Combined exposure of carps (*Cyprinus carpio* L.) to cyanobacterial biomass and white spot disease

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Abstract

OBJECTIVES: Under environmental conditions, fish can be exposed to multiple stressors including natural toxins and infectious agents at the same time. This study brings new knowledge on the effects of controlled exposure to multiple stressors in fish. The aim of this study was to test the hypothesis that influence of cyanobacterial biomass and an infection agent represented by the white spot disease can combine to enhance the effects on fish.

METHODS: Common carps were divided into four groups, each with 40 specimens for 20 days: control group, cyanobacterial biomass exposed group, *Ichthyophthirius multifiliis*-infected fish (Ich) and cyanobacterial biomass-exposed fish + *Ichthyophthirius multifiliis*-infected fish. During the experiment we evaluated the clinical signs, mortality, selected haematological parameters, immune parameters and toxin accumulation.

RESULTS: There was no mortality in control fish and cyanobacterial biomass-exposed fish. One specimen died in *Ichthyophthirius multifiliis*-infected fish and the combined exposure resulted in the death of 13 specimens. The whole leukocyte counts (WBC) of the control group did not show any significant differences. Cyanobacteria alone caused a significant increase of the WBC on day 13 ($p \leq 0.05$) and on day 20 ($p \leq 0.01$). Also, *I. multifiliis* caused a significant elevation of WBC ($p \leq 0.01$) on day 20. Co-exposition resulted in WBC increased on day 13 and decrease on day 20, but the changes were not significant. It is evident from the differential leukocyte counts that while the increase of WBC in the group exposed to cyanobacteria was caused by elevation of lymphocytes, the increase in the group infected by *I. multifiliis* was due to the increase of myeloid cells. It well corresponds with the integral of chemiluminescence in the group infected by *I. multifiliis*,

ORIGINAL ARTICLE

which is significantly elevated on day 20 in comparison with all other groups.

CONCLUSIONS: We can confirm additive action of different agents on the immune system of fish. While single agents seemed to stimulate the immune response, the combination of both caused immunosuppression.

Abbreviations:

Adda	- 3-amino-9-methoxy-2,6,8-trimethyl-10-fenyldeca-4,6-dien acid
ANOVA	- analysis of variance
B	- group exposed to cyanobacteria
B + Ich	- group with combined exposure
BE	- brutto energy
C	- control group
CE	- collision energy, expressed as volts, for MS detection
ELISA	- enzyme immunoassay
ESI	- electrospray ionization
FBS	- fetal bovine serum
HPLC	- high performance liquid chromatography
Ig	- immunoglobulin
Ich	- *Ichthiophthirius multifiliis*
LC-MS/MS	- liquid chromatography with double mass spectrometry
LOQ	- limit of quantification
LSD test	- the least significant difference test
MC, MCs	- microcystin, microcystins
MCH	- mean corpuscular haemoglobin
MCHC	- mean corpuscular haemoglobin concentration
MCV	- mean corpuscular volume
MDL	- method detection limit
MRM	- multiple reaction monitoring mode
m/z	- represents mass divided by charge number of detected molecule
NCC	- nonspecific cytotoxic cells
NFE	- nitrogen free extract
OD450	- optical density
PBS	- phosphate buffer saline
PBST	- phosphate buffer saline Tween-20
PPI	- protein phosphatase inhibition
PCV	- haematocrit
RBC	- erythrocyte count
RLU	- relative luminescence unit
ROS	- reactive oxygen species
T 7	- 7 days after start of the experiment
T 13	- 13 days after start of the experiment
T 20	- 20 days after start of the experiment
TMB	- tetramethylbenzidine
WBC	- leukocyte count

INTRODUCTION

Under environmental conditions, fish can be exposed to multiple stressors including natural toxins and infectious agents at the same time. This study brings new knowledge on the effects of controlled exposure to multiple stressors in fish.

