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Effect of arsenic and cyanobacterial co-exposure on pathological, haematological and immunological parameters of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

OBJECTIVES: Under environmental conditions, fish are simultaneously exposed to multiple stressors. This study provides new knowledge on the effects of controlled exposure to multiple stressors, namely cyanobacterial biomass and food contaminated with arsenic.
METHODS: Rainbow trout were divided into six groups of 25 fish and exposed to different contaminant combinations for 30 days: 1) control group, 2) cyanobacterial biomass, 3 & 4) two groups exposed to arsenic at concentrations of 5 $mg.kg^{-1}$ and 50 $mg.kg^{-1}$ fish feed, and 5 & 6) two groups exposed to cyanobacterial biomass and arsenic combined. We then evaluated pathological, haematological and immunological parameters at 10, 20 and 30 days after exposure.
RESULTS: Marked gross pathological findings were present in groups exposed to arsenic and arsenic/cyanobacteria after 30 days. A strong decrease in haemoglobin concentration was observed in all experimental groups receiving arsenic after 10 days exposure. Total leukocyte count increased markedly in fish exposed to cyanobacterial biomass, and to higher arsenic concentrations by the end of the experiment. Neutrophils decreased significantly at the end of exposure. Similarly, exposure to cyanobacteria and/or arsenic led to suppression of opsonised zymosan particle-induced neutrophil respiratory bursts.
CONCLUSIONS: Our results demonstrate that the effects of exposure to toxic cyanobacterial biomass and arsenic on fish are enhanced when the contaminants are combined. In particular, long-term exposure led to disturbances in the white blood-cell count. Modulation of phagocytosis, which is the first line of defence against invading pathogens, suggests that the combined action leads to a decreased ability to control infection.

ORIGINAL ARTICLE

Abbreviations

ANOVA	- analysis of variance
As	- arsenic
As 5; As 50	- groups fed with addition of arsenic
B	- group fed with addition of cyanobacterial biomass
B + As 5	- group with combined feeding
B + As 50	- group with combined feeding
C	- control group
CL	- chemiluminescence
DW	- dry weight
HPLC	- high performance liquid chromatography
LC-MS/MS	- liquid chromatography with double mass spectrometry
MC, MCs	- microcystin, microcystins
MC LR	- microcystin LR
MC RR	- microcystin RR
MC YR	- microcystin YR
MCH	- mean corpuscular haemoglobin
MCHC	- mean corpuscular haemoglobin concentration
MCV	- mean corpuscular volume
MRM	- multiple reaction monitoring mode
PCV	- packed cell volume - haematocrit
RBC	- red blood cell count
SVC	- spring viraemia of carp
t10, t20, t30	- 10, 20, 30 days after start of the experiment
OZP	- opsonised zymosan particles

INTRODUCTION

Cyanobacterial toxins are amongst the most common negative factors affecting fish. Cyanobacteria, also known as blue-green algae, are natural components of marine and freshwater bacteria and occur worldwide. Under favourable eutrophic conditions (i.e. calm water, high temperatures and an abundance of nutrients), they often form huge blooms. Such cyanobacterial overgrowths constitute a real health risk as the toxins produced by cyanobacteria can exert a variety of adverse effects on human and animal health (e.g. Carbis *et al.* 1997; Dawson 1998). Microcystins are amongst the most common cyanobacterial toxins, and hence are the most studied group (Welker & von Dohren 2006).

Microcystins produce a range of adverse effects on both terrestrial and aquatic organisms (Palíková *et al.* 1998, 2004; Pikula *et al.* 2010; Paskova *et al.* 2011; Ondracek *et al.* 2012; Adamovsky *et al.* 2013; Palikova *et al.* 2013). In fish, microcystins are directly consumed with cyanobacteria. Microcystins are also released into the water following the death and decay of cyanobacterial biomass, therefore, they can also enter the fish's body through the gills (Sieroslawska *et al.* 2012). Thus, even predaceous fish, which do not normally consume plant matter, are unceasingly exposed to microcystins during the occurrence of cyanobacterial blooms. Moreover, microcystins can persist in fish tissue and be passed on through the food chain (Kopp *et al.* 2013).

