

# Neonatal withdrawal syndrome and perinatal asphyxia. How to manage the patient?

Ingrid BRUCKNEROVÁ<sup>1</sup>, Mojmír MACH<sup>2</sup>, Michal DUBOVICKÝ<sup>2</sup>, Eduard UJHÁZY<sup>2</sup>

<sup>1</sup> Neonatal Department of Intensive Medicine, Faculty of Medicine, Comenius University in Bratislava and Children's Hospital in Bratislava, Slovak Republic

<sup>2</sup> Institute of Experimental Pharmacology & Toxicology, Slovak Academy of Sciences, Bratislava, Slovak Republic

*Correspondence to:* Assoc. Prof. Ingrid Brucknerová, MD., PhD.  
Neonatal Department of Intensive Care, Faculty of Medicine  
Comenius University in Bratislava and Children's Hospital in Bratislava  
Limbova 1, 833 40 Bratislava, Slovak Republic.  
TEL: +421-259371232; E-MAIL: ingrid.brucknerova@fmed.uniba.sk

*Submitted:* 2015-07-18 *Accepted:* 2015-09-09 *Published online:* 2015-10-15

*Key words:* **asphyxia; neonatal withdrawal syndrome; heroin; methadone; hypertonicity**

Neuroendocrinol Lett 2014;**35**(Suppl. 1):53–56 PMID: 26757122 NEL360915C01 © 2015 Neuroendocrinology Letters • [www.nel.edu](http://www.nel.edu)

## Abstract

The aim of this work is to present the pitfalls of management of newborns with neonatal withdrawal syndrome (NWS) of different forms, which were complicated with the presence of severe perinatal asphyxia.

The authors present some case reports of asphyxiated newborns of different gestational age with different forms of NWS.

Prenatal and perinatal asphyxia determines the prognosis of future development of newborn. The combination of the asphyxia and NWS is stressful not only for the patient, but also for the physician. The most important step in management of this group of patients is to know the detailed mother's and patient's history and to perform detailed physical investigation. The optimal prenatal, perinatal and postnatal management with good cooperation between gynecologist and neonatologist can improve the quality of newborn's life. Care of newborn requires all the time teamwork.

## Abbreviations

NWS - neonatal withdrawal syndrome

## INTRODUCTION

The quality of newborn's life is under strong influence of many prenatal and perinatal factors. Sometimes we can change or diminished their influence (nutrition of the mother, illness, drugs, abuses, method of delivery, management after delivery, etc.). Some of them are not changeable (genetic predisposition, function of placenta, etc.) (Brucknerová 2014). Spectrum of complications depends

on gestational age, nutritional status, intensity of duration of risk factor, as well as on perinatal, and postnatal management.

Asphyxia is still the most frequent complication in newborn just behind prenatal infections. The newborn who is not breathing within 30 seconds after birth, or if a regular rhythmic breathing activity is not present within 90 seconds of life, is considered asphyxiated. Asphyxia is a condition in which the body is subjected to a time of

foetal hypoxia and/or reduces tissue perfusion. When the imbalance between the total amount of antioxidants and free radicals is presented, the oxidative stress is activated (Brucknerová *et al.* 2005a,b; Tsukahara 2007). Complications are due to hypoxaemia, ischaemia and later to injury caused by reperfusion and reoxygenation. Consequences of asphyxia depend on several factors (intensity, duration of the influence of “asphyxiated impulse”, gestational age of the foetus, quantity of antioxidant substances in the newborn, level of first neonatal treatment provided, i. e. resuscitation, after-care of the newborn). A more precise characteristic of the newborn's status is obtained using Apgar scoring system (Brucknerová *et al.* 2012; Brucknerová 2014).

Neonatal withdrawal syndrome (NWS) is mostly present in term newborn of mother who was dependent on addictive substances during pregnancy. Some hours after birth, typical neurological symptomatology appears (Brucknerová 2014). Clinical manifestation of NWS may be presented after several hours of life up to 2 weeks (narcotics: heroin 1–144 hours, methadone 1–14 days, morphine 1–7 days; sedatives: barbiturates 1–14 days, diazepam 2–6 hours) (Kliegman *et al.* 2011). The most clinical manifestations are neurological symptoms (neuromuscular irritability; tremors; restlessness, which increases during investigation of the newborn; hypertonicity; exaggerated Moro reflex; high-pitched cry), respiratory symptoms (apnoea; dyspnoea; tachypnoea), gastrointestinal symptoms (problem with food intake due to impaired coordination of swallowing; distractibility; vomiting; increased frequency of stools) and others (sweating; yawning; increasing of body temperature; skin abrasions; self skin injury). The onset of clinical manifestations depends on the type and dose of addictive substances, on the length, the time interval between the last dose and delivery, from combination of drugs as well as on the maturity of newborn. The presence of clinical consequences is confirmation of transport of the drug through placenta (Brucknerová 2015).