Mass development of cyanobacteria has become a serious problem in waters in many parts of the world. Their secondary metabolites, especially cyanotoxins, have been shown to cause adverse effects in various organisms including fish (for instance Råberg *et al.* 1991; Tencala *et al.* 1994; Bury *et al.* 1996; Carbis *et al.* 1996, 1997; Fischer & Dietrich 2000; Best *et al.* 2002; Jos *et al.* 2005; Malbrouck & Kestemont 2006). Other reports have also indicated that the toxicity of MCs may influence the immune system of fish (Palíková *et al.* 1998; Wright *et al.* 2004; Palíková *et al.* 2004; Sieroslawska *et al.* 2007; Rymuszka *et al.* 2007; 2008). It is also known, that the modulation of immune response is dose-dependent and the low concentrations of toxins may stimulate the immune system (Palíková *et al.* 1998; Sieroslawska *et al.* 2007).

The parasitic ciliate *Ichthyophthirius multifiliis* (Ich) infects a range of freshwater fish species causing a significant economic loss to the aquaculture industry (Dickerson 2006). It parasites on the skin and gills. Both specific and non-specific host defence mechanisms are responsible for the protection of fish against challenge infections with this ciliate. The specific humoral components comprise at least antibodies that agglutinate the parasites *in vitro* (Dickerson 2006; Hines & Spira 1974; Sigh & Buchmann 2001). Cellular immune responses play a major role in the defence against ichthyophthiriasis. Trophonts could be exposed to cell mediated responses as localised leukocytic infiltration of the epidermis and elevated leukocyte levels within peripheral blood (Houghton & Matthews 1993; Graves *et al.* 1985). The peripheral blood of Ich infected fish contained an increased percentage of active NCC with increased killing capacity and target cell affinity compared to peripheral blood NCC activity of uninfected fish (Graves *et al.* 1985).

Low concentrations of individual agents would not result in mortality on a separate basis and the combined exposure of experimental fish to individual stressors is ecologically realistic. The aim was to test the hypothesis that influence of cyanobacterial biomass and an infection agent represented by *Ichthyophthirius multifiliis* can combine to enhance the effects on fish. For this purpose we compared the effects of single and combined exposures and evaluated the clinical signs, mortality, selected haematological parameters, immune parameters and toxin accumulation.

MATERIALS AND METHODS

Fish

Fish with average weight of 233 ± 66 g were obtained from Pohořelice Fishery. Fish were placed to the laminated circular tanks with own recirculation with volume 1m^3. The acclimatisation lasted 14 days. Fish were fed by commercial granulated food.

Experimental design

Fish were divided into four groups, each with 40 specimens. Fish in the control group and the group infected by *Ichthyophthirius multifiliis* were fed by a commercial food Dibaq Carpio Plus (Spain, 35% proteins, 9% fat, 29.5% NFE, BE 24.4 MJ/kg). The other two groups were fed by the same food with addition of 1% of lyophilised toxic cyanobacterial biomass of natural origin. The whole amount of microcystins was 27 mg/kg of food, i.e. 0.4 mg /kg of fish weight and day. Feeding was twice

daily in the whole amount of 1.5% of fish stock. An adaptation to the feed ration was made for a week on the basis of actual fish weight.

The fish were infected by *Ichthyophthirius multifiliis* (Ich) in 1 group with commercial food and 1 experimental group exposed to cyanobacterial biomass. The intensity of infection was controlled weekly by the microscopic examination of the gills and it was expressed in absolute numbers of trophonts in high power fields (magnification 40 times).

Four groups of fish with all possible combinations of the above stressors and controls were employed in the study, i.e. control (C), cyanobacterial biomass-exposed fish (B), *Ichthyophthirius multifiliis*-infected fish (Ich) and cyanobacterial biomass-exposed fish + *Ichthyophthirius multifiliis*-infected fish (B + Ich).

The exposure lasted 20 days. Five to seven specimens from each group was euthanised, necropsied and samples for the evaluation were taken on days 7, 13 and 20 (T7, T13, T20).

The experiment was performed in compliance with the laws for the protection of animals against cruelty as approved by the Ethical Committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic.