Arsenic is one of the most toxic environmental contaminants. In the aquatic environment, it occurs both naturally, from geological sources, and as a result of human activity (Bhattacharya *et al.* 2007). Arsenic is readily accumulated in the tissues of marine fish and other marine organisms (e.g. Zhang *et al.* 2011; Ruttens *et al.* 2012). Freshwater fish can face high arsenic exposures when fed commercial feed mixtures containing marine fish meal. In the Czech Republic, the arsenic content in complete fish feed is limited to 6 $mg.kg^{-1}$ under Czech Decree No. 356/2008 on feedstuffs.

It is already known that the toxic effect of a substance can be modified through simultaneous exposure to other agents and that combined exposure to multiple toxins often has a different result than would be expected from simply adding the effects of the individual components (Silins & Högberg 2011). This also applies to cyanobacterial toxins, as shown by our previous work (Palikova *et al.* 2012) on the combined exposure of carp (*Cyprinus carpio* L.) to cyanobacterial biomass and the white spot disease agent, which had an additive effect on the fish's immune system. While the individual agents appeared to stimulate an immune response, the combination of both caused immunosuppression (i.e. a decrease in leukocyte count and the intensity of phagocytic activity along with a non-significant decrease in total immunoglobulin level). On the other hand, combined exposure to cyanobacteria and SVC virus led to stimulation of the specific immune response in carp, apparently due to optimal water temperatures. This process probably plays an important role in accumulation of toxins in the hepatopancreas, as well as their elimination (Soukupová *et al.* 2014). Kopp *et al.* (2014) has shown that co-exposure to arsenic and cyanobacteria can lead to either a significant decrease or increase in electrolytes in rainbow trout. While studying combined exposure of cyanobacterial biomass, lead and Newcastle virus in Japanese quail (*Coturnix coturnix japonica*), Pikula *et al.* (2010) observed no mortality and no clinical signs of toxicity exposed to cyanobacterial biomass alone, but mortality following combined exposure, with acute effects observed around 10 days after exposure.

In this work, we examine the effect of combined exposure of cyanobacteria and inorganic arsenic (5+) on haematological and immunological parameters of rainbow trout (*Oncorhynchus mykiss* W.). The aim of this study was to test the hypothesis that both stressors could combine, enhancing their effect on fish.

MATERIALS AND METHODS

Fish

All rainbow trout for this experiment (average weight 288±59 g) were obtained from the Skalní Mlýn commercial fishery, Czech Republic. The fish were divided into six experimental groups (see below) and each group was placed into individual 1 m^3 tanks with their own recirculation systems. These tanks were kept under controlled light and temperature conditions throughout the experiment. Water temperature, pH and oxygen saturation were monitored every day and were as follows: temperature 15.4±0.5 °C, oxygen saturation 89.0±2.2%, pH 8.20±0.14. The fish were left to acclimatise for 20 days prior to the start of the experiment.

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Experimental design

Fish were divided into six groups of 25 fish each. A control group (C) was fed with EFICO Enviro 920 commercial complete food (Biomar, Denmark), which contained arsenic at trace levels (1.55 $mg.kg^{-1}$) below the maximum allowable limits. Five experimental groups each received the same food but with the addition of 1) 3% lyophilised cyanobacterial biomass (B), 2) arsenic at 5 $mg.kg^{-1}$ (As 5), 3) arsenic at 50 $mg.kg^{-1}$ (As 50), and 4) & 5) a combination of cyanobacterial biomass and arsenic at B + As 5 and B + As 50. Fish were fed twice a day for 30 days at 0.8% of fish weight for the first 10 days and then at 1% of fish weight from then on (experimental design and daily doses of arsenic and microcystins are provided in Table 1).

Cyanobacteria and arsenic exposure

Cyanobacterial biomass (identified microscopically as a monoculture of *Microcystis aeruginosa*) was obtained from ponds of the Pohořelice Fishery, Czech Republic. The cyanobacterial biomass was lyophilised and stored at –20 °C for further use. Microcystin concentration was measured using a modified LC-MS/MS method according to Kohoutek *et al.* (2010). The microcystins present were identified as MC-RR (1462.4 $\mu g.g^{-1}$ DW), MC-LR (1087.9 $\mu g.g^{-1}$ DW), MC YR (95.9 $\mu g.g^{-1}$ DW) and unidentified (51.7 $\mu g.g^{-1}$ DW). Total microcystin concentration was 2697.9 $\mu g.g^{-1}$ DW; hence 3% of cyanobacterial biomass added to the feed corresponded to 81 mg $MCs.kg^{-1}$ feed (Table 1).

