed to provide a nutritional basis for the toxic algae to bloom again after chemical treatment. After treatment, many immotile algal cells were observed, probably temporary cysts.

3.2. The GUDP plant, Hirtshals

The GUDP plant is an experimental plant maintained by the Technical University of Denmark in conjunction with a number of private companies, including the company which runs the Ejsing farm. The dinoflagellates occurred in a 5-meter deep outdoor tank of 2500 cubic meters (Fig. 2S). The water had a salinity of 27 psu and the temperature during the fish kill was 16–17 °C. The water was re-circulated 1–2 times every hour. During the fish kill the plant contained about 25–30 tons of rainbow trout (*Oncorhynchus mykiss*), which received 300 kg of food daily. The production of fish was nearly 300 kg trout per day. In addition to *Luciella masanensis*, the water of the plant contained some phytoplankton diatoms, but no potentially harmful diatoms, dinoflagellates or other algae were observed.

Since the fish kill began in June 2012, the plant was treated regularly with peracetic acid (Divosan) at a concentration of 3 mg l^{-1}, and this treatment usually resulted in the fish recovering and remaining fresh for several days, after which time the treatment was repeated. Following the treatment, many immotile dinoflagellate cells were observed, probably temporary cysts.

Treatment with chloramine-T was also attempted, at concentrations of 0, 0.5, 1, 2 and 4 mg l^{-1}. Only the highest concentration had an effect on the dinoflagellates, which stopped moving after a few hours' exposure. However, swimming of the dinoflagellates resumed after 18 h, and they were as active as before treatment began. The cells were found to be morphologically different from those of the Ejsing farm, and identification as *Luciella masanensis* was done using molecular sequence determination of LSU rDNA. In mid-July the fish kill became very serious, despite treatment with peracetic acid and chloramine-T, and for a few days about 100 kg of dead fish had to be removed every other hour. It became clear that the plant could not be rescued and it was closed down.

3.3. Identification of the dinoflagellates to species level

Nuclear-encoded LSU rDNA was determined of two cells isolated from the Ejsing farm and four cells from the farm at Hirtshals. The sequences of the different cells from a fish farm were identical. Using Genbank, we did nucleotide blast searches of 1408 base pairs of LSU rDNA of the dinoflagellate from Ejsing Seafarm and 1263 base pairs of dinoflagellate from the fish farm at

Hirtshals. The fish killer from Ejsing Seafarm was 99.3% identical to *Pfiesteria shumwayae* (accession number AY245694). This comparison was based on 848 base pairs. The six base-pair difference could not be justified, as they were all undetermined nucleotides in the sequence of *P. shumwayae* available in Genbank (i.e. 1×(G-R), 1×(G-S), 2×(T-Y) and 2×(C-Y). The LSU rDNA sequences of the dinoflagellate from Hirtshals were 100% identical to three isolates of *Luciella masanensis* ribotype group 1 sensu Mason et al. (2007; isolates VIMS 1041, VIMS 1050 and HR1NovC5 with accession numbers EU048552, EU048553 and AY590482, respectively). This comparison included 803 base pairs for the VIMS isolates and 768 for the HR1NovC5 isolate.

3.4. Microscopy

Light microscopy was insufficient to determine the material to species level. However, cells from the two locations differed, those from Ejsing being almost round (Figs. 2 and 3), and those from Hirtshals more elongate, sometimes slightly fungiform (Figs. 4 and 5). This agrees with previous descriptions from the literature. Details of plate pattern could not be seen in live material.

Details of the plate pattern were generally difficult to see also in the SEM, but in some specimens the suture demarcations made it possible to see the key plates. Thus, the presence of a small four-sided intercalary plate confirmed the molecular identification of the Ejsing material as *Pfiesteria shumwayae* (Fig. 3B). SEM also revealed the partly protruding peduncle from the peduncle cover plate (pc), the first apical plate and the relatively long and narrow canal plate (Fig. 3A).

Fluorescence microscopy of the material from Hirtshals clearly revealed the presence of two small 4-sided intercalary plates confirming its identification as *Luciella masanensis* (Fig. 5A and B). Scanning electron microscopy only barely revealed the plates, but a peduncle was also here partly protruding from the pc-plate (Fig. 5C).

Based on the suite of methods applied here, we therefore conclude that the cause of the fishkills at Ejsing Seafarm was *Pfiesteria shumwayae* and at Hirtshals *Luciella masanensis*.

