Physiol. Res. 69 (Suppl. 4): S595-S606, 2020 *https://doi.org/10.33549/physiolres.934608*

Could the Musk Compound Tonalide Affect Physiological Functions and Act as an Endocrine Disruptor in Rainbow Trout?

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Received October 8, 2020
Accepted October 24, 2020

Summary

In the present study, the effect of polycyclic musk compound tonalide (AHTN) in two concentrations was studied in male rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792). A feeding trial was conducted with AHTN incorporated into feed granules. One concentration was environmentally relevant (854 µg/kg); the second one was 10× higher (8699 µg/kg). The fish were fed twice a day with the amount of feed at 1 % of their body weight. After an acclimatization period, the experimental phase in duration of six weeks followed. At the end of the experiment, fish were sampled and the biometrical data were recorded. Subsequently, hematological and biochemical tests, histopathological examination, analysis of oxidative stress markers and evaluation of endocrine disruption using plasma vitellogenin were performed. In conclusion, an increase of hematocrit for both AHTN concentrations was found, but no significant changes were observed in biochemical profile. Moreover, AHTN caused lipid peroxidation in caudal kidney tissue, which was confirmed by histopathological images. The long-lasting AHTN exposure could thus be harmful for maintaining homeostasis in the rainbow trout organism. However, the vitellogenin concentration seemed not to be affected by AHTN.

Key words

Hematology • Biochemistry • Histology • Lipid peroxidation • *Oncorhynchus mykiss*

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Introduction

The issue of water pollution caused by many different substances raises questions about their impact on the non-target aquatic organisms (Carlsson *et al.* 2013, Freitas *et al.* 2019, Sehonova *et al.* 2019). The musk compounds are widely used in various industries as a fragrance carrier – in perfumes, personal care products, washing powders, household detergents and sprays, candles, etc. (Nakata *et al.* 2015). They have been reported to have a potential to adversely affect non-target organisms, such as fish (Yamauchi *et al.* 2008), amphibians (Carlsson and Norrgren 2014, Pablos *et al.* 2015) and other aquatic organisms as summarized by Tumova *et al.* (2019). Both estrogenic and anti-estrogenic effects of musk have been described (Schreurs *et al.*

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2004, Luckenbach and Epel 2005, Simmons *et al.* 2010) as well as genotoxicity (Parolini *et al.* 2015), neurotoxicity (Heberer 2002), photosensitive effects (Fang *et al.* 2017), the potential to cause oxidative stress (Blahova *et al.* 2018) or a change in gene expression (Shi *et al.* 2013). Due to their reported bio-cumulative effects and long-term environmental persistence caused by lipophilicity, many of them were forbidden or their usage was limited (Rimkus 1999, European Commission 2009).

In industrial sector, synthetic musk compounds are replacing the natural ones (Li *et al.* 2020). One of the main musk groups used are polycyclic musk compounds (PCMs), while tonalide (AHTN) and galaxolide (HHCB) are most abundant (Vallecillos *et al.* 2015). Their ability to enter surface water and sediment was documented in many studies (Rimkus 1999, Wong *et al.* 2019, Zhang *et al.* 2020) and the waste water treatment plant's (WWTP) effluent was detected as the main environmental source of PCMs (Ramierz *et al.* 2009, Homem *et al.* 2015). At WWTP, the purification of the musk compounds is less than 100 % effective and depends on the applied technology. For example, the cleaning process for AHTN was reported not to be higher than 84 % (Ren *et al.* 2013) or 89 % (Wombacher and Hornbuckle 2009). Based on the study by Peck and Hornbuckle (2004), AHTN is adsorbed to sludge during the WWTP process. The AHTN concentration in WWTP influent was found to range between 49.9 and 64.6 µg/l (Chen *et al.* 2007, Vallecillos *et al.* 2015), while in the sludge the concentration was up to 169.3 mg/kg (Zeng *et al.* 2005).

