



Association of the alga *Cladogonium* sp. with a multifactorial disease outbreak in dwarf shrimp (*Neocaridina davidi*)

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ABSTRACT: This study outlines a multifactorial disease outbreak in a population of the freshwater shrimp *Neocaridina davidi*, with the focus on a rarely described parasitic alga. Within this multifactorial disease outbreak, low but consistent mortality was observed. During microscopic examination, an infection of the shrimp with bacterial and fungal-like agents was diagnosed. Furthermore, the green alga *Cladogonium* sp. was found in pleopodal regions. The alga compromised the body surface of the shrimp, and its rhizoids penetrated the chitin shell and reached into the subcutaneous tissue. This might be a first indication of a parasitic lifestyle. In addition to a morphological description, sequencing data are presented which allow the taxonomic classification of the organism within the order Trentepohliales.

KEY WORDS: Freshwater shrimp · *Neocaridina* · Green alga · *Cladogonium* · Trentepohliales

1. INTRODUCTION

Shrimp of the genus *Neocaridina* are among the most popular shrimp in freshwater aquaristics and can be purchased in almost every aquaristics store or online shop. Multiple colour variants (Lucas et al. 2019) of the species *Neocaridina davidi* (Bouvier, 1904) are available, including Red Cherry (Red Fire), Yellow Fire, Black and Red Sakura, Blue Jelly or Rili Shrimp. The natural habitats of the wild type are inland flowing freshwater reservoirs mainly in China and Korea (De Grave 2013). Current observations show that this species may also establish stable populations in thermally polluted temperate waters, e.g. the Gillbach stream in North Rhine-Westphalia, Germany (Klotz et al. 2013, Schoolmann & Arndt 2018) or the Oder River near Gryfino, Poland (Maciaszek et al. 2021), as these animals can withstand water temperatures between 6 and 30°C (Klotz et al. 2013). Therefore, diseases affecting these animals are not

only of concern for aquarists but also for the native crustacean fauna in temperate waters. As a neozoon, this species might bring new pathogens into its natural habitats.

A number of relevant parasites affect *N. davidi*, and several organisms have been described to colonize the body surfaces of the shrimp. For example, various epibionts, including rotifers, nematodes, ciliates and others, can be found on freshwater shrimp, as these organisms normally populate the same habitats and benefit from a cooperative lifestyle (Corliss 1979, Fernandez-Leborans et al. 2006, Fernandez-Leborans & Von Rintelen 2007, Maciaszek et al. 2018). Fungi, fungal-like organisms and bacteria may colonize the shrimp carapace as epibionts and, because many species are opportunists, they can sometimes cause infections as secondary pathogens (Gozlan et al. 2014). Because they are opportunistic, facultative pathogens, they usually do not cause any

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signs of disease as long as husbandry conditions, especially water quality, are kept within optimal ranges (Yanong 2003). Many bacteria are epibiotic and belong to the physiological microbiome of the body surface or digestive tract of shrimp. Bacteria assigned to the phyla *Proteobacteria*, *Bacteroidetes* and others were previously described to colonize the hepatopancreas of healthy *N. denticulata* (Cheung et al. 2015). The relationship between shrimp and some bacteria can even be classified as symbiotic; for example, *Paucibacter toxinivorans* can degrade toxins produced by cyanobacteria, thereby protecting the host from hepatopancreatic damage caused by these toxins (Rapala et al. 2005). Most bacteria of the aquatic environment may act as potentially pathogenic bacteria to freshwater shrimp. In most cases they are involved in a multifactorial cause of disease, yet some bacteria are able to cause more damage than others due to specific traits like virulence factors or being able to produce toxins or enzymes that can be harmful to the host. For example, the Photorhabdus insect related toxin PirA/B is a primary virulence factor for acute hepatopancreatic necrosis disease in *Litopenaeus vannamei*, caused by the bacterium *Vibrio parahaemolyticus* (Tinwongger et al. 2016). Furthermore, chitinolytic bacteria are able to degrade chitin and cause melanised lesions on the cuticle. Therefore this disease is commonly called 'rust disease', 'black spot disease' or 'brown spot disease' (Bower et al. 1994).

Fungal-like oomycetes like *Saprolegnia* are ubiquitous in aquatic freshwater habitats and soil (Yanong 2003). Like other epibionts and most ubiquitous bacteria, they are considered not to harm their hosts under optimal conditions. In freshwater crayfish, *S. parasitica* mostly acts as a secondary pathogen that infects damaged tissue and can lead to chronic systemic infections as well as mortalities (Diéguez-Uribeondo et al. 1994).