Experimental procedures and laboratory analyses

Blood samples were collected for haematology and serology. Briefly, blood (2 mL) was collected by cardiac puncture to heparinised (1.5 mL) and non-heparinised (0.5 mL) syringes. Whole heparinised blood was used for the evaluation of the phagocytic activity by luminol enhanced chemiluminescence by using the modified method according to Kubala *et al.* (1996) and for the evaluation of white blood cell counts (WBC) and red blood cell counts (RBC) according to Svobodová *et al.* (1986). Blood smears on glass slides, prepared immediately after blood collection, were air-dried and stained using May-Grünwald and Giemsa-Romanowski stains. Two hundred leukocytes were counted for each smear and classified as neutrophils, lymphocytes and monocytes. Non-heparinised blood was centrifuged and serum removed and frozen. The total immunoglobulins were evaluated in serum by ELISA method. Microtitration plate (GAMA Ceske Budejovice) wells were filled with 100 µl of monoclonal antibody 1E10/2A8 against carp Ig (Vesely *et al.* 2006) at a working dilution of 1:10 000 in binding bicarbonate buffer pH 9.6 and incubated overnight in a refrigerator at 4±2 °C. Upon binding of the antibody, the plates were washed three times with PBST wash buffer (0.1% Tween 20 in PBS; pH 7.2) and all wells were blocked for 1 h at 37 °C by adding 100 µL/well blocking buffer (2% FBS in PBST). After washing in PBST three times, incubation of the tested carp sera followed. Carp sera were examined in duplicate at four dilutions expressed as log 2 values (1:500–1:4000). In each microtitration plate, purified carp Ig at concentrations of 0.25–15.9 µL/mL was included as a positive control. Then, the samples were incubated for 60 min at 37±2 °C in a moist chamber. After washing with PBST three times, 50 µl of monoclonal antibody 1E10/2A8 conjugated to horseradish peroxidase by using the periodate method (Boorsma & Streefkerk 1979) was dispensed into each well. After incubation at 37±2 °C for 1 hour, the plates were washed again three times with PBST wash buffer and 100 µl substrate, prepared immediately before use by mixing a two-component substrate solution containing chromogentetramethylbenzidine (TMB), substrate buffer and hydrogen peroxide (Test-Line, Ltd.) was dispensed into each well. The enzymatic reaction was carried out for 10 min at room temperature. Then, the reaction was stopped by adding 100 µl of 1M sulphuric acid. The absorbance of the colour produced was measured usingan ELISA reader at a wave length of 450 nm (OD_{450}). For the quantification of carp serum Ig levels, seven different dilutions of purified carp Ig in the concentration range from 0.25 to 15.9 µg/mL were used. A calibration curve was constructed and used to convert the values of the examined sera detected by ELISA (OD_{450}) to the carp Ig levels (mg/mL).

Microcystin concentrations in liver were analysed by LC-MS/MS method according to Kohoutek *et al.* (2010). Tissue (frozen sample; 0.5 g fresh weight) was homogenised 3 times with methanol (3 mL), sonicated in an ultrasonic bath for 30 min, and centrifuged at 2 900 *g* for 10 min. Supernatants were pooled and extracted repeatedly (3 times) with hexane (1 mL) to remove lipids. Extract was evaporated at 50 °C and the residue was dissolved in 300 µL of 50% aqueous methanol (v/v) and used for LC-MS/MS analyses. Analyses were based on Liquid Chromatography Mass Spectrometry (MS/MS) with MRM (multiple reaction monitoring mode) using the HPLC apparatus Agilent 1200 series (Agilent Technologies, Waldbronn, Germany), which consisted of a vacuum degasser, a binary pump, an autosampler, and a thermostatted column compartment kept at 30 °C. The column was a Supelcosil ABZ+Plus RP-18 endcapped (5 µm) 150 × 4.6 mm i.d. (Supelco). A SecureGuard C18 (Phenomenex, Torrance, CA, USA) guard column was used. The mobile phase consisted of 5 mM ammonium acetate in water, pH 4 (A) and acetonitrile (B). The binary pump gradient was linear (increase from 20% B at 0 min to 59% B at 30 min, then 90% B for 15 min); the flow rate was 0.4 mL/min. 20 µL of individual sample was injected for the analyses. The mass spectrometer was an Agilent 6410 Triple Quad mass spectrometer (Agilent Technologies, Waldbronn, Germany) with electrospray ionization (ESI). Ions were detected in the positive mode. The ionization parameters were as follows: capillary voltage, 5.5 kV; desolvation temperature, 350 °C; desolvation gas flow, 11 L/min. The transitions from the protonated molecular ion to a fragment of amino acid Adda (unusual aminoacid present only in microcystins and related nodularins - (2S,3S,8S,9S)-3-amino-9-me-

thoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) at m/z 135.2 and fragment at m/z 127.1 were monitored in multiple reaction monitoring (MRM) mode. Collision energies (CE) used for fragmentation were 50V for MC-RR and respective conjugates, and 40V for MC-YR and -LR and respective conjugates. Quantification of analytes was based on external standards of MC-RR, MC-YR, MC-LR in matrix (final extract of microcystin-free fish tissue). Method detection limit (MDL; per gram of tissue, fresh weight) was 3 ng/g in MRM mode.