Side effects are different (of nicotine abuse – prenatal hypotrophy and prematurity; of opiates – prematurity, prenatal growth retardation and microcephaly; of cocaine and amphetamines – congenital anomalies of heart, kidneys and brain; of barbiturates – hypertonicity, problems with food intake and irritability; of benzodiazepines – congenital anomalies of the heart, decreased metabolism, clefts; of antidepressants – irritability, hypertonicity, high-pitched cry; of alcohol abuse in pregnant mother – foetal alcohol syndrome, etc.) (Schaefer *et al.* 2014).

For indirect confirmation of drug abuse in newborn of the mother who was dependent on addictive substances we can use various scoring schemes (Finnegan's score) and for direct confirmation we can use urine, blood and hair.

Treatment consists of some rules which are quiet, dark and thermoneutral environment, minimal handling, optimal position and medicamentous treatment.

## CASE REPORTS

### Patient 1

The child is from the second risk pregnancy. Mother (30 years old) was positive for hepatitis C and syphilis. She has a long lasting abuse of heroin. During pregnancy she was treated with methadone (10 mL per os.day<sup>-1</sup>). The delivery was in the 39<sup>th</sup> week of gestation, spontaneously (birth weight 3,650 grams; birth length 52 cm; the value of Apgar score 3/7). During the birth mother did not cooperate with obstetrician. During the delivery problems with giving newborn's arms and right hand were present. The newborn after delivery was atonic, bradycardic and without signs of spontaneous breathing activity. He was resuscitated.

From the first day of life the newborn had signs of neonatal withdrawal syndrome (irritability, increased muscle tonus, cry, and problems with feeding). Due to development of NWS we started therapy with morphine syrup (45 days in orally form). Blood count, biochemical parameters and serology (hepatitis A, B, C, HIV, toxoplasmosis, herpes virus simplex and zoster, cytomegalovirus, rubella, syphilis) were negative. His neurological status confirmed the persistency of neuromuscular irritability and increases muscle tonus. Ultrasound of the brain was negative. Based on social investigation of the family we could send him back to his family. Final diagnoses were Neonatal withdrawal syndrome (methadone) and Perinatal asphyxia.

### Patient 2

The child is from the first pregnancy of hepatitis C positive 30 years old mother, who had abuse of heroin and the last 6 months of pregnancy she was treated with methadone. Daily she smoked 40 cigarettes. The delivery was in 37<sup>th</sup> week of gestation, spontaneously, by head position, the value of Apgar score was 5/9 points, and birth weight was 2,850 grams. During the second phase of delivery the mother did not cooperate with the obstetrician. The newborn had the umbilical cord wrapped around the neck. After birth resuscitation of the newborn was started because of development of cyanosis, grunting and signs of respiratory insufficiency. The newborn had a severe stagnating cyanosis on the face.

At the time of admission he had nasal continuous positive airway pressure ventilation, gradually consumption of oxygen has increased, more severe was the bradypnoea, that's why the newborn was intubated and further ventilated. On thorax X-ray we confirmed the small right-sided pneumothorax.

Due to development of NWS we started with the therapy of morphine syrup (45 days in orally form), in combination with phenobarbital (2–5 mg.kg<sup>-1</sup>) and chloral hydrate (10–50 mg.kg<sup>-1</sup>). Up to social investigation the child was given back to family. Final diagnoses were Neonatal withdrawal syndrome (methadone) and Perinatal asphyxia.

Patient 3

The child is from the first pregnancy, delivery was in term in head position (birth weight 3,500 grams, birth length 52 cm, value of Apgar score 3/6 points). During the second phase of delivery the alteration of the heart beats was presented. The using of forceps was indicated due to not progressing labour. Amniotic fluid was greenish and mushy. The newborn was very atonic after delivery; from the respiratory airways the thick amniotic fluid was aspirated. The respiratory insufficiency was immediately treated using surfactant, inhalation of 100% oxygen, NO and high frequency ventilation (31 days). Hemodynamic instability required continuous inotropic treatment (dopamine, dobutamine; 13 days) and continuous intravenous morphine sedation. Due to development of iatrogenic NWS after interruption of morphine sedation we started orally therapy of morphine syrup for 10 days. Neurological investigation at the end of patient's stay at hospital confirmed increased neuromuscular reactivity. Final diagnoses were Neonatal iatrogenic withdrawal syndrome and Prenatal and perinatal asphyxia.