Specific algae can also colonize the body surface of shrimp. Algae usually colonize pond or aquarium water or submerged surfaces and react to changes in water quality and nutrient availability. For example, in koi, green algae can colonize damaged body surfaces similar to most fungi and fungal-like organisms. Green algae of the genus *Cladogonium* seem to be an exception. *C. ogishimae* was first described in Japan from freshwater shrimp and was suspected to be a parasitic alga that attaches to shrimp in the pleopodal region (Ogishima 1950, Hirose & Akiyama 1971). It was subsequently described as an epibiont in *Neocaridina davidi* (Maci-

aszek et al. 2018) and as an ectoparasitic alga on the freshwater shrimp species *Paratya compressa improvisa*, *Macrobrachium longipes* de Haan, and *Caridina leukosticta* Stimpson, 1860 (Hirose & Akiyama 1971), as well as *Neocaridina denticulata* (Matsuyama-Serisawa et al. 2014). Previous studies have suggested that the genus *Cladogonium* may belong to either the Trentepohliales (Singh 1962), the Chaetophorales (Ogishima 1950) or the Cladophorales (Hirose & Akiyama 1971) within the phylum Chlorophyta. Molecular data to support these theories are still missing.

This report provides molecular data on this algal species collected from *N. davidi* and suggests the presence of an additional species within the genus *Cladogonium*. Furthermore, it provides histological images which support the assumption of a parasitic nature of *Cladogonium*.

2. MATERIALS AND METHODS

Twenty specimens of *Neocaridina davidi* of different colour variants were sent by a wholesaler, who regularly imports shrimp from Southeast Asia, to the Fish Disease Research Unit (FDRU) for examination. Four animals died during transport. All shrimp were carrying green structures in the area of the pleopods. Previously, the owners observed a low but consistent mortality rate, although most animals recovered after moulting. However, the disease signs reoccurred sporadically, warranting an investigation into the reason for the symptoms and the mortalities.

After a clinical examination, including a visual assessment of behaviour and morphological abnormalities, the shrimp were stunned by quickly lowering the body core temperature in iced water and subsequently killed by a longitudinal cut through the central ganglia in order to perform a necropsy. Wet mounts of carapaces, gills and hepatopancreatic tissue were analysed microscopically. Affected tissue was sterilely sampled and submitted to microbiological examination.

Four of the examined animals were fixed in 4% buffered formalin for patho-histological examination. Fixed samples were infiltrated, blocked with paraffin and cut into 2 µm sections, which were stained with haematoxylin and eosin or Giemsa (Romeis 1989).

For molecular biological analysis, 2 samples of the green club-shaped structures were removed and the DNA was extracted according to a previous study using the Qiagen DNeasy blood and tissue

kit (Eland et al. 2012). Samples were analysed via PCR using the primers SR1 SSU and SS11H SSU (Hanyuda et al. 2002, Leliaert et al. 2007) targeting the small subunit (SSU) of the 18S gene locus, which resulted in 2 identical sequences (983 bp). Sequences were compared to sequences deposited in the nucleotide collection database of the National Center for Biotechnology Information (NCBI) and analysed using the basic local alignment search tool (BLAST). A phylogenetic tree of the samples and of additional 18S SSU sequences from green algae (NCBI GenBank) of the classes Chlorophyceae and Ulvophyceae was constructed using the Kalign Multi Sequence Alignment Tool (Lassmann & Sonnhammer 2005) and visualised using FigTree v1.4.4.

3. RESULTS

3.1. Clinical examination

Upon arrival at the FDRU, the shrimp were in good to moderate general condition. However, whitish discoloured muscle tissue could be observed in the tail region in some animals. The preliminarily reported green structures were the predominant finding (Fig. 1). These structures were club-shaped and pedunculated, hanging out of the interpleopodal spaces, and several stages of colonization could be observed (Fig. 2). The zoosporangial surfaces showed different stages of development, with either a smooth surface or a more granular, spore-like surface structure and a green colour. The peduncle filament was colourless, segmented, branched and vacuolated (Fig. 1E).

The microscopic examination of wet mounts of gill tissue revealed minor colonization by turbellarians, possibly belonging to the order Temnocephalida (Fig. 3). In the pleopodal area, a low number of eggs were found in female shrimp. Also, some single nematodes and rotifers were detected, and high colonization by hyphae, possibly from the oomycete *Saprolegnia* sp. and bacteria, was identified in some animals (Fig. 1F).

Wet mounts of the hepatopancreas showed moderately reduced fat droplets. No parasites could be found in the gut.