The aim of the present study was to assess whether oral exposure to AHTN could affect hematological and biochemical indices in rainbow trout. Moreover, its effects on oxidative stress were evaluated, as well as the effects on vitellogenin (VTG) concentrations in male fish plasma due to its possible xenoestrogenic potential. The impact on selected organs was evaluated by histopathological analysis. The AHTN concentrations tested in our experiment were selected according to the concentrations detected in surface water as discussed above and summarized, for example, by Blahova *et al.* (2008) and Tumova *et al.* (2019). As well, the maximum concentrations detected in wild fish tissues were taken into consideration, while they were determined up to 52.1 µg/kg and 349 µg/kg in common carp's (*Cyprinus carpio,* Linnaeus 1758) muscle and liver tissue, respectively; or even up to 3700 µg/kg lipid weight in sea trout's (*Salmo trutta,* Linnaeus 1758) muscle (Fromme *et al.* 2001, Kannan *et al.* 2005, Lange *et al.* 2015, Yao *et al.* 2018). The bioconcentration and bioaccumulation factors in wild fish species were considered for these concentrations to be environmentally relevant and also for testing the dose-dependent effects.

Methods

Ethical statement

After approval by the institutional ethical committee, the experiment was carried out in accordance with institutional guidelines and national legislation (Act No. 246/1992 Coll., on the Protection of Animals against Cruelty, as amended).

Experimental design & fish condition

The trial was conducted at the Dpt. of Ecology & Diseases of Zooanimals, Game, Fish and Bees at the University of Veterinary and Pharmaceutical Sciences Brno, Czechia. A total of 60 male juveniles of rainbow trout from *Skalni Mlyn* hatchery were obtained (total length 32.2±2.2 cm, weight 516.8±102.9 g) and acclimated to the laboratory conditions for two weeks. The aquaria (n=6) were connected to a recirculation system, while the condition of water was monitored every 24 h and was as follows: temperature 14.5-15.6 °C, dissolved oxygen >70 %, pH 7.9-8.3, total ammonia <0.03 mg/l, chlorides 26.71 mg/l, ammonia 0.23 mg/l, nitrites 0.12 mg/l, nitrates 45.44 mg/l. Every aquarium was individually aerated. Fish were held at a 12 h/12 h light to dark photoperiod during both the acclimatization and experimental phase. The probe Oxi 340i and pH 340i digital sampling systems were applied for measurement of O_2 and pH in water (WTWGmbH, Germany). The spectrophotometric methods according to the Animal and Plant Health Agency (APHA 2017) methodology was used for determination of ammonia and nitrites in water samples (method No. 4500-NH_3, 4500-NO_2).

During the acclimatization period, fish were fed with commercial pellets EFICO Enviro 920 Advance 4.5 mm (Biomar, Denmark) twice a day with the amount of feed at 1 % of their body weight. The composition of feeding pellets was as follows: 41-44 % crude protein, 28-31 % crude lipid, 13-16 % carbohydrates, 0.7-2.2 % crude fiber, 4-7 % ash, 0.9 % total phosphorus. During the experimental phase, the same amount of feed was given to the fish, but with addition of AHTN. Every 14 days of the experimental phase, the fish were re-weighed and the amount of feed per day was recalculated.

At the beginning of the experimental phase, the fish were randomly divided into three groups – control, AHTN1 and AHTN2 (n=20/per group); each group was divided and placed into two aquaria. The AHTN was tested in two concentrations which were incorporated into fish feed using a specific methodology described in detail by Modra *et al.* (2020). The first tested concentration was environmentally-relevant: 854 µg/kg, thereafter designated as AHTN1. The second concentration, thereafter labelled as AHTN2, was ten times higher: 8699 µg/kg. The feed for the control group was prepared in the same way, but without the use of AHTN. After six weeks of the experimental phase, all fish were sampled.

The monitored biometrical data were as follows: total and standard length (cm), wet weight (g) and weight of liver and gonads (g). For the measurement, a digital balance with an accuracy of up to 0.01 g was used. The wet weight and standard length were used for setting the Fulton's condition factor (CF) according to formula: CF=(wet weight [g]/standard length [cm]^3)×100 (Froese 2006). The hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated as: HSI (GSI)=(weight of tissue [g]/fish wet weight [g])×100 (Mazlan and Rohaya 2008).