4. Discussion

Pfiesteria shumwayae has been found in geographically widely separated localities (Rublee et al., 2004). Following the incident in Denmark, somewhat similar cells were observed over the sediment in the coastal lagoon near Aveiro, Portugal and these were identified by molecular methods as *Cryptoperidiniopsis brodyi* (Craveiro and Calado, unpubl. observations). Most recently a culture in the Scandinavian Culture Collection for Algae and Protozoa, isolated from Danish waters some years ago, was found to belong to this species too (G. Hansen, N. Lundholm, unpubl.). Our impression is therefore that in addition to the two species of *Pfiesteria*, which have been found almost worldwide, pfiesteriacean dinoflagellates in general may be widespread in the marine environment, in plankton or near the bottom, at least in temperate waters. In freshwater, the pfiesteriacean species *Tyrannodinium edax* is one of the commonest dinoflagellates. The Ejsing material was grown at a salinity of 25 psu as well as in freshwater, and in both cases cells grew rapidly. When the Ejsing material was fed pieces of live fish kills, the cells displayed no interest in the food, but the gills began producing mucilage. In the laboratory we fed the cells a punctured nematode, in which the gut and its contents had oozed out, but the algae were not attracted, in contrast to our experience with the related species *Tyrannodinium* (Calado and Moestrup, 1997; Calado et al., 2009). Similarly with the Hirtshals material: when cells were offered pieces of fresh gill tissue, they showed no interest, but rather seemed to avoid the tissue. This is in apparent contrast to the report of Vogelbein et al. (2002), who observed cells swarming around and attaching to skin of fish larvae, and rapidly denuding the larvae of the epidermis. However, when the dinoflagellates from Ejsing were starved for two days and then offered a punctured nematode, the picture changed dramatically. The algae were then strongly attracted to the nematode, attached to the damaged part of the animal with a filament and started sucking up material from the nematode through a peduncle. When fully fed, the attached dinoflagellates started moving again, withdrew the peduncle and swam away. This indicates that as in *Tyrannodinium*, *Pfiesteria* attaches to its prey by means of a thin thread, which is cut or retracted and reabsorbed once feeding has been completed. When the starved dinoflagellates were offered a damaged rotifer, they swarmed around the rotifer like mosquitoes around their prey, and attached to the rotifer. Vogelbein et al. (2002) interpreted the fish kill to be caused by the attack of *Pfiesteria* on the fish, rather than by the production of toxin. In contrast, Burkholder and Marshall (2012) concluded that in addition to nontoxic strains, three functional types of toxigenic *Pfiesteria* strains occur, one of which (TOX-A) is capable of killing fish when grown together with fish, the other (TOX-B) producing enough toxin to kill larval, but not mature fish, and the third strain (NON-IND) incapable of killing fish with toxin. The TOX-A functional type attached to fish but showed no attraction to cryptomonads, while the two other types were attracted to cryptomonad prey. Our material obviously fell into functional group TOX-A. Cells grew very readily in culture when fed cryptomonads and, as mentioned above, after being starved, also fed on nematodes and rotifers, and in this respect acted as a TOX-B strain. The related freshwater species *Tyrannodinium edax* (syn. *Tyrannodinium berolinense*) is also a vigorous feeder on organic material, including weakened cells of its own kind (Calado et al., 2009). *Tyrannodinium* is strongly chemically attracted to the organic material, as reported also in several other species of the Pfiesteriaceae such as *Paulsenella* (Schnepf and Drebes, 1986), *Cryptoperidiniopsis brodyi* (Steidinger et al., 2006), *Pfiesteria piscicida* (e.g. Burkholder and Marshall, 2012), '*Gymnodinium*' *fungiforme* (Spero, 1985), and *P. shumwayae* (present report). *P. shumwayae* has been associated with neurological effects in humans. We have so far observed no such effects, whether in the fish farms nor in our mixed cultures of flagellates in the laboratory, and this was therefore not examined further.