3.2. Microbiological examination

A microbiological examination of macro- and microscopically altered muscle tissue revealed profound bacterial colonization by *Chryseobacterium* sp., *Paucibacter* sp. and motile aeromonads. Resistance testing revealed a high grade of bacterial resistance towards multiple antibiotic substances; doxycycline and chlortetracycline were the only 2 antibiotics to which all isolates were sensitive (Table 1).

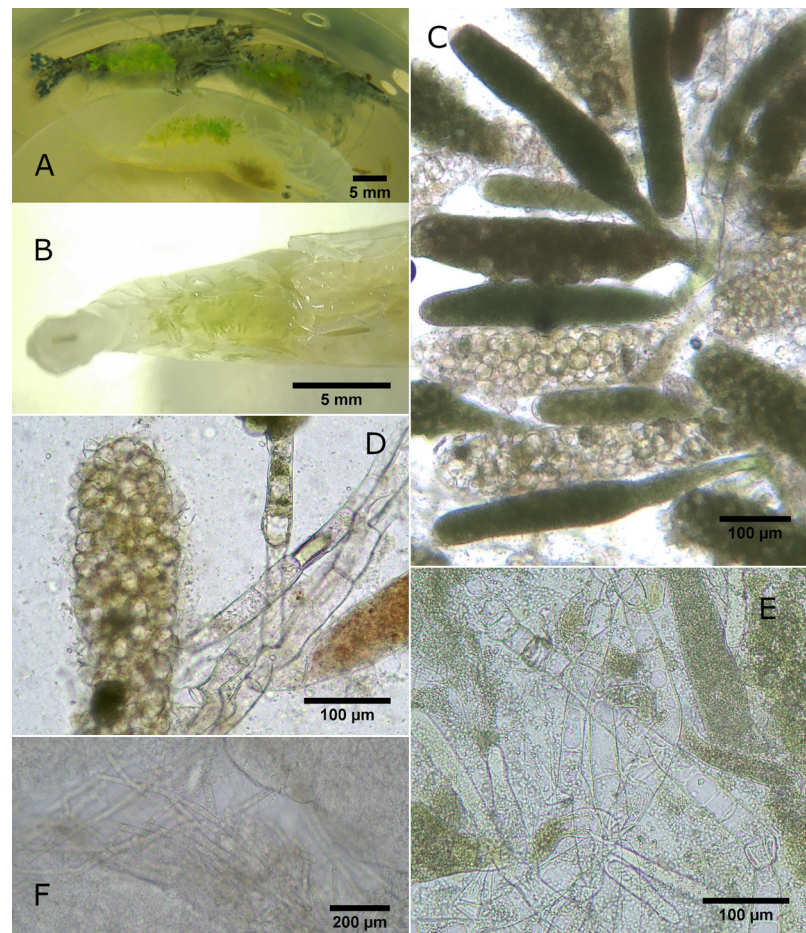


Fig. 1. *Cladogonium* sp. in dwarf shrimp. (A) Anaesthetised shrimp showing green club-shaped appendages in the pleopodal area. (B) Pleopodal area of a shrimp at higher magnification. Also shown are wet mounts of *Cladogonium* sp.: (C) zoosporangia of different development stages; (D) fully developed zoosporangium showing mature flagellated zoospores; (E) filaments showing multiple vacuoles; (F) fungal-like hyphae