Patient 4

The child is from the first risk pregnancy. The mother (26 years old) had before pregnancy a long lasting abuse of heroin, and during the whole pregnancy she has had a long lasting treatment with methadone. The delivery was in the 36<sup>th</sup> week of gestation (caesarean section) due to prenatally confirmed gastroschisis, poor blood perfusion through placenta and growth retardation. The birth weight was 1,700 grams, amniotic fluid was greenish. Value of Apgar scoring system was 3/5. The newborn was resuscitated immediately after birth due to symptoms of respiratory insufficiency. Due to congenital anomaly of gastrointestinal tract the child underwent surgery of intestine (resection of terminal ileum together with caecum; ileocolonoanastomosis according to Bishop-Coop with opened enterostomy) during the 1<sup>st</sup> day of life. Clinical status was complicated by development of NWS. Sonography of the brain was negative. Neurological investigation confirmed irritability and increased neuromuscular reactivity. The newborn was treated with phenobarbital (2–5 mg.kg<sup>-1</sup>). Final diagnoses were Neonatal withdrawal syndrome (methadone) and Prenatal and perinatal asphyxia.

Patient 5

The 25-years old mother had a long lasting abuse of **heroin** before pregnancy, and during pregnancy she was treated with methadone. She was hepatitis C positive. The course of pregnancy was not checked regularly. Delivery was in the 38<sup>th</sup> week of gestation (birth weight was 3,520 grams, birth length 54 cm, values of Apgar score was 1/2 points) extra muros. Rapid health care found cyanotic newborn lying on placenta without ligation of umbilical cord. He had immediately cardiopulmonary resuscitation. Then he was admitted

to our Neonatal department at the age of 30 minutes. The patient was hypotonic, apathetic, with poorly presented neonatal reflexes, but with prominent tremor activity. Respiratory effort was irregular with the tendency to apnoea. Blood pressure was stabilized, but combined with bradycardia. The biggest problem was that we were not able to distinguish between presentation of withdrawal syndrome and severe neurological damage. The controlled hibernation was indicated for 72 hours. During hibernation the patient was ventilated and had sedation. When we finished on 5<sup>th</sup> day of life an intensive treatment, we continued due to presence of NWS with morphine syrup (33 days). On the 38<sup>th</sup> day of life we finished this treatment according to values of Finnegan score. Screening investigation of drugs in urine confirmed negative results for methadone, positive confirmation of opiates, barbiturates and benzodiazepines. Neurological investigation at the end of patient's stay at hospital confirmed the hypertonicity of muscles with mild tremor of extremities during handling, ultrasound of the brain did not confirm any pathology. The child is followed by outpatient neurological department. Final diagnoses were Neonatal withdrawal syndrome (methadone) and Perinatal asphyxia of severe degree.

**DISCUSSION**

Asphyxia has many reasons. Some of them are well known and some of them are unknown. Sometimes we can only assume the presence of prenatal or perinatal asphyxia due to indirect postnatal findings (Brucknerová & Ujházy 2014). Neonatal withdrawal syndrome can sometimes be present with the presence of prenatal hypotrophy of the foetus, rarely with premature delivery. Neonatal withdrawal syndrome has its typical postnatal presentations.

The combination of these two serious diagnoses is stressful not only for the patient, but also for the physician. The most important step in management of patient is to know the detailed patient's history (pregnancy, mother, abuses, delivery, and gestational age of the newborn and postnatal phase of adaptation) focused on asphyxia and drug abuse.

The physical investigation can only confirm the wide variability of clinical symptoms, which can mask presence of severe neurological damage of the newborn.

Common aim of treatment of newborn after asphyxia in combination with development of NWS is neuroprotection therapy. The most important points are to obtain stability of the internal environment and haemostasis, to minimize the influence of environmental factors, to regulate the body temperature within the recommended range (managed, controlled, short-termed hibernation), to have normoxaemias when giving only the necessary amount of oxygen, and in the presence of seizure activity (Electroencephalogram monitoring) to use anticonvulsant therapy (Guidelines

for management of Infants with Suspected Hypoxic Ischaemic Encephalopathy 2011; Cavallaro *et al.* 2013).

Treatment of the newborn with neonatal withdrawal syndrome can be nonpharmacological (thermoneutral environment, darkness, minimal handling, optimal position) and pharmacological (morphine syrup; buprenorphine; phenobarbital) (Kandall *et al.* 1999; Kakko *et al.* 2008; Kimberly *et al.* 2012; Selby & Turner 2012). An integral part of therapy is monitoring of vital functions (heart rate and breathing frequency, blood oxygen saturations, blood pressure and body temperature) as well as to check the urinary output.

In presented case reports we tried to describe different patients and present individual attitude in their treatment. The future is opened for new specific drugs in treatment of asphyxia. Various biomodels, model situations in animals, results of methods for measuring biomarkers in peripheral blood will help us not only to explain and understand many postashyxiated changes, but also bring us new possibility in treatment (Dubovický *et al.* 2008; Ujházy *et al.* 2008; Adamovsky *et al.* 2013; Chromcova *et al.* 2013; Balcerczyk *et al.* 2014; Gasparova *et al.* 2014; Sedláčková *et al.* 2014; Szychta *et al.* 2014).

