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Scientific Report

Gill lesions and mortality in common carp (*Cyprinus carpio*) with a dense bloom of *Heterosigma*-like algae in Khuzestan province, Iran

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Summary

Background: Widespread common carp (*Cyprinus carpio*) mortalities have been recorded in Khuzestan province fish farms in recent years. In summer of 2017 two cases of harmful algal bloom were encountered that led to massive mortality in common carp in Khuzestan, Iran. **Aims:** The aim of this study was to identify the possible etiologic agent of two mortalities with characteristic symptoms of gill lesions due to harmful algae. **Methods:** Water samples were collected and 5 moribund fishes were examined by histophatologic, scanning electron microscopic and PCR examination. **Results:** In wet smear preparations, a lot of algal cells and fragments, and sloughed, necrotic epithelial cells were observed between the lamellae. In histopathologic examination of gills, hyperplasia, necrosis and algal cells surrounded by hyperplastic cells were seen in tissue sections. No inflammatory cell aggregation was noticed. In scanning electron microscopic examination the algae was found attached to the gill surface (cell diameter: 8.5 ± 4.2 µm) with 2 equal flagella. **Conclusion:** Phytoplankton analysis using direct microscopy and electron microscopy, morphologically resembling *Heterosigma* was identified, however, in PCR tests, *Heterosigma* analysis showed negative results, therefore the causative agent was called "*Heterosigma*-like" algae.

Key words: Common carp, Gill lesions, *Heterosigma* sp., Khuzestan province, Mortality

Introduction

The considerable economic and ecological damages incurred from carp mortality across Khuzestan province (unpublished data) provide a strong incentive to formulate effective diagnosis and control strategies. Previous investigations have focused on the viral origin of disease, i.e. koi herpes virus disease (KHV) or carp fungal gill necrosis etiology (branchiomycosis). By using PCR methods, virus etiology was excluded by the local veterinary department. Other than viral etiology, our work also focused on harmful algal cells and noticed evidence of water quality changes before and during mortality by fish farmers. Algal origin deserves special attention, since viral has previously been reported as negative by the local veterinary authority and no sign of fungal etiology (branchiomycosis) was seen in our survey.

A well-known species, *Heterosigma akashiwo* is one of the most important toxic algae which can cause acute or chronic toxicity which leads to fish mortality in various species of fishes. It is a swimming alga that episodically forms toxic surface aggregations known as harmful algal bloom (Yu *et al*., 2010). Formation of *H. akashiwo* which bloom in marine and estuary waters have been associated with freshwater runoff, usually followed by a reduction in salinity (Taylor and Haigh, 1993). The species name *akashiwo* is from the Japanese for "red tide".

Case history

We visited a fish farm near Abadan, Khuzestan province, Iran, following a report by the farm owner of a watercolour change to dark green and increased fish mortality. The physicochemical parameters of water (pH and salinity) were not found to be higher or lower than the normal limits. The diseased fish were inspected by routine methods and their gross pathological changes were recorded. Then gills and visceral organs from 5 moribund fish were preserved in phosphate buffered formalin fixative and processed for histopathology.

Two water samples were collected for phytoplankton and physicochemical evaluations during blooming phenomenon with a three day interval. The phytoplankton analysis was done by using direct microscopy and staining. Physical-chemical analysis of water was measured with a digital portable instrument. Water quality parameters including water temperature, dissolved oxygen, salinity and pH were measured in the affected fish ponds at the same time as fish sampling.

The five samples of moribund common carps

Table 1: Forward and reverse primers used in the present study

Primer name	Sequence (5´-3´)	Length	References
KHV	5´-GACGACGCCGGAGACCTTGTG-3´ 5´-CACAAGTTCAGTCTGTTCCTCAAC-3´	484 bp	Gilad *et al*. (2002)
KHV	5´-GGGTTACCTGTACGAG-3´ 5´-CACCCAGTAGATTATGC-3´	409 bp	Siwicki *et al*. (2006)
Heterosigma akashiwo	5´-GTCAACATCATTTCGGGTTTG-3´ 5´-CGCTGATTTGCTTCAAACTCTTG-3´	1186 bp	Higashi *et al*. (2017)
Heterosigma akashiwo	5´-GTGCGATTCATTACTGTAG-3´ 5´-CCCATTTTAGCGTAAGGGATT-3´	1320 bp	Akase *et al*. (2004)

KHV: Koi herpesvirus disease

(*Cyprinus carpio*) were collected from the affected pond during the mortality phase. Total body length and weight range of the fish samples were 25 to 35.5 cm and 570 to 780 g.

Fish tissue specimens including gills, liver and kidney were collected and fixed in 10% phosphate-buffered formalin for haematoxilin and eosin, giemsa and PAS staining, and in 2% glutaraldehyde solution for scanning electron microscopic (SEM) evaluation and also fixed in 70% ethanol solution for PCR test. PCR tests were carried out using 4 primers selected for *Heterosigma* and koi herpes virus (Gilad *et al*., 2002). Specific primers for KHV detecting and *H. akashiwo*, have been demonstrated in Table 1.