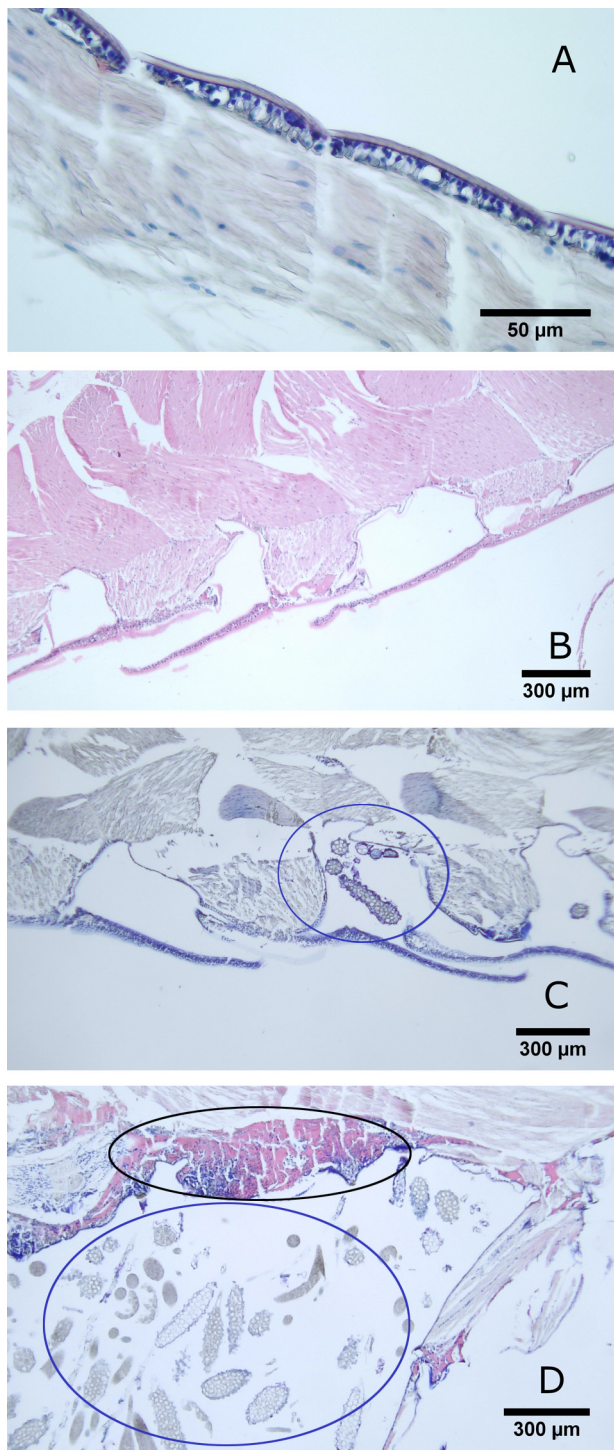


Fig. 2. Histological sections of (A) cuticle of healthy shrimp (Giemsa); sections through the pleopodal area showing (B) healthy shrimp (HE); (C) minor infestation with *Cladogonium* sp. (blue circle) (Giemsa) and (D) severe infestation with *Cladogonium* sp. (blue circle), showing a broadened subcutaneous area with accumulation of haemolymph and haemocytes as well as spreading of algal rhizoids (black circle) (Giemsa)



Fig. 3. (A–C) Wet mounts of the gill showing a temnocephalid turbellarian with a distinct tentacle region (A) and eye spots (B)