## CONCLUSION

Asphyxia (prenatal, perinatal, and postnatal) is a syndrome, which can significantly affect quality of future development of the newborn. Neonatal withdrawal syndrome can also complicate not only prenatal period of development, but also the process of postnatal adaptation as well as the quality of life. Some time the consequence of intensive treatment can cause development of iatrogenic abstinence syndrome. Common aim of treatment of newborn after asphyxia in combination with development of NWS is effective neuroprotection therapy.

With the good cooperation among gynecologist and neonatologist, as well as with the optimal prenatal, perinatal and postnatal management we can improve the quality of newborn's life.

## ACKNOWLEDGEMENTS

The work was supported by Grant VEGA 2/0129/15.

## REFERENCES

- Adamovsky O, Palikova M, Ondrackova P, Zikova A, Kopp R, Mares J, *et al.* (2013). Biochemical and histopathological responses of wistar rats to oral intake of microcystins and cyanobacterial biomass. *Neuroendocrinol Lett.* **34**(Suppl. 2): 11–20.
- Balcerczyk A, Bartosz G, Drzewinska J, Piotrowski L, Pulaski L, Stefek M (2014). Antioxidant action of SMe1EC2, the low-basicity derivative of the pyridoinole stobadine, in cell free chemical models and at cellular level. *Interdisc Toxicol.* **7**: 27–32.
- Brucknerová I (2014). Neonatology simple & easy. Part I. Comenius University in Bratislava. 145 pp.
- Brucknerová I (2015). Neonatology simple & easy. Part 2. Comenius University in Bratislava. 100 pp.
- Brucknerová I, Benedeková M, Holomáň K, Bielíková E, Kostrová A, Ujházy E, Dubovický M (2005a). Delivery as “physiological stress” and its influence on liver enzymatic systems in asphyxial newborns. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.* **149**: 409–411.
- Brucknerová I, Benedeková M, Pecháň I, Franková E, Ujházy E, Dubovický M (2005b). Influence of oxidative stress on liver cell function on 1<sup>st</sup> and 5<sup>th</sup> day of life in asphyxiated newborns. *Biologia, Bratislava.* **60**: 25–28.
- Brucknerová I, Holomáňová A, Ujházy E, Mach M (2012). Sixty-year anniversary of the Apgar scoring system and 100-year anniversary of “Moro” reflex. *Neuroendocrinol Lett.* **33**: 729–730.
- Brucknerová I, Ujházy E (2014). Asphyxia in newborn – risk, prevention and identification of a hypoxic event. *Neuroendocrinol Lett.* **35**(Suppl. 2): 201–210.
- Cavallaro G, Filippi L, Raffaelli G, Cristofori G, Schena F, Agazzani E, *et al.* (2013). Clinical Study. Heart rate and arterial pressure changes during whole-body deep hypothermia. *IRSN Pediatrics.* 2013, Article ID 140213, 6 pages.
- Dubovický M, Kovačovský P, Ujházy E, Navarová J, Brucknerová I, Mach M (2008). Evaluation of developmental neurotoxicity: some important issues focused on neurobehavioral development. *Interdisc Toxicol.* **1**: 206–210.
- Gasparova Z, Stara V, Stolic S (2014). Effect of antioxidants on functional recovery after in vitro-induced ischemia and long-term potentiation recorded in the pyramidal layer of the CA1 area of rat hippocampus. *General Physiology and Biophysics.* **33**: 43–52.
- Guidelines for Management of Infants with Suspected Hypoxic Ischaemic Encephalopathy. 5. 9. 2011. East of England Perinatal Networks. 2011: 11–34.
- Chromcová L, Blahová J, Pihalová L, Živná D, Štěpánová S, Prášková E, *et al.* (2013). The effects of atrazine exposure on early life stages of common carp (*Cyprinus carpio*). *Neuroendocrinol Lett.* **34**(Suppl. 2): 95–101.
- Kakko J, Heilig M, Sarman I (2008). Buprenorphine and methadone treatment of opiate dependence during pregnancy: comparison of fetal growth and neonatal outcome in two consecutive case series. *Drug Alcohol Depend.* **96**: 69–78.
- Kandall SR, Doberczak TM, Jantunen M, Stein J (1999). The methadone-maintained pregnancy. *Clin Perinatol.* **29**: 173–83.
- Kimberly D, Ordean A, Murphy-Oikone J, Pereira J, Koren G, Roukema H, *et al.* (2012). Attenuation of autoimmune responses to oxidative specific epitopes, but not nitroso-adducts, is associated with a better clinical outcome in Myalgic Encephalomyelitis/chronic syndrome. *Neuroendocrinol Lett.* **35**: 577–585.
- Kliegman RM, *et al.* (2011). *Nelson textbook of Pediatrics.* 19<sup>th</sup> Edition. ELSEVIER. 2610 pp.
- Sedláčková N, Krajčiová M, Koprudová R, Ujházy E, Brucknerová I, Mach M (2014). Subchronic perinatal asphyxia increased anxiety- and depression-like behaviors in the rat offspring. *Neuroendocrinol Lett.* **35**(Suppl. 2): 214–220.
- Selby P, Turner R (2012). Neonatal abstinence syndrome. Clinical practice guidelines for Ontario. *J Popul Ther Clin Pharmacol.* **19**: e488–e506.
- Schaefer Ch, Peters PWJ, Miller RK (2014). *Drugs during pregnancy and lactation. Treatment options and risk assessment.* Second edition. Academic Press, Elsevier Amsterdam 875 pp.
- Szychta P, Zadrozny M, Lewinski A, Karbownik-Lewinska M (2014). Increased oxidative damage to membrane lipids after surgery for breast cancer. *Neuroendocrinol Lett.* **35**: 602–607.
- Tsukahara H (2007). Biomarkers for oxidative stress: clinical application in pediatric period. *Curr Med Chem.* **14**: 324–328.
- Ujházy E, Dubovický M, Navarová J, Sedláčková, L. Danihel, Brucknerová I, *et al.* (2008). Subchronic perinatal asphyxia in rats: Embryo foetal assessment of a new model of oxidative stress during critical period of development. *Food Chem Toxicol.* **61**: 233–239.