A standardised arsenic solution (Astasol, Analytika Praha, Czech Republic) was used for preparation of arsenic-contaminated food, with the stock solution containing 1.000±0.002 $g.L^{-1}$ As^{5+} in 2% nitric acid.

Fish sampling

Seven fish from each group were sampled after 10, 20 and 30 days of exposure. Blood samples (2 mL) were taken by cardiac puncture using heparinised syringes. The fish were then sacrificed by stunning with a blow to the back of the head followed by spinal cord transaction. Each fish was dissected and pathological changes evaluated (see below). The heparinised blood was used for evaluation of haematological parameters and phagocyte activity.

All experiments were performed in compliance with Czech laws for the protection of animals against cruelty (Act No 246/1992 and amendments), as approved by the Ethical Committee of the University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic.

Haematological parameters, plasma iron and phagocyte activity analysis

Red and white blood cell counts, haematocrit values, haemoglobin concentration and mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV) were assessed according to Svobodová *et al.* (2012). Blood smears were stained using May-Grünwald and Giemsa-Romanowski stains. Two hundred leukocytes were counted for each smear and classified as neutrophils, lymphocytes and monocytes. Phagocyte activity (OZP-induced respiratory burst of neutrophils) was measured by luminol-enhanced chemiluminescence using the modified method of Kubala *et al.* (1996). Iron (Fe) was determined photometrically with ferene (ferroin-type reagent) without deproteination (Higgins 1981).

Statistical analysis

All statistical analyses were performed using Statistica for Windows® 7.0 (StatSoft, Tulsa, OK, USA). Data were evaluated using ANOVA and post-hoc analysis using the least significant difference test.

RESULTS

Pathological changes

No mortality was observed in experimental fish over the entire 30 day exposure period. Furthermore, no macroscopic pathological changes were observed over the first 10 and 20 days. After 30 days exposure, however, marked pathological changes were observed, especially in fish co-exposed to cyanobacteria and arsenic. Of the seven fish in the B + As 5 group, one had an enlarged spleen, two showed gill haemorrhaging and

Tab. 1. Experimental design. Microcystin and arsenic levels in feed and daily dose (first 10 days/following 20 days) of microcystins and arsenic in individual experimental groups.

Group	Microcystins in feed ($mg.kg^{-1}$)	Microcystin daily dose ($mg.kg^{-1}$ fish weight)	Arsenic in feed ($mg.kg^{-1}$)	Arsenic daily dose ($mg.kg^{-1}$ fish weight)
C	–	–	–	–
B	81	0.65/0.81	–	–
As 5	–	–	5	0.04/0.05
As 50	–	–	50	0.4/0.5
B + As 5	81	0.65/0.81	5	0.04/0.05
B + As 50	81	0.65/0.81	50	0.4/0.5

three liver haemorrhaging. In the As 50 group, five showed gill haemorrhaging, one focal gill necrosis and two liver haemorrhaging. Pathological changes were even more pronounced in the B + As 50 group, with all seven fish displaying gill haemorrhaging. In addition, one displayed focal gill necrosis, four liver haemorrhaging and three an enlarged spleen.

Haematological parameters and plasma iron

No significant changes were observed in erythrocyte count or haematocrit values after 10 days exposure. There was, however, a strong decrease in haemoglobin concentration, with MCH and MCHC observed in all experimental groups receiving arsenic (Table 2). Interestingly, these changes were no longer recorded after 20 and 30 days exposure (Table 3 and 4). Iron concentrations decreased significantly after 20 days exposure in the B + As 50 group, and in the As 5, B + As 5 and B+ As 50 groups after 30 days (Table 5). Total leukocyte counts increased markedly in group B + As 50 after 30 days due to a decrease in neutrophil count, which affected the lymphocytes. Indeed, neutrophils decreased significantly against the control in all experimental groups except for As 50 (Figure 1).