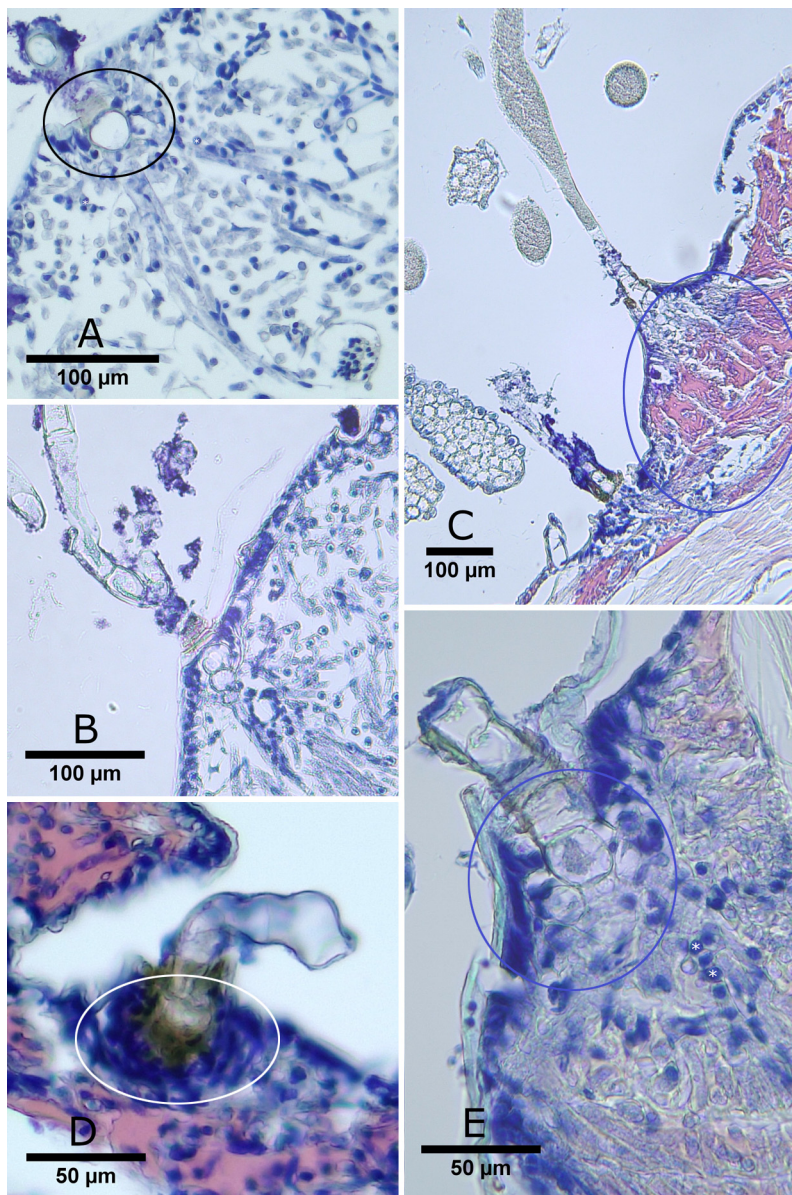
3.3. Histological examination

The histological examination of organ tissue revealed minor swelling of the gill epithelial tissue in some animals, whereas the gill structure appeared to be unchanged in others. Some sections of the muscle tissue showed a severe infiltration of hyphal structures of *Saprolegnia* sp. as well as bacterial colonization.

The green club-shaped structures in the pleopodal area were compact and pedunculated, and some showed a more granulated structure. The connections to the animals' bodies were located at the abdominal

Table 1. Results of the microbiological examination showing isolated species, grade of bacterial growth (number of bacterial colonies on agar plate; +: low; ++: moderate; +++ high) and results of resistance testing. R: resistant; S: sensitive; I: intermediate

Isolated bacteria	Grade	Trimethoprim/ Sulphamethoxazole	Enrofloxacin	Neomycin	Oxytetracycline	Erythromycin	Doxycycline	Gentamicin	Ampicillin	Chlortetracycline	Tylosin	Florfenicol	Kanamycin	Tulathromycin
Motile aeromonads	++	S	R	S	S	R	S	S	R	S	R	S	S	R
<i>Paucibacter</i> sp.	+++	S	R	I	S	S	S	R	I	S	S	S	S	S
<i>Chryseobacterium</i> sp.	+++	R	S	R	R	R	S	R	R	S	R	R	I	R



wall at the basis of the pleopods. The connecting peduncles were rhizoidal and reached into the subcutaneous tissue (Fig. 4). The subcutaneous area was widened, and in severely affected animals, high amounts of haemolymph and haemocytes were detected subcutaneously (Figs. 2 & 4). The structures of the thallus, rhizoid and zoosporangia were similar to a previous description of *Cladogonium ogishimae* (Hirose & Akiyama 1971), although some differences could be observed (Table 2). The fully developed zoosporangia measured $329.9 \pm 13.8 \mu\text{m}$ in length (mean \pm SD) and $102.7 \pm 11.6 \mu\text{m}$ in width. The early development stages showed a smooth green surface with only fine granular pigmentation, whereas further developed stages began to show a sporulated surface. Fully developed zoosporangia were colourless, consisting of mature, flagellated zoospores that measured $10.2 \pm 0.87 \mu\text{m}$ in diameter and contained multiple nuclei of $2.0 \pm 0.3 \mu\text{m}$ in diameter. Cells of the thallus measured $44.6 \pm 8.1 \mu\text{m}$ in length and $29.6 \pm 1.5 \mu\text{m}$ in width (Fig. 5).

3.4. Molecular biological analyses

Molecular biological identification of 18S rRNA SSU sequences using BLAST and the NCBI nucleotide collection database yielded no sequence matches exceeding 85% similarity. The closest relationship was seen to species within the genera *Trentepohlia* and *Cephaleurus*. The phylogenetic tree constructed using 18S rRNA SSU

Fig. 4. Histological sections of connecting peduncles of *Cladogonium* sp. (A,B) Root-like spreading of rhizoids (black circle) in broadened subcutaneous area (Giemsa). (C) Root of *Cladogonium* sp. and broadened subcutaneous area with accumulation of haemolymph (blue circle) and haemocytes appearing as blue-stained cells (Giemsa). (D,E) High magnification of penetrating rhizoidal basal cell of *Cladogonium* sp. (D) with no spreading of hyphal rhizoids (white circle) and (E) imbedded in the subcutaneous tissue (blue circle) (Giemsa)

Table 2. Comparison of morphological descriptions and measurements of *Cladogonium* sp. (mean \pm SD) and *C. ogishimae*