Determination of AHTN in the feed

The measurement of AHTN was performed by gas chromatography coupled with ion trap tandem mass spectrometry (GC/MS/MS). A 5 g representative portion of fish feed granules was homogenized and extracted into cyclohexane (10 ml). The extract was filtered through a 0.7 µm glass filter (Millipore, USA) and used for GC/MS/MS analysis. The separation, identification, and quantification of AHTN were carried out using a Varian 450-GC gas chromatograph with a Varian 220-MS ion trap mass spectrometer equipped with electron ionization and VF-5ms (30 m×0.25 mm) column (Varian, Inc., USA). A 1 µl aliquot of sample extract was injected in a split mode. The injector temperature was 250 °C. The initial oven temperature was set at 50 °C for 1 min, increased at a rate of 35 °C/min to 280 °C, held for 1 min, increased at a rate of 20 °C/min to 300 °C and held for 5 min. The total run time was 14.57 min. The ion trap mass spectrometer was operated in MS/MS mode. The most abundant ion (m/z=243) was selected as a parent ion and ion m/z=187 was selected as a product ion. A standard of AHTN was purchased from Sigma-Aldrich (USA). Cyclohexane was GC/MS-grade purity (Chromservis, Czech Republic). For our QA/QC program, the instrument was calibrated daily with multi-level calibration curves. Procedural blank and solvent blank were analysed for every set of 10 samples. The spiked recovery was 103 %. The reported concentrations after corrections are based on the recovery. The coefficient of variation for between-series was 6.1 %. The limit of detection was determined as 3:1 signal versus noise value and was 0.8 µg/kg.

Hematological analysis

The blood was taken from each fish from the caudal vein using an aspiration syringe and was stabilized with sodium heparin (50 IU per 1 ml of blood). After that, the fish were immediately stunned with a blow in the head, killed by spinal transection and submitted to autopsy. Each fish was weighed and measured, while the weight of the organs was also recorded. The whole blood was used for the red blood cell (RBC) and white blood cell (WBC) count determination with the use of the Burker hemocytometer to which the heparinized blood diluted with Natt-Herrick solution at a 1:200 ratio was applied. For the hematocrit (PCV) determination, the microhematocrit method with centrifugation of blood in capillary tubes was employed. The photometric cyanohemoglobin method at 540 nm was applied for the determination of hemoglobin concentration (Hb). Additionally, the mean erythrocyte hemoglobin (MCH), mean erythrocyte volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated with using the PCV, Hb and RBC data. All hematological analyses were performed based on the methodology by Svobodova *et al.* (2012).

Biochemical analysis

One half of each fish blood sample was centrifuged (800×g, 4°C, 10 min). In the obtained plasma, albumin (ALB), ammonia (NH_3), total protein (TP), glucose (GLU), triglycerides (TG), cholesterol (CHOL), lactate (LACT), calcium (Ca), phosphorus (PHOS), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were evaluated. Biochemical parameters were determined using the commercial kit (BioVendor PCL, Czech Republic) and biochemical analyser Konelab 20i (Thermo Fisher Scientific, Czech Republic) following the manufacturer's instructions.

VTG analysis

The VTG concentration was detected in plasma

samples using a commercial ELISA kit (Biosense Laboratories, Norway) following the manufacturer's instructions. The samples were taken from all fish (n=60, 20 pcs/group), while males in the control group without exposure to AHTN served as a negative control.

Oxidative stress analysis

For oxidative stress evaluation, the activity of ceruloplasmin (CP) and ferric reducing ability of plasma (FRAP) were determined in plasma samples. The CP was determined spectrophotometrically using a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific Inc., USA) according to Ceron and Martinez-Subiela (2004) methodology. The results are expressed as an amount of absorbance increase per minute ×10000. The FRAP was analysed according the Benzie and Strain method (1996) with the use of a biochemical analyser Konelab 20i.

The analysis of oxidative stress enzymes was performed in organs, specifically in liver, caudal kidney, gill and gonad tissue. The monitored parameters were glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST) and thiobarbituric acid-substances (TBARS). The organs were individually homogenized and the homogenates were then divided into two parts. The first part was used for TBARS determination and the second part was centrifuged (11000×g, 4 °C for 20 min) and the obtained supernatant was submitted to GR, GPx and GST analysis. The catalytic activities were determined spectrophotometrically with use of a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific Inc., USA). The GR activity was measured by determination of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm according to the method of Carlberg and Mannervik (1975). The GPx activity determination was based on the NADPH oxidation rate and the reaction with GR at 340 nm (Flohe and Gunzler 1984). Both GR and GPx were expressed as nmol of NADPH consumption per min per mg of protein. The GST activity was detected using the Habig *et al.* (1974) methodology based on conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione at 340 nm and was expressed as nmol of the formed product per min per mg of protein. The Bicinchoninic Acid Protein Essay Kit (Sigma-Aldrich, USA) was employed for establishing of protein concentration with the use of bovine serum ALB as a standard (Smith *et al.* 1985). The levels of TBARS were determined by malondialdehyde measurement at 535 nm (Lushchak *et al.* 2005) and were expressed as TBARS in nmol per gram of tissue. The TBARS concentrations were not determined in gonads due to a lack of tissue.