Phagocyte activity (OZP-induced respiratory burst of neutrophils)

Thirty days exposure to cyanobacteria and/or arsenic led to a clear suppression in OZP-induced respiratory bursts of neutrophils. This was most pronounced in the B + As 50 group (p=0.003; Figure 2). A similar trend was observed after 20 days exposure but with no statistical significance. Interestingly, when the chemiluminescence signal was normalised to $1*10^9$ neutrophils, arsenic stimulated a respiratory burst at 5 mg.kg^{-1} (As 5), though not at a statistically significant

Tab. 2. Selected haematological parameters for trout after 10 days exposure (mean ± standard deviation, N=7).

haematological parameters	C	B	As 5	As 50	B + As 5	B + As 50
Hb (g.L^{-1})	115.0±35.6	104.4±34.1	69.3±18.1**	78.4±12.2*	73.9±5.5**	70.9±14.3**
MCH (pg)	90.5±32.4	83.2±29.4	54.7±12.3*	78.1±21.4	55.4±9.5*	60.7±12.5*
MCHC (L.L^{-1})	0.38±0.08	0.27±0.08	0.20±0.05*	0.23±0.04*	0.19±0.03**	0.18±0.04**

Asterisks indicate statistically significant changes compared to the control at p<0.01 (**) and p<0.05 (*).

Tab. 3. Selected haematological parameters for trout after 20 days exposure (mean ± standard deviation, N=7).

haematological parameters	C	B	As 5	As 50	B + As 5	B + As 50
Hb (g.L^{-1})	89.4±15.6	82.1±13.6	82.0±6.6	84.1±6.6	80.5±9.1	85.1±13.9
MCH (pg)	72.5±11.2	65.7±16.5	65.9±11.5	70.0±13.2	62.7±13.3	77.8±25.8
MCHC (L.L^{-1})	0.22±0.04	0.22±0.04	0.21±0.04	0.24±0.03	0.19±0.03	0.24±0.07

Tab. 4. Selected haematological parameters for trout after 30 days exposure (mean ± standard deviation, N=7).

haematological parameters	C	B	As 5	As 50	B + As 5	B + As 50
Hb (g.L^{-1})	85.1±13.9	75.4±5.5	94.8±14.3	86.7±11.7	79.8±12.4	85.1±9.7
MCH (pg)	69.1±18.0	63.0±8.9	77.6±18.3	69.0±13.3	61.9±13.6	64.0±14.3
MCHC (L.L^{-1})	0.16±0.04	0.16±0.03	0.22±0.05*	0.18±0.03*	0.17±0.03*	0.18±0.03*

Asterisks indicate statistically significant changes compared to the control at p<0.05 (*).

Tab. 5. Plasma iron levels (µmol. L^{-1}) after 10, 20 and 30 days exposure (mean ± standard deviation, N=7).

Days exposure	C	B	As 5	As 50	B+As 5	B+As 50
t 10	21.3±4.0	25.5±1.3	23.4±8.3	24.3±7.6	22.2±3.6	20.8±1.3
t 20	22.6±1.0	24.0±3.2	20.7±4.2	21.4±6.2	23.9±1.3	15.9±2.5*
t 30	25.4±2.2	20.6±4.3	19.7±2.3*	23.48±3.0	18.9±5.2*	19.8±1.4*

Asterisks indicate statistically significant changes compared to the control at p<0.01 (**) and p<0.05 (*).

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level (Figure 3). Cyanobacteria and arsenic had no significant effect on OZP-induced respiratory burst after 10 days exposure.

DISCUSSION

In fish, haematological parameters can be affected by many factors, including toxins (Palíková *et al.* 2010; Kopp *et al.* 2011). As disturbances in the complete blood count occur early, usually long before any clinical and histopathological changes, haematological examination can be useful when evaluating toxic effects (Benarjee *et al.* 2010). In the present study, pathological changes were observed after 30 days exposure yet the first haematological disturbances occurred after just 10 days, with the most marked changes being a decrease in haemoglobin accompanied by decreased MCH and MCHC. In this case, there was no significant difference between experimental groups when cyanobacteria and arsenic were taken simultaneously.