		<i>Cladogonium</i> sp. (present study)	<i>C. ogishimae</i> (Hirose & Akiyama 1971)
Target species		<i>Neocaridina davidi</i>	<i>Paratya compressa improvisa</i> <i>Macrobrachium longipes</i> <i>Caridina leucosticta</i>
Zoosporangium	Colour	Green	Green
	Surface structure	Highly irregular surface of mature zoosporangia	Rather flat surface of mature zoosporangia
	Width	102.7 \pm 11.6 μ m	130–(181.5)–210 μ m ^a
	Length	329.9 \pm 13.8 μ m	440–(561.5)–790 μ m ^a
Zoospores	Colour	Green	Green
	Ciliated	Yes	Yes (4)
	Diameter	10.2 \pm 0.87 μ m	8–12 μ m
	Nuclei	Yes, multiple	Not determined
Erect filament of thallus	Colour	Colourless	Colourless
	Width	29.6 \pm 1.5 μ m	17–(26.5)–35 ^a
	Length	44.6 \pm 8.1 μ m	40–(99)–180 ^a
	Nuclei	Not determined	4–8
Rhizoidal cells	Vacuolation	Yes	Yes
	Colour	Colourless	Colourless
	Basal cell	Yes	Yes

^aFormat used by Hirose & Akiyama (1971); assumed to show 'min.–(mean)–max.'

sequences of related species showed a clustering of the 2 samples near the family Trentepohliaceae, possibly within the order Trentepohliales (Fig. 6). The sequence was deposited in NCBI GenBank (accession number MT789773).

4. DISCUSSION

In this case report, a disease outbreak in *Neocaridina davidi* caused by oomycete and bacterial infections is described. The most remarkable alteration seen in the shrimp were the green structures in the pleopodal area. On the basis of morphological traits, these structures were identified as green algae, probably belonging to the genus *Cladogonium*. An association of the green alga *Cladogonium* sp. with this multifactorial disease was seen.

There are previous descriptions of *Cladogonium* in *N. davidi* which were identified as *C. ogishimae* (Maciaszek et al. 2018) or left undetermined as *Cladogonium* sp. (Matsuyama-Serisawa et al. 2014). On the basis of photographs and measurements, both of the previously described cases of *Cladogonium* colonization in *N. davidi* show more morphological similarities to the organism observed herein than to *C. ogishimae*. The main differences between the organism that we observed and *C. ogishimae* were the length and width of zoosporangia as well as the surface

structure of mature zoosporangia. Furthermore, the filament cells were longer in *C. ogishimae*.

Molecular data of the *Cladogonium* sp. observed here showed a close relationship to specimens from the family Trentepohliaceae, but our isolates did not cluster directly with members of this family (*Trentepohlia*, *Cephaleurus*, *Printzina*, *Stomatochroon* and *Phycopeltis*), suggesting that *Cladogonium* sp. forms a second family within the order Trentepohliales (Fig. 6). The structure of the algae that we examined shows differences to the previously described *C. ogishimae*. We suggest that *Cladogonium* sp. might be a second species in the genus *Cladogonium*, along with *C. ogishimae*, within the order Trentepohliales, possibly as a second family. In future, the identification could be more focussed on molecular data, which will help to differentiate individual species and determine target species with more certainty.

This case report indicates that *Cladogonium* sp. might have persisting stages that remain in the bodies of shrimp after moulting. The hyphal attachment of the algae to the shrimp in this and previous cases (Hirose & Akiyama 1971) suggest severe damage of the body surface. The histological alterations of the cuticle and subcutaneous area that we observed, including thickening and infiltration with rhizoids, haemolymph and haemocytes, suggest that *Cladogonium* sp. might act as a parasitic pathogen in these dwarf shrimp.