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

Miroslava PALIKOVA<sup>1</sup>, Ivana PAPEZIKOVA<sup>1</sup>, Radovan KOPP<sup>2</sup>, Jan MARES<sup>2</sup>,  
Zdenka MARKOVA<sup>1</sup>, Stanislav NAVRATIL<sup>1</sup>, Ondrej ADAMOVSKY<sup>3</sup>,  
Jiri KOHOUTEK<sup>3</sup>, Lukas NAVRATIL<sup>1</sup>, Ludek BLAHA<sup>3</sup>

<sup>1</sup> Department of Ecology and Diseases of Game, Fish and Bees, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic

<sup>2</sup> Department of Zoology, Fishery, Hydrobiology and Bee Keeping, Faculty of Agronomy, Mendel University in Brno, Brno, Czech Republic

<sup>3</sup> RECETOX, Faculty of Science, Masaryk University, Brno, Czech Republic

*Correspondence to:* Assoc. Prof. MVDr. Miroslava Palikova, PhD.  
Department of Ecology and Diseases of Game, Fish and Bees  
Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho tr. 1/3, 612 42 Brno, Czech Republic.  
TEL: +420-541 562 654, E-MAIL: palikovam@vfu.cz

*Submitted:* 2015-07-18 *Accepted:* 2015-09-09 *Published online:* 2015-10-15

*Key words:* **microcystin; multiple exposure; lymphocytosis; neutropenia; phagocytic activity; fish**

Neuroendocrinol Lett 2015;36(Suppl. 1):57-63 PMID: 26757114 NEL360915A14 © 2015 Neuroendocrinology Letters • www.nel.edu

## 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<sup>-1</sup> and 50 mg.kg<sup>-1</sup> 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.

**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 (Paliková *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<sup>-1</sup> 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<sup>3</sup> 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.



### 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 ( $1.55 \text{ mg.kg}^{-1}$ ) 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  $5 \text{ mg.kg}^{-1}$  (As 5), 3) arsenic at  $50 \text{ mg.kg}^{-1}$  (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  $-20^\circ\text{C}$  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 ( $1462.4 \mu\text{g.g}^{-1}$  DW), MC-LR ( $1087.9 \mu\text{g.g}^{-1}$  DW), MC YR ( $95.9 \mu\text{g.g}^{-1}$  DW) and unidentified ( $51.7 \mu\text{g.g}^{-1}$  DW). Total microcystin concentration was  $2697.9 \mu\text{g.g}^{-1}$  DW; hence 3% of cyanobacterial biomass added to the feed corresponded to  $81 \text{ mg MCs.kg}^{-1}$  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  $1.000 \pm 0.002 \text{ g.L}^{-1} \text{ As}^{5+}$  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 ( $\text{mg.kg}^{-1}$ )	Microcystin daily dose ( $\text{mg.kg}^{-1}$ fish weight)	Arsenic in feed ( $\text{mg.kg}^{-1}$ )	Arsenic daily dose ( $\text{mg.kg}^{-1}$ 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  $1 \times 10^9$  neutrophils, arsenic stimulated a respiratory burst at  $5 \text{ mg kg}^{-1}$  (As 5), though not at a statistically significant

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

haematological parameters	C	B	As 5	As 50	B + As 5	B + As 50
Hb (g.L <sup>-1</sup> )	115.0 $\pm$ 35.6	104.4 $\pm$ 34.1	69.3 $\pm$ 18.1**	78.4 $\pm$ 12.2*	73.9 $\pm$ 5.5**	70.9 $\pm$ 14.3**
MCH (pg)	90.5 $\pm$ 32.4	83.2 $\pm$ 29.4	54.7 $\pm$ 12.3*	78.1 $\pm$ 21.4	55.4 $\pm$ 9.5*	60.7 $\pm$ 12.5*
MCHC (L.L <sup>-1</sup> )	0.38 $\pm$ 0.08	0.27 $\pm$ 0.08	0.20 $\pm$ 0.05*	0.23 $\pm$ 0.04*	0.19 $\pm$ 0.03**	0.18 $\pm$ 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  $\pm$  standard deviation, N=7).