Statistical analysis

Statistical analyses were performed with Statistica for Windows® 7.0 (StatSoft, Tulsa, OK, USA). Results from different treatment groups were compared by one-way analysis of variance (ANOVA) and post-hoc analysis of means using the LSD test.

RESULTS

Only minimal concentrations of microcystins in several specimens were detected but the values were under the LOQ (limit of quantification).

There was no mortality in control fish (C) and cyanobacterial biomass-exposed fish (B). One specimen died in *Ichthyophthirius multifiliis*-infected fish (Ich) on day 19 of exposure. The combined exposure to cyanobacterial biomass-exposed and *Ichthyophthirius multifiliis*-infected fish (B+Ich) resulted in the death of 13 specimens. Fish died on days 18, 19 and 20 of exposure. The examined fish of groups Ich and Ich+B showed increased amounts of mucus on skin and gills and presence of necrotic lesions on gills in T 20. The intensity of Ich infection during the experiment is presented in Table 1.

Tab. 1. Intensity of *Ichthyophthirius multifiliis* infection on the gills of fish (absolute numbers of trophonts in the microscope field of view at magnification 40 times).

group	Days of exposure	*Ichthyophthirius multifiliis* (trophonts numbers, magnification 40x)	n
C	T7	0	5
B	T7	0	5
Ich	T7	0–4	5
B + Ich	T7	0–4	5
C	T13	0	5
B	T13	0	6
Ich	T13	0–7	5
B + Ich	T13	0–50	6
C	T20	0	7
B	T20	0	7
Ich	T20	10–100	7
B + Ich	T20	20–80	5

No significant differences were found in the red blood cell counts, the values ranged between 1.3–1.9 T/L. The whole leukocyte counts (WBC) of the control group (C) did not show any significant differences during the experiment (40.3±4.0 G/L in T7, 34.8±5.1 G/L in T13 and 34.0±14.0 G/L in T20). Cyanobacteria alone (group B) caused a significant increase of the WBC on $p \leq 0.05$ in T13 (from 34.2±6.6 in T7 to 44.0±7.9 G/L) and on $p \leq 0.01$ in T20 (to 51.4±11.8 G/L). Also *I. multifiliis* (Ich) caused a significant elevation of WBC on $p \leq 0.01$ in T20 (to 74.3±32.8 G/L) in comparison with T7 (29.1±13.4 G/L) and T13 (33.2±4.7 G/L). By the influence of co-exposition (B + Ich) WBC increased in T13 and decreased in T20 (from 35.6±8.2 G/L in T7 and 40.7±7.5 G/L in T13 to 31.6±10.4 G/L), but the changes were not significant (Figure 1). It is evident from the differential leukocyte counts that while the increase of WBC in group B treated by cyanobacteria was caused by elevation of lymphocytes (T7 31.5±6.7 G/L, T13 40.8±8.2 G/L, T20 44.8±7.8 G/L), the increase in group Ich infected by *I. multifiliis* was due to increase of myeloid cells, i.e. neutrophile myelocytes, metamyelocytes and bands (T7 3.1±1.5 G/L, T13 4.0±2.1 G/L, T20 44.1±28.6 G/L). The great increase may be created by reaction of the immune system of infected fish and by flooding out of these cells to peripheral blood. It well corresponds with the integral of chemiluminescence in the group infected by *I. multifiliis* which is significantly elevated in T20 in comparison with all other groups (Figure 2). As clearly shown in Figure 3, the kinetics of opsonised phagocytosis of this group was much higher in T20 when compared against the other groups. The whole immunoglobulins did not show any significant change, the ranges of average values of single groups during the whole experiment were as follows: C: 4.93–10.73 g/L, B: 2.34–10.15 g/L, Ich: 3.54–5.0 g/L, B+Ich: 2.0–3.73 g/L.