Histopathological analysis

The tissue of liver, cranial and caudal kidney, gill (upper limb of arch), spleen, gonads and skin (above the ventral fin) was dissected from each fish and submitted to histological analysis. Samples were fixed in buffered 10 % neutral formalin, thereafter dehydrated and embedded in paraffin wax. Samples were sliced at a thickness of 4 µm using the microtome tool. All samples were stained with hematoxylin-eosin dye and examined with light microscope.

Statistical analysis

The statistical software Unistat for Excel 6.5 (Unistat Ltd., UK) was employed for performing of the statistical analysis. Firstly, all data were tested for normality distribution using the Shapiro-Wilk test. In case of normality achievement, the analysis of variance and Tukey-HSD test were employed; while the Kruskal-Wallis nonparametric test was applied for data with non-normal distribution. The control, AHTN1 and AHTN2 groups were compared and data were considered as statistically significant when $p<0.05$ (*) and $p<0.01$ (**). Data are expressed as mean ± standard deviation (SD).

Results

Mortality, behaviour and health status

No mortality of fish was observed and the fish behaviour was not changed during any part of the experiment. Fish subjected to the trial were without bacterial, viral or parasitic infection at the beginning and at the end of the experiment. No significant changes were found in biometric indices thus these data are not presented.

Hematological profile

The only observable changes of hematological profile were found for PCV where a significant increase was detected for both concentrations of AHTN. The PCV increase in both concentrations was significantly different from the control group. However, the AHTN1 and AHTN2 groups did not differ between each other. Consequently, the changes in PCV parameter caused changes in MCV and MCHC levels, specifically the

MCV increased after exposure to AHTN and, in contrast, the MCHC decreased. The MCH levels were also significantly different; a decrease in both AHTN concentrations was observed when compared to the control group. The other hematological parameters were not changed. An overview of hematological analysis after the induction of changes with AHTN is given in Table 1.

Table 1. Hematological and biochemical indices after oral exposure to two different concentrations of tonalide (AHTN): AHTN1 (854 µg/kg) and AHTN2 (8699 µg/kg).

Parameter [unit]	Control	AHTN1	AHTN2
Hematological indices			
RBC [T/l]	1.37 ± 0.06^{a}	1.54 ± 0.05^{a}	1.46 ± 0.06^{a}
Hb [g/l]	83.87 ± 3.08^{a}	80.72 ± 1.73^{a}	78.21 ± 2.25^{a}
PCV [l/l]	0.38 ± 0.03^{b}	0.45 ± 0.02^{a}	$0.44 \pm 0.02^{a,b}$
MCV [fl]	262.07 ± 11.75^{b}	$295.90 \pm 11.44^{a,b}$	307.18 ± 11.44^{a}
MCH [pg]	60.97 ± 2.72^{a}	52.92 ± 1.98^{b}	$54.45 \pm 1.87^{a,b}$
MCHC [g/l]	0.24 ± 0.03^{a}	0.18 ± 0.01^{b}	$0.18 \pm 0.01^{a,b}$
WBC [G/l]	18.03 ± 1.43^{a}	19.54 ± 2.17^{a}	17.36 ± 1.45^{a}
Biochemical indices			
ALB [g/l]	18.21 ± 0.86^{a}	20.24 ± 0.60^{a}	18.58 ± 0.69^{a}
NH_3 [µmol/l]	240.30 ± 16.24^{a}	245.17 ± 18.09^{a}	262.13 ± 21.52^{a}
TP [g/l]	36.37 ± 1.32^{a}	39.68 ± 0.96^{a}	36.41 ± 1.06^{a}
GLU [mmol/l]	4.28 ± 0.17^{a}	4.89 ± 0.20^{a}	4.20 ± 0.09^{a}
TG [mmol/l]	2.54 ± 0.31^{a}	4.29 ± 0.67^{a}	3.62 ± 0.51^{a}
CHOL [mmol/l]	7.47 ± 0.38^{a}	8.49 ± 0.26^{a}	7.73 ± 0.37^{a}
LACT [mmol/l]	2.36 ± 0.27^{a}	2.48 ± 0.32^{a}	2.58 ± 0.26^{a}
Ca [mmol/l]	2.56 ± 0.04^{a}	2.69 ± 0.05^{a}	2.64 ± 0.05^{a}
PHOS [mmol/l]	3.93 ± 0.10^{a}	3.98 ± 0.09^{a}	4.07 ± 0.09^{a}
ALP [µkat/l]	1.06 ± 0.14^{a}	1.52 ± 0.17^{a}	1.08 ± 0.09^{a}
ALT [µkat/l]	0.29 ± 0.04^{a}	0.23 ± 0.02^{a}	0.23 ± 0.01^{a}
AST [µkat/l]	6.30 ± 0.76^{a}	5.80 ± 0.44^{a}	6.08 ± 0.28^{a}
LDH [µkat/l]	12.04 ± 1.03^{a}	9.88 ± 0.78^{a}	12.46 ± 1.76^{a}