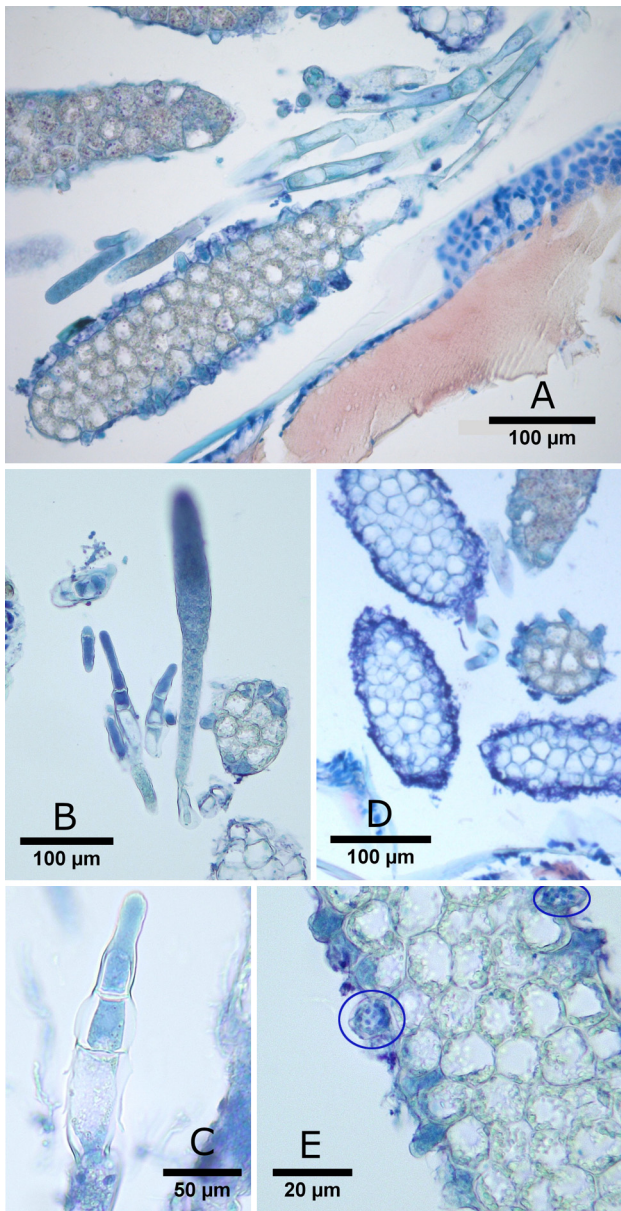


Fig. 5. Histological sections of developmental stages of *Cladogonium* sp. (A) Cross section showing an overview of different development stages and an erect filament of *Cladogonium* sp. (Giemsa). (B,C) Young zoosporangia showing growth of terminal cells (Giemsa). (D) Fully sporulated zoosporangia (Giemsa). (E) Sporulating zoosporangium with multinucleated zoospores (blue circles) (Giemsa)

Unfortunately, the eradication of this alga might be difficult, especially in rearing facilities of distributors, because of its spreading via numerous flagellated spores. We therefore suggest that affected animals should be quarantined until after the next moulting and then be monitored separately for reoccurrence. After each moult, the quarantine tank should be

cleaned and disinfected using a saturated sodium chloride solution for 24 h before reuse. Commonly used medication for shrimp can certainly reduce the superficial parts of *Cladogonium*. However, medications against algae usually contain copper. The LD_{50} for copper in *Caridina* sp. is $281 \mu\text{g l}^{-1}$ (Ghate & Mulherkar 1979), and a standard treatment dosage of copper sulphate with $1500 \mu\text{g l}^{-1}$ would probably be highly toxic to *Neocaridina* sp. An effective treatment that also eliminates systemic stages of *Cladogonium* has yet to be developed and tested.

In the shrimp showing signs of disease, multiple additional epibionts (nematodes and rotifers) were detected. Nematodes and rotifers are considered to be non-parasitic, although they benefit from a close relationship with shrimp, and they usually do not disrupt the integrity of the host body surface. The pleopodal area of shrimp provides a protected environment with usually high concentrations of nutrients. Epibionts do not typically lead to diseases in shrimp. However, a decline in water quality and nutritional overload may lead to a rapid increase in epibionts, which can increase stress in shrimp and substantially weaken them (Wahl 2008). Therefore, optimizing the husbandry conditions can help to prevent excessive colonization of the body surface of shrimp with epibiotic organisms and thus reduce stress in the shrimp.

The identified bacterial species belong to the normal flora of the gut and hepatopancreas of dwarf shrimp (Cheung et al. 2015), but the bacterial colonization and colour change of muscle tissue indicated a bacterial infection. As there are no reports on the detection of bacteria in the muscle tissue of healthy shrimp, we conclude that these bacteria are not usually found in the muscle of healthy shrimp. Infections with bacteria of the normal flora of the digestive tract or body surface can occur when animals are weakened due to poor water quality or other infections, or when the integrity of the shrimp body surface is damaged, which allows bacteria to enter the shrimp muscle. Aquaculture facilities which keep and breed various shrimp or fish species usually have disinfection protocols to lower the bacterial load in the tank water. Even though bacteria are killed by disinfection, not all resistance genes are destroyed. Therefore, dissemination of antibiotic resistance by horizontal gene transfer via antibiotic resistance genes in the aquatic environment remains possible, as shown previously for chlorine and UV disinfection (McKinney & Pruden 2012, Liu et al. 2018). The bacteria identified here showed a high level of antibiotic resistance to most of the commonly used antibiotic