In humans, chronic arsenic poisoning almost always results in anaemia (Guha Mazumder 2008), usually in the form of normocytic normochromous anaemia caused by haemolysis (Lee *et al.* 2004) or suppression of erythropoiesis (Subcommittee on Arsenic in Drinking Water 1999). Erythrocyte counts were unchanged in our experimental fish and no signs of haemolysis were observed. Based on the results of the complete blood count, the anaemia observed in our experimental fish could be classified as normocytic hypochromous anaemia, which is most frequently caused by a lack of iron due to low iron intake or high iron loss. Loss of iron through the gastrointestinal tract (bleeding) was excluded in the present study, despite gastrointestinal bleeding being associated with arsenic poisoning (Ratnaike 2003). Similarly, no signs of bleeding and no defects in the gastrointestinal mucosa were observed after 10 days. Moreover, we observed no significant decrease in plasma iron levels after 10 days. Interestingly, we did not observe any hypochromous anaemia following prolonged exposure for 20 and 30 days, despite a decrease in plasma iron levels in most groups receiving arsenic. These results imply an increase in the consumption of iron, necessary for the recovery of haemoglobin levels. While our fish were all fed using a complete commercial feed with a balanced content of nutrients and minerals, it is possible that iron intake was not sufficient to fulfil demand during increased erythrocyte turnover. The enlarged spleens found after 20 and 30 days makes an increase in erythrocyte turnover highly plausible. Another possible cause of hypochromous anaemia is some form of disturbance to haemosynthesis. In humans, arsenic has been shown to affect more than 200 different enzymes (Subcommittee on Arsenic in Drinking Water 1999), including those involved in haemosynthesis. Haemosynthesis suppression is unlikely in our experimental fish as hypochromous anaemia was not observed at later dates (i.e. after

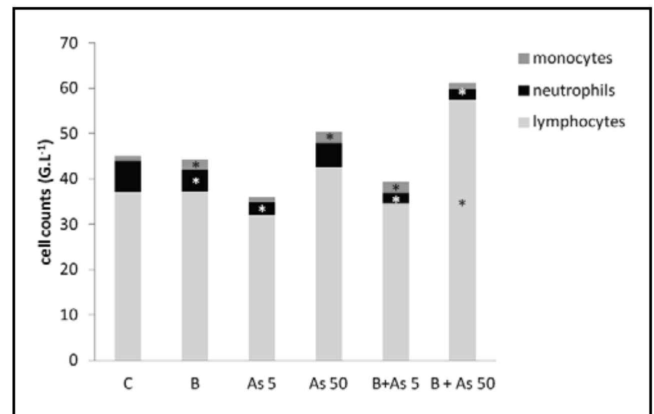

Fig. 1. Mean white blood cell counts after 30 days exposure. Statistically significant changes compared to the control ($p<0.05$) are marked with asterisks.

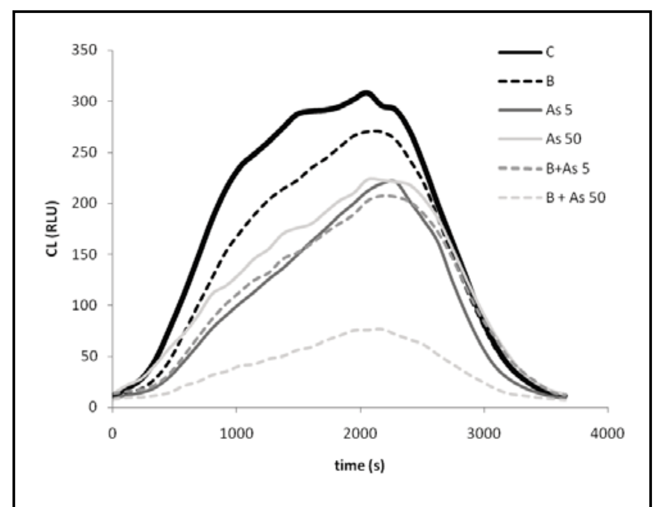

Fig. 2. Phagocyte kinetic activity. OZP-induced chemiluminescence (CL) expressed in relative luminescence units (RLU) following 30 days exposure. Each curve represents the calculated mean for seven experimental fish.