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

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

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

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

**Tab. 5.** Plasma iron levels ( $\mu\text{mol L}^{-1}$ ) after 10, 20 and 30 days exposure (mean  $\pm$  standard deviation, N=7).

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

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

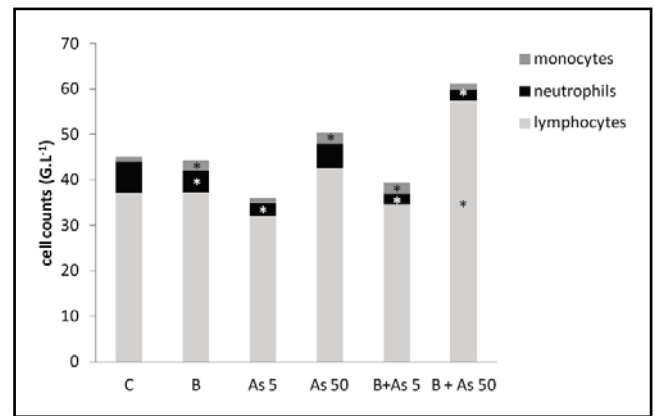


level (Figure 3). Cyanobacteria and arsenic had no significant effect on OZP-induced respiratory burst after 10 days exposure.

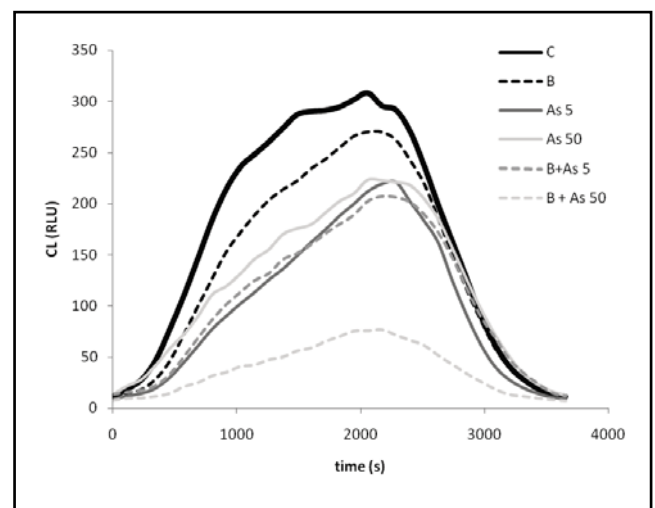
## DISCUSSION

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.

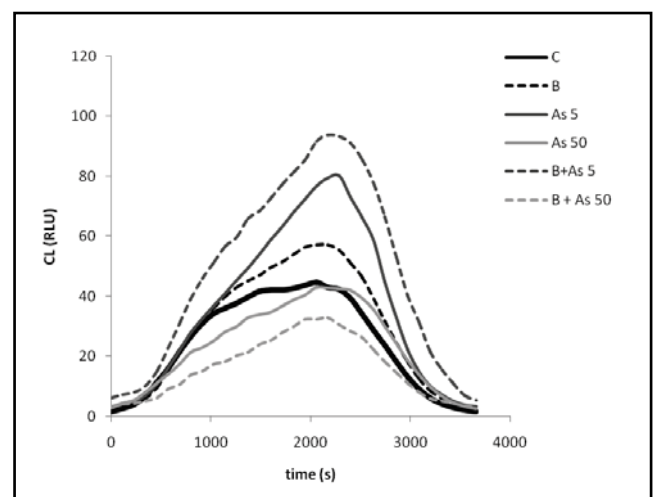
In humans, chronic arsenic poisoning almost always results in anaemia (Guha Mazumder 2008), usually in the form of normocytic normochromous anaemia caused by haemolysis (Lee *et al.* 2004) or suppression of erythropoiesis (Subcommittee on Arsenic in Drinking Water 1999). Erythrocyte counts were unchanged 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  $1 \times 10^9$  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  $1 \times 10^9$  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 equal amounts of leukocytes and observed that arsenic enhanced both respiratory burst and phagocytic activity. Our results indicate that  $5 \text{ mg} \cdot \text{kg}^{-1}$  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  $50 \text{ mg} \cdot \text{kg}^{-1}$  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 combina-

tion 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.