DISCUSSION

Some authors reported very high concentrations of microcystins accumulated in fish tissues (Magalhães *et al.* 2001; Xie *et al.* 2005; Ibelings *et al.* 2005). However, these concentrations were found in fish living in the natural environment with massive development of the toxic water bloom. At the experimental level various concentrations of microcystins were found in fish tissues following intraperitoneal injection (Malbrouck *et al.* 2004, Li *et al.* 2007). The presence of microcystins was also detected in fish tissues following oral application (Li *et al.* 2004; Xie *et al.* 2004; Soares *et al.* 2004; Shen *et al.* 2005; Zhao *et al.* 2006; Zhang *et al.* 2007). However, these authors mostly used different methods for detection of microcystins, namely the ELISA method, PPI and HPLC method. In our study no concentrations of microcystins exeeding the LOQ were found in the liver of fish. The method used in our study with tandem mass-spectrometric detection is more

selective and does not provide false-positive responses (Kohoutek *et al.* 2010). Moreover, the overall doses of microcystins in most above-mentioned studies were several times higher in comparison with our study. We can assume that the daily intake of microcystins in dose of 0.4 mg /kg of fish weight in food was low to relinquish the detectable residues in the liver.

The above-mentioned low dose did not cause mortality in the fish exposed to cyanobacteria only. In the group infected by Ich one specimen died on day 19 of the experiment. However, in the combined group 13 fish died at the same period. The fish also showed patho-anatomic changes typical for ichthyophthiriasis. It is obvious from Table 1 that a substantially higher increase of trophonts count appeared on the gills at this time. The intensity of Ich was 7 times higher in this group. We assume that the pathogen had better conditions in an organism exposed to multiple stressors. This assumption was supported by the leukocyte increase in the group infected by Ich only in comparison to the combined group where the decrease in WBC was found in T20. Our results show that the alimentary intake of cyanobacterial mass led to the elevation of leukocytes. This effect was obvious after 13 days and lasted during following days of treatment. The elevation was induced by the significant increase of lymphocytes.

Development of Ich infection led also to the elevation of leukocytes. However, in this case the elevation was caused by the elevation of myelocytes and was accompanied with an increase of OZP-activated phagocytic activity. This effect was obvious after 20 days. The additive action of both agents caused, on the other hand, a decrease of the studied immune parameters. Our previous work shows that even a 24h exposure to cyanobacteria can lead to a decrease of OZP-activated phagocytic activity, but the low concentrations of toxins may stimulate the immune system (Palíková *et al.* 1998; Sieroslawska *et al.* 2007).

Also, the concentrations of cyanobacteria used in our study stimulated the studied immune parameters, as well as the Ich. On the other hand, the additive action of both agents resulted in their reduction.

Integral values of chemiluminescence obtained from kinetic curves give us information about the efficacy of the oxidative burst on the whole organism level. To obtain more precise information about the effect of cyanobacteria on phagocytes themselves, integral values were corrected to absolute counts of myeloid cells. Immature cells were included, as it was shown previously that myeloid progenitors participate on ROS production during the oxidative burst. After the correction, the inhibitory effect remained unchanged. The lack of the effect on spontaneous activity excludes decreased viability as well as non-specific effects on phagocytic function. This suggests that the combined exposure really led to the suppression of the oxidative burst. Altogether, this suggests that the inhibitory effect was the result of influencing the signalling pathway leading to the activation of neutrophil NADPH oxidase and ROS production. However, the exact mode of action on neutrophil signalling remains to be elucidated.

In eutrophic water bodies, fish are commonly exposed to various concentrations of cyanobacteria.

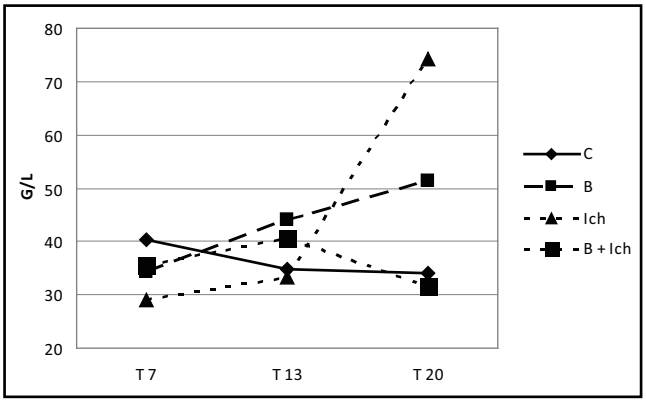

Fig. 1. White blood cell counts during the experiment (C - control, B - cyanobacterial biomass-exposed fish, Ich – *Ichthyophthirius multifiliis*-infected fish, B + Ich - cyanobacterial biomass-exposed fish + *Ichthyophthirius multifiliis*-infected fish.