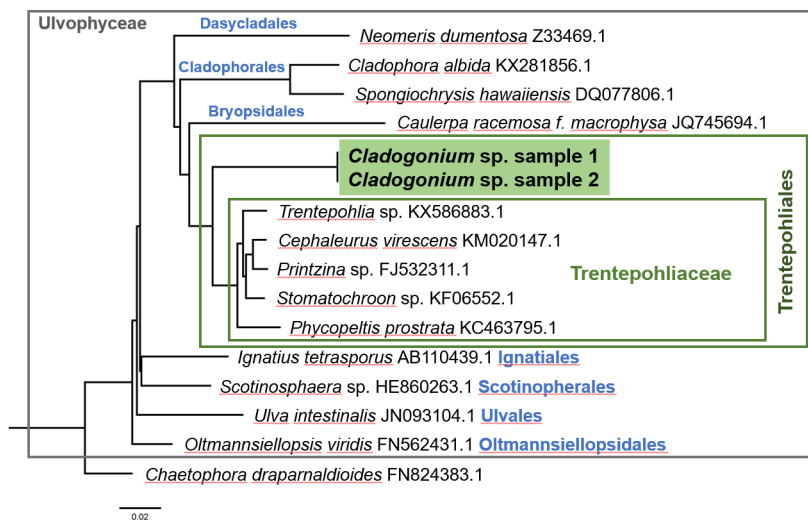


Fig. 6. Phylogenetic tree constructed using Kalign Multi Sequence Alignment Tool with sequences of the 18S rRNA small subunit including the newly sequenced *Cladogonium* sp. within the order Trentepohliales. Sequences of additional isolates were obtained from NCBI GenBank, and accession numbers are indicated

substances in fish and shrimp culture; thus, an effective treatment of severely affected animals is highly problematic.

Minor infections with gill flukes regularly occur in freshwater shrimp, and animals with low infestation levels usually show no health impairment. Severe infestations can easily be treated, and animals usually recover fast. Nevertheless, shrimp with parasitic infections like gill flukes are more vulnerable to other pathogens or colonization by epibionts. Infected animals show gill symptoms including swelling and in severe cases gill necrosis. The most frequently diagnosed gill flukes in freshwater shrimp are *Scutariella* sp. In this case, the tentacle region, the eye spots and the absence of an opisthaptor (Fig. 3) indicate an undetermined temnocephalid turbellarian, which most likely acted as an epibiont rather than a parasite.

Overall, severe infestations of either bacteria, fungi or parasites can occur when animals are weakened by poor rearing conditions, long transport durations or intensified stress due to medication or disinfection. Multifactorial diseases with a combination of primary and secondary pathogens enhance the severity of either infection. In general, shrimp health can easily be monitored by macroscopic examination and microscopic examination using wet mounts of the carapace and gills. The examination of wet mounts of hepatopancreatic tissue can also be useful, as a reduced amount of fat droplets in the hepatopancreatic tissue indicates an affected health status (Gomes et al. 2011).

The results of our examinations show that the reported low mortality was probably caused by a systemic bacterial infection of the shrimp. It can be reasoned that shrimp colonized by *Cladogonium* sp. are more likely to be infected with other pathogens, because *Cladogonium* sp. compromises the integrity of the body surface, as previously described for *C. ogishimae* (Hirose & Akiyama 1971) and observed for the *Cladogonium* sp. described in the present study. Treatment of secondary infections like other parasites, fungi, fungi-like organisms or bacteria and optimizing the rearing conditions and water quality can help to reduce stress and mortalities.

Within this multifactorial disease outbreak, *Cladogonium* sp. might have acted as the primary pathogen followed by secondary systemic bacterial

and fungal-like infestations. It may be possible that *Cladogonium* sp. provides nutrients to bacteria and fungi, thereby enhancing a secondary bacterial or fungal infection. Our results suggest that *Cladogonium* sp. might act in a parasitic rather than epibiotic fashion, massively injuring the shrimp cuticle and causing a profound cellular reaction in subcutaneous areas. Therefore, it may allow secondary pathogens to enter the body of the shrimp.

Several factors prevented a proper description of the observed *Cladogonium* as a new species. The material was collected from shrimp that were submitted by a wholesaler for diagnostic examinations, and no further information regarding the type locality was provided. All remaining specimens were embedded in paraffin, and the DNA of the analysed alga was stored for further analysis at the FDRU. The requirements set out by the International Code of Nomenclature for Algae, Fungi, and Plants ('Shenzhen Code') 2018 could not be met, and further research is necessary to evaluate the possible infectious nature and possible pathogenicity of *Cladogonium* sp. in general. Possible transmission mechanisms and the possible persistence of *Cladogonium* sp. in the subcutaneous tissue after moulting must also be investigated. Nevertheless, the present study provides further information about *Cladogonium* sp. in *N. davidi*, which is of great concern because of the wide distribution of this shrimp species in aquaria, in multiple aquatic environments and even as a neozoon (Klotz et al. 2013, Schoolmann & Arndt 2018, Maciaszek et al. 2021). In

this paper, we have provided the first molecular data that group the *Cladogonium* sp. described here within the order Trentepohliales.

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