## ACKNOWLEDGEMENTS

This study was supported through Grant NAZV QJ 1210013 and a grant of the Ministry of Education of the Czech Republic (LO1214). The authors would like to thank Mr. Kevin Roche for his linguistic assistance.

## REFERENCES

- Adamovsky O, Palikova M, Ondrackova P, Zikova A, Kopp R, Mares J, *et al.* (2013). Biochemical and histopathological responses of Wistar rats to oral intake of microcystins and cyanobacterial biomass. *Neuroendocrinol Lett.* **34**(Suppl. 2): 11–20.
- Bhattacharya P, Welch AH, Stollenwerk KG, Mc Laughlin MJ, Bundschuh J, Panaullah G (2007). Arsenic in the environment: Biology and Chemistry. *Sci Total Env.* **379**: 109–120.
- Benarjee G, Narayana R, Srikanth K, Ramu G (2010). Haematological changes in the fresh water fish, *Channa punctatus* due to the effect of rayon industry effluents. *Pol Res.* **29**: 63–68.
- Carbis CR, Rawlin GT, Grant P, Mitchell GF, Anderson JW, McCauley I (1997). The study of feral carp, *Cyprinus carpio*, L., exposed to *Microcystis aeruginosa* at Lake Mokoan, Australia, and possible implications for fish health. *J Fish Dis.* **20**: 81–91.
- Dawson RM (1998). The toxicology of microcystins. *Toxicol.* **36**: 953–962.
- Ghosh D, Bhattacharya S, Mazumder S (2006). Perturbations in the catfish immune responses by arsenic: Organ and cell specific effects. *Comp Biochem Physiol C: Toxicol Pharmacol.* **143**: 455–463.
- Guardiola FA, Gónualez-Párraga MP, Cuesta A, Meseguer J, Martínez S, Martínez-Sánchez MJ, *et al.* (2013). Immunotoxicological effects of inorganic arsenic on gilthead seabream (*Sparus aurata* L.) *Aquat Toxicol.* **134–135**: 112–119.
- Guha Mazumder DN (2008). Chronic arsenic toxicity & human health. *Indian J Med Res.* **128**: 436–447.
- Higgins T (1981). Novel chromogen for serum iron determinations. *Clin Chem.* **9**: 1619–1620.
- Islam LN, Nabi AH, Rahman MM, Khan MA, Kazi AI (2004). Association of clinical complications with nutritional status and the prevalence of leukopenia among arsenic patients in Bangladesh. *Int J Environ Res Pub Health.* **1**: 74–82.
- Islam MT, Parvin S, Pervin M, Bari ASM, Khan MAHNA (2011). Effects of chronic arsenic toxicity on the haematology and histo-architecture of female reproductive system of black Bengal goat. *Bangl J Vet Med.* **9**: 59–66.
- Kohoutek J, Adamovský O, Oravec M, Šimek Z, Palíková M, Kopp R, *et al.* (2010). LC-MS analyses of microcystins in fish tissues over-estimate toxin levels-critical comparison with LC-MS/MS. *Anal Bioanal Chem.* **398**: 1231–1237.
- Kopp R, Mares J, Soukupova Z, Navratil S, Palikova M (2014). Influence of arsenic and cyanobacteria co-exposure on plas-matic parameters of rainbow trout (*Oncorhynchus mykiss* W.) *Neuroendocrinol Lett.* **35**(Suppl. 2): 57–63.
- Kopp R, Palikova M, Adamovsky O, Zikova A, Navratil S, Kohoutek J, *et al.* (2013). Concentrations of microcystins in tissues of several fish species from freshwater reservoirs and ponds. *Environ Monit Assess.* **185**: 9717–9727.