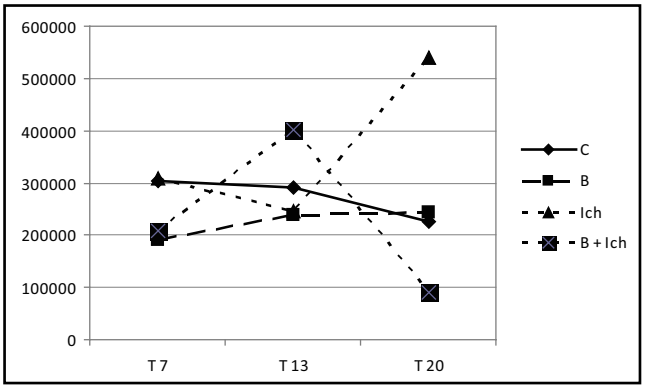

Fig. 2. Integral of chemiluminescence (mV/s) during the experiment (C - control, B -cyanobacterial biomass-exposed fish, Ich – *Ichthyophthirius multifiliis*-infected fish, B + Ich - cyanobacterial biomass-exposed fish + *Ichthyophthirius multifiliis*-infected fish.

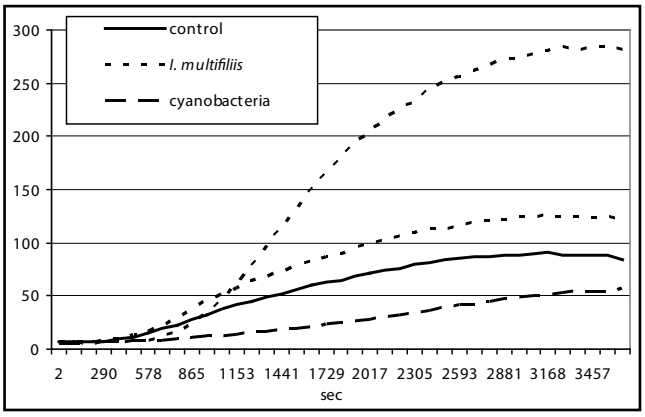

Fig. 3. Kinetics of opsonised phagocytosis (RLU/s) in the T20.

During summer, this exposure can last for weeks. It was shown previously that exposure to cyanobacteria or their released toxins can cause various health problems in animals. However, the fish can be affected by multiple stressors in the environment and while mostly single agents in sub-lethal doses do not involve visible changes or mortality, their co-exposure does. The studies of co-exposure to various agents including cyanobacteria are lacking in fish.

Paskova *et al.* (2011) and Pikula *et al.* (2010) studied the combined exposure of cyanobacterial biomass, lead and the Newcastle virus in Japanese quails (*Coturnix coturnix japonica*). The cyanobacterial biomass was applied orally in dose 46μg MCs per day for 30 days. Cyanobacterial biomass-exposed birds received a total of 1381.32 μg of microcystins over 30 days of exposure. No mortality and no clinical signs of toxicity were found in birds exposed to cyanobacterial biomass only, but mortality occurred in combined exposures and acute effects were observed around day 10 in combined exposure. Paskova *et al.* (2011) confirmed a general stimulation of the antioxidative system with the greatest modulations of sub-lethal parameters in specimens from the groups with combined exposures. These results support the hypothesis of higher energy demand to counteract adverse effects of multiple exposures.

This work originally shows that while the single agents in sub-lethal doses do not cause visible changes or mortality and may stimulate the immune parameters, their co-exposure leads to the inhibition of the oxidative burst of fish phagocytes. As phagocytosis is the first line defence against invading pathogens, it can be supposed that the additive action of Ich and cyanobacteria can have a strong impact on the non-specific immune response of fish and can contribute to the increased susceptibility to infectious diseases.

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Potential Conflicts of Interest: *None disclosed.*

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