Nuclease-free water was used as the negative control. PCR products were visualized by electrophoresis in 1% agarose gel (Max Pure, Spain) and stained with safe stain (×1) (cinaclon) using the UV transilluminator (UVtech, Germany) (Gilad *et al*., 2002; Siwicki *et al*., 2006).

Results

According to the history of the studied two ponds, the mortality rate is estimated as 15% in 2 week intervals. In one of the cases, about 10 percent mortality in common carp at the farm was noted by the farmer during the first day of the bloom and mortality continued for 1 week. The only symptoms of the fish were lethargy and hypoxia symptoms. In examination of moribund fishes, excessive mucus secretion, multifocal white lesions and rot were visible in the gills. In wet smear preparations a lot of algal cells and fragments and sloughed, necrotic epithelial cells were observed between the lamellae (Fig. 1). After dissection, in internal organs examination, the intestines were empty and hyperemic with no other internal gross lesions.

In the water, the phytoplankton analysis was done by using direct microscopy the flagellated phytoplankton (cell diameter: 8.5 ± 4.2 µm) with 2 equal flagella. The morphology of dominant algal cell in water specimens using a light microscope is shown in Fig. 2. The algal cells resembled *Heterosigma* sp in shape.

In scanning electron microscopic examination the algae was found attached to the gill surface. Scanning electron micrographs of the attached algae to gills were then taken. A conical-shaped alga was clearly visible on the cell surface of the gill lamella (Fig. 3).

In histopathologic examination of gills, hyperplasia, necrosis and algal cells surrounded by hyperplastic cells were seen in tissue sections (Fig. 4). These cells had eosinophilic organelles that were PAS positive in PAS staining (Figs. 5 and 6). No white blood cell aggregation was noticed.

The result of PCR assay of KHV and *H. akashiwo* using the gill samples of carps affected with gill necrosis were found to be KHV and *H. akashiwo* DNA negative. More molecular investigation for identification of the causative algae species is needed.

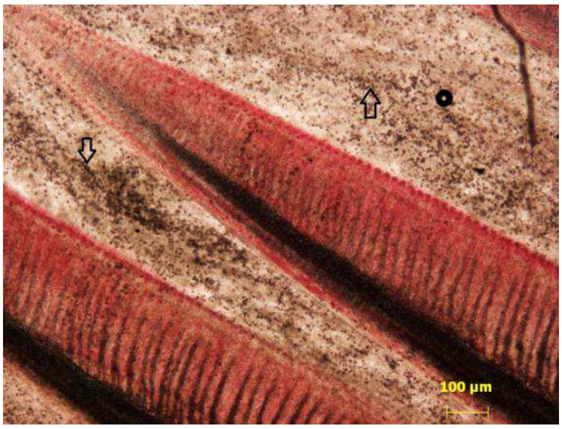

Fig. 1: Algal aggregation between gill lamella in a fresh smear from moribund common carp (arrows)

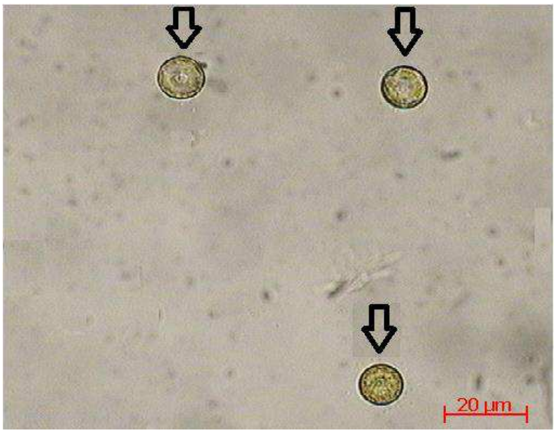

Fig. 2: *Heterosigma*-like alga in water smear from a plankton bloom associated with mortality of common carp farm (arrows)

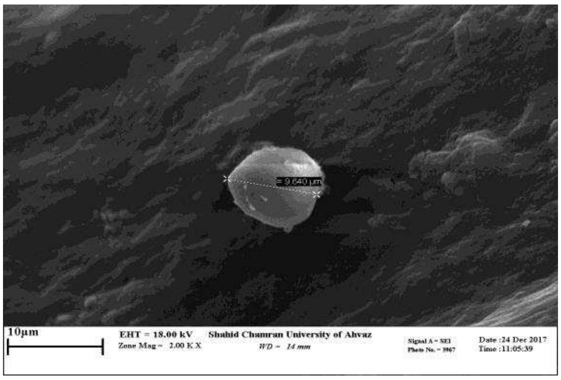

Fig. 3: Scanning electron micrograph of trophont attached to gill epithelium. Note the absence of surface patterning in the affected hyperplastic gill lamella