- 15 Kopp R, Palíková M, Mareš J, Navrátil S, Kubíček Z, Ziková A, *et al.* (2011). Haematological indices are modulated in juvenile carp (*Cyprinus carpio* L.) exposed to microcystins produced by cyanobacterial water bloom. *J Fish Dis.* **34**: 103–114.
- 16 Kotsanis N, Iliopoulou-Georgudaki J, Kapata-Zoumbos K (2004). Changes in selected haematological parameters at early stages of the rainbow trout, *Oncorhynchus mykiss*, subjected to metal toxicants: arsenic, cadmium and mercury. *J Appl Ichthyol.* **16**: 276–278.
- 17 Kubala L, Lojek A, Číž M, Vondráček J, Slavíková H (1996). Stanovení fagocytární aktivity v plné krvi kapra (*Cyprinus carpio* L.) chemiluminiscenci zesílenou luminolem. *Vet Med-Czech* **41**: 323–327 (in Czech).
- 18 Lee JJ, Kim YK, Cho SH, Park KS, Chung IJ, Cho D, *et al.* (2004). Hemolytic anemia as a sequel of arsenic intoxication following long-term ingestion of traditional Chinese medicine. *J Korean Med Sci.* **19**: 127–129.
- 19 Nayak AS, Lage CR, Kim CH (2007). Effects of low concentrations of arsenic on the innate immune system of the zebrafish (*Danio rerio*). *Toxicol Sci.* **98**: 118–124.
- 20 Ondracek K, Bandouchova H, Damkova V, Hilscherova K, Kral J, Osickova J, *et al.* (2012). Risk of combined exposure of birds to cyanobacterial biomass containing microcystins, acetylcholinesterase inhibitor and anticoagulant. *Neuroendocrinol Lett.* **33**(Suppl. 3): 155–160.
- 21 Palikova M, Adamovsky O, Blaha L, Mares J, Kopp R, Navratil S, *et al.* (2013). Fish tapeworm *Khawia sinensis*: an indicator of environmental microcystins? *Neuroendocrinol Lett.* **34**(Suppl. 2): 21–24.
- 22 Palíková M, Kopp R, Mareš J, Navrátil S, Kubíček Z, Chmelář L, *et al.* (2010). Selected haematological and biochemical indices of Nile tilapia (*Oreochromis niloticus*) reared in the environment with cyanobacterial water bloom. *Acta Vet Brno.* **79**: S63–S71.
- 23 Palíková M, Kovářů F, Navrátil S, Kubala L, Pešák S, Vajcova V (1998). The effects of pure microcystin LR and biomass of blue-green algae on selected immunological indices of carp (*Cyprinus carpio* L.) and silver carp (*Hypophthalmichthys molitrix* Val.). *Acta Vet Brno.* **67**: 265–272.
- 24 Palíková M, Navrátil S, Krejčí R, Štěrba F, Tichý F, Kubala L, *et al.* (2004). Outcomes of repeated exposure of the carp (*Cyprinus carpio* L.) to cyanobacteria extract. *Acta Vet Brno.* **73**: 259–265.
- 25 Palikova M, Navratil S, Papezikova I, Ambroz P, Vesely T, Pokorova D, *et al.* (2012). Combined exposure of carps (*Cyprinus carpio* L.) to cyanobacterial biomass and white spot disease. *Neuroendocrinol Lett.* **33**(Suppl. 3): 77–83.
- 26 Paskova V, Pikula J, Bandouchova H, Sedlackova J, Hilscherova K (2011). Combined exposure of Japanese quails to cyanotoxins, Newcastle virus and lead: Oxidative stress responses. *Ecotox Environ Safe.* **74**: 2082–2090.
- 27 Pikula J, Bandouchova H, Hilscherova K, Paskova V, Sedlackova J, Adamovsky O, *et al.* (2010). Combined exposure to cyanobacterial biomass, lead and the Newcastle virus enhances avian toxicity. *Sci Total Environ.* **408**: 4984–4992.
- 28 Ratnaike RN (2003). Acute and chronic arsenic toxicity. *Postgrad Med J.* **79**: 391–396.
- 29 Ruttens A, Blanpair AC, De Temmerman L, Waegeneers N (2012). Arsenic speciation in food in Belgium: Part 1: Fish, molluscs and crustaceans. *J Geochem Explor.* **121**: 55–61.
- 30 Sieroslawska A, Rymuszka A, Velisek J, Pawlik-Skowronska B, Svobodova Z, Skowronski T (2012). Effects of microcystin-containing cyanobacterial extract on hematological and biochemical parameters of common carp (*Cyprinus carpio* L.). *Fish Physiol Biochem.* **7**: 106–117.
- 31 Silins I, Högberg J (2011). Combined Toxic Exposures and Human Health: Biomarkers of Exposure and Effect. *Int J Environ Res Pub Health.* **8**: 629–647.
- 32 Soukupová Z, Veselý T, Pokorová D, Mareš J, Kopp R, Kohoutek J, *et al.* (2014). Influence of cyanobacteria and spring viraemia of carp co-exposition on common carp (*Cyprinus carpio* L.) Interdisciplinary Toxicol. **7**(Suppl. 1): 76–77.
- 33 Subcommittee on Arsenic in Drinking Water; Commission on Life Sciences; Division on Earth and Life Studies; National Research Council: Arsenic in drinking water. The National Academic Press 1999.
- 34 Svobodová Z, Pravda D, Modrá H (2012). Methods of haematological examination of fish. Faculty of fishery and water protection JU in České Budějovice. Edition Methods. **122**: 38 pp. (in Czech)
- 35 Welker M, von Dohren H (2006). Cyanobacterial peptides – Nature's own combinatorial biosynthesis. *Fems Microbiol Rev.* **30**: 530–563.
- 36 Zhang W, Huang L, Wang WX (2011). Arsenic bioaccumulation in a marine juvenile fish *Terapon jarbua*. *Aquat Toxicol.* **105**: 582–588.