Our conclusions of the observations are: (1) if conditions are right, pfiesteriaceans may occur in areas where they have not been observed before. The lack of observations may be due to difficulty in identification, and our evidence confirms that pfiesteriaceans occur naturally in marine plankton or bottom waters of temperate waters in many parts, if not throughout, the world. A life cycle comprising sexual fusion of gametes and subsequent formation of resting spores has been observed in *Pfiesteria piscicida* (Parrow et al., 2002) and, when sought for, such resting stages of *P. piscicida* occurred commonly in sediments in Delaware, USA (Coyne et al., 2006). (2) *Pfiesteria shumwayae* and *Luciella masanensis* are favored by high organic content in the water, such as fish aquaculture plants with high concentrations of fish, and this may apply to most if not all pfiesteriaceans. Skelton et al. (2008) were capable of feeding *P. shumwayae* with egg yolk and particles present in the biphasic medium used for culturing the algae. (3) Fish farms utilizing re-circulated water are at risk, unless the amount of organic material in the water can be kept low. (4) A concentration of 2–3 million cells l^{-1} of *P. shumwayae* resulted in fish kills in the Danish fish farms. (5) The fish kills could to some extent be controlled by treatment of the water, but economic, reliable methods for controlling the number of algal cells need to be developed. Other protists and copepods feed on pfiesteriaceans, including *P. piscicida* and *L. masanensis* (Jeong et al., 2007b), and it

may be worthwhile to investigate whether this can be used to control the algae in the aquaculture farms. (6) We observed none of the effects of the dinoflagellates on humans described by Grattan et al. (1998), neither in laboratories nor in open or covered aquaculture plants, where people worked on a daily basis. (7) Pfiesteriaceans are chemically attracted to organic material. (8) All species of the Pfiesteriaceae appear to be heterotrophs. *P. piscicida* has been shown to be able to retain chloroplasts from prey as kleptochloroplasts, which are photosynthetically active (Lewitus et al., 1999a,b) and this may also apply to other species of the family (e.g. *Cryptoperidiniopsis* sp.: Eriksen et al., 2002).

To our knowledge this is the first time that toxic algae have been shown to be a potential risk in closed re-circulated aquaculture (RAS) systems, and this information may have implications on the planning of location of new farms and the necessary water management measures needed to control outbreaks of toxic algae. In events of otherwise unexplained mortality in RAS systems, an investigation of algae in the water should be included in the management plan.

We have presently no new information on the possible production of toxins by *Pfiesteria shumwayae* or *Luciella masanensis*. A toxin was identified in *Pfiesteria piscicida* by Moeller et al. (2007) who found a highly volatile toxin, whose chemical structure remains to be determined in detail. The toxigenic potential of the other species of the Pfiesteriaceae is unknown. The many behavioral similarities between the species indicate that the species have similar toxigenic potential but this obviously needs to be examined.

The heterotrophic condition separates pfiesteriaceans from other fish-killing algae, which are autotrophic or mixotrophic. It makes them relatively easy to identify to family level, at least in the living condition. However, identification to species level presently requires fluorescence microscopy, scanning electron microscopy or molecular methods. During the regular monitoring schemes from the Baltic Sea, pfiesteriaceans have not been reported before, but they are probably hidden in the lists as 'heterotrophic *Glenodinium*' (e.g. Hällfors et al., 2013).

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Appendix A. Related species

Six of the seven known genera of the family Pfiesteriaceae occur in marine or brackish water. Two of the genera, *Paulsenella* Chatton and *Amyloodinium* Brown and Hovasse were described during the first half of the 1900s (Chatton, 1920; Brown and Hovasse, 1946) and their pfiesteriacean affinity was discovered only recently (Litaker et al., 1999; Kühn and Medlin, 2005). The remaining four marine genera *Pfiesteria* Steidinger & Burkholder, *Stoeckeria* Jeong et al., *Cryptoperidiniopsis* Steidinger et al. and *Luciella* Mason et al. were described in rapid succession in 1996–2007 (Steidinger et al., 1996; Jeong et al., 2005; Litaker et al., 2005; Steidinger et al., 2006; Mason et al., 2007), following the first fish kills on the US East Coast. *Peridiniopsis salina* may also belong in the family, but it was described to be phototrophic with numerous chloroplasts (Trigueros, 2000). The single representative of the family in freshwater, *Tyrannodinium* Calado et al. (Calado et al., 2009) is the most recent member of the family to be discovered. It was previously considered to belong to *Glenodinium* or *Peridiniopsis* (Peridiniaceae). *Tyrannodinium* comprises a single, often very numerous, freshwater species, *Tyrannodinium edax* (also known as *Peridiniopsis berolinensis*) (Calado et al., 2009; Calado, 2011). Fish kills have previously been attributed to the two species of *Pfiesteria* only. However, species of the family are closely related and many are morphologically very similar, indicating that many are potential fish killers when conditions are suitable, i.e. if the amount of food, live or dead organic material, is available. As mentioned above, identification presently requires molecular studies or scanning electron microscopy, and a comparative study of all known species by light microscopy is strongly needed.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.hal.2013.12.002.

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