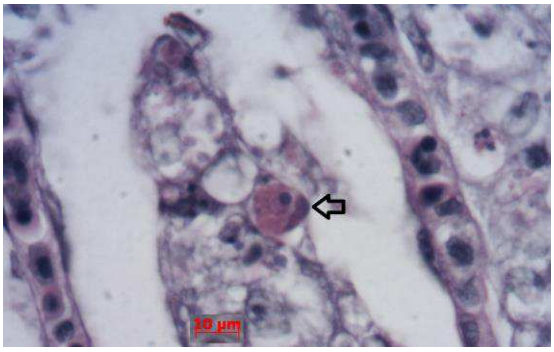

Fig. 4: Common carp gill. algae (arrow) entrapped in mucous cell. Note associated hyperplasia of the gill epithelium (PAS staining)

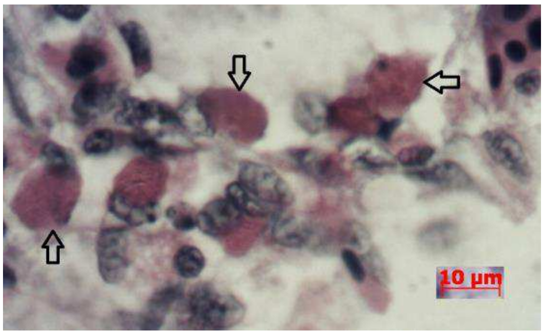

Fig. 5: Common carp gill lesions associated with algal bloom. Algal cells (arrows) between hyperplastic gill cells (PAS staining)

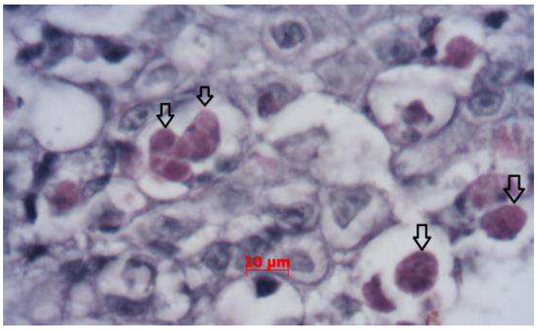

Fig. 6: Common carp gill. Algal cells containing eosinophilic organelles (arrows) between hyperplastic gill cells (PAS staining)

Discussion

In the affected ponds the water quality was apparently inappropriate and due to lethargy the fish mostly aggregated near the water outlet and sides of the pond. According to the history, despite the bad quality of the water, the farmers insisted on adding fertilizers to the water which deteriorated the situation. Organic fertilizers, such as bird droppings and inorganic fertilizers (i.e. superphosphate and urea) were used, usually without enough water circulation or artificial aeration. These mentioned situations may be considered as one of probable causes of unwanted bloom. Mortality rates associated with algal problems are largely dependent on the degree of bloom, the duration of exposure and husbandry methods used during recovery. In several such cases involving carp farms in Khuzestan province, affected populations continue to exhibit mortalities directly attributed to the bloom or may succumb to secondary infections caused by stress or bad management (Shikata *et al*., 2008; Abou El-Gheit *et al*., 2012).

The histological changes suggested that the mortality may be due to gill lesions. In wet smear no other pathogens (e.g. Flavobacteria, protozoa) were observed in the affected gills, and presence of algae in gill lesions was suggested so that the lesion may directly associate with accumulations of algae. These species seem to be cytotoxic and it may be associated with fish die-offs as a result of the aggregation of the alga in pond bottom where the common carp is mostly found. The gill lesion and accumulation of mucus on gill causes fish therein to suffocate.

In our cases the exact mode of bloom toxicity is currently unknown. Gill hyperplasia showed us that this species seems to be cytotoxic and it may be associated with fish die-offs as a result of the aggregation of the alga in pond bottom where the common carp is mostly found. Also, the gill lesion and accumulation of mucus on gill causes fish therein to suffocate. The alga may produce toxins, but we suggest the accumulation of the alga in gills is too high for fish, therefore gill irritation by algae may account for such a large pathologic effect on fish gills. Some local experts have argued the production of hydrogen sulfide gas (H_2S) in the bottom may be responsible for gill damage, however, water quality sampling suggest H_2S concentrations were far too low to have significant effects on fish.

Algal measurements were 8.5 ± 4.2 µm. We found that the shape of the *Heterosigma*-like algae in our study is similar to *Heterosigma* isolated from the Gulf of Mexico (Martinez *et al*., 2010). Cells of alga occurring in our survey were similar to the descriptions and illustrations of the species by Hara *et al*. (1985). Blooms of *H. akashiwo* are usually recorded in brackish waters (Martinez *et al*., 2010). However, the formation of *H. akashiwo* blooms has also been also associated with freshwater runoff (Taylor and Haigh, 1993), in agreement with the tolerance of this species to low salinities previously reported in the literature (Storm *et* *al*., 2013). Based on their SEM morphology, the algae appeared as the naked type in which its flagella may embed in the gill tissue.

It should be emphasized that, the assessment and definite identification of a toxic algae or other detrimental environmental impact on fish farm mortality usually is a complex task and it is not easy to prove it as the causative agent. This report constitutes a new phenomenon for the region where the algal mortality is the main cause and other factors may affect the mortality rate due to the presence of a wide range of possible causes.

Acknowledgements

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Conflict of interest

Authors declare no conflict of interest.

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