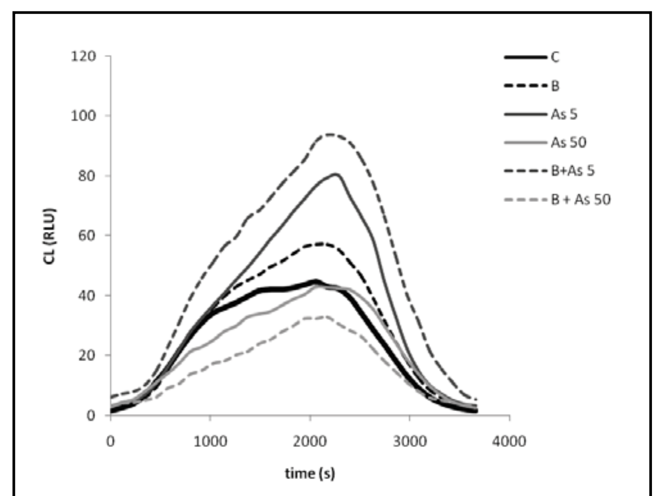

Fig. 3. Phagocyte kinetic activity. OZP-induced chemiluminescence (CL) expressed in relative luminescence units (RLU) and corrected to $1*10^9$ neutrophils following 30 days exposure. Each curve represents the calculated mean for seven experimental fish.

20 and 30 days). Although all haematological abnormalities associated with arsenic poisoning have been shown to be reversible (Subcommittee on Arsenic in Drinking Water 1999), recovery always requires cessation of arsenic intake.

Leucopenia is also a common finding associated with chronic arsenic poisoning (Islam *et al.* 2004). In this experiment, severe neutropenia developed in all arsenic-treated fish after 30 days exposure. Neutropenia from arsenic intake was aggravated by a simultaneous intake of cyanobacterial biomass. It came as no surprise that phagocyte activity (i.e. respiratory burst) was also decreased in this case. Such an arsenic-induced decrease in respiratory burst was also described by Nayak *et al.* (2007), though the authors took no account of the actual amount of neutrophils present in the blood in their study. In our work, correction of chemiluminescence signal to $1*10^9$ neutrophils revealed that the decrease in phagocyte activity was caused by neutropenia only, neither arsenic nor cyanobacteria really inhibited respiratory burst. In our study, the stimulatory trend in neutrophil response to OZP was even observed in the As 5 experimental group. This is consistent with the results of Guardiola *et al.* (2013) who used equable amounts of leukocytes and observed that arsenic enhanced both respiratory burst and phagocytic activity. Our results indicate that $5\,mg.kg^{-1}$ arsenic stimulated a respiratory burst (though at a non-significant level) but that the combination with severe neutropenia resulted in a decreased chemiluminescence signal. The actual consequence for live fish, therefore, was immunosuppression. Simultaneous intake of cyanobacterial biomass enhanced the effect of arsenic on respiratory burst, implying either an additive or slight potentiating effect of these two agents.

Lymphocyte count was also affected by co-exposure to arsenic and cyanobacteria. Although arsenic alone caused mild non-significant lymphocytosis, co-exposure to cyanobacteria and $50\,mg.kg^{-1}$ arsenic caused a marked increase in lymphocyte counts after 30 days. Several authors have previously described the effect of arsenic on lymphocyte count. For example, the work of Islam *et al.* (2004) revealed neutropenia and lymphocytosis in humans chronically exposed to arsenic. On the other hand, others have observed lymphocyte depletion in animal species exposed to arsenic, e.g. the walking catfish (*Clarias batrachus*) (Ghosh *et al.* 2006), rainbow trout (Kotsanis *et al.* 2004) and Bengal goat (Islam *et al.* 2011). These discrepancies probably result from differences in arsenic dose, form of intake and length of exposure, as well as species-related differences in sensitivity to arsenic burden.

In conclusion, this work shows that prolonged combined exposure to arsenic and cyanobacteria causes marked pathological changes and disturbances in white blood cell count (lymphocytosis and neutropenia) in rainbow trout. Although the lower dose of arsenic significantly stimulated respiratory burst, in combination with severe neutropenia it resulted in a decreased intensity of phagocytosis. As phagocytosis is the first line of defence against invading pathogens, the combined action of both cyanobacteria and arsenic lead to a decreased ability to control infection. At this point, the question remains as to whether arsenic and cyanobacterial toxins interact or whether the combined effect of both agents is simply additive.

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