Data are given as mean ± SD; n=20/group. Means in the same row lacking a common letter of superscript (**a, b**) differ significantly ($p<0.05$). RBC – red blood cells count, Hb – hemoglobin concentration, PCV – hematocrit, MCV – mean erythrocyte volume, MCH – mean erythrocyte hemoglobin, MCHC – mean corpuscular hemoglobin concentration, WBC – white blood cell count.

Biochemical profile

Surprisingly, there were no significant changes in any observed biochemical parameter and all the indices were at physiological levels. An overview of biochemical analysis results is presented in Table 1.

VTG

The VTG concentration was measured in plasma samples of both AHTN exposure groups in comparison with the control group. Surprisingly, even though the control group consisted of males, which should have served as a negative control, 20 % samples were positive with VTG levels between 303 and 985 ng/ml. With the lower concentration (AHTN1), the VTG concentration ranged between 173 and 945 ng/ml in 25 % samples. However, in the higher concentration (AHTN2), positivity was detected in 15 % cases with a concentration range from 362 to 609 ng/ml. The results are shown in Figure 1.

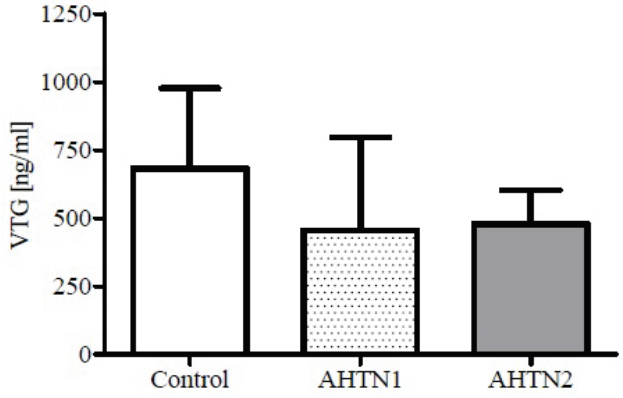

Fig. 1. The concentration of vitellogenin (VTG) in ng/ml in plasma samples after oral exposure to two different concentrations of tonalide (AHTN): AHTN (854 µg/kg) and AHTN2 (8699 µg/kg). Data are given as mean ± SD; n=20/group. No significant changes were found among the tested groups (p>0.05).

Oxidative stress profile

Oxidative stress was measured in both plasma and tissue samples. In the case of plasma, CP and FRAP did not show any significant changes. For tissue samples, significant changes were detected in the caudal kidney tissue where TBARS control levels significantly differed from both AHTN1 and AHTN2. In AHTN1, a decrease was observed as compared with the control, while, in contrast, a significant increase was detected for AHTN2. The results are given in Table 2.

Histopathological profile

The histopathological changes in AHTN1 and AHTN2 groups showed congestion and mixed hepatodystrophy in all liver tissues. In the control group,

Table 2. Oxidative stress parameters in plasma, liver, caudal kidney, gill and gonads after oral exposure to two different concentrations of tonalide (AHTN): AHTN1 (854 µg/kg) and AHTN2 (8699 µg/kg). Data are given as mean ± SD; n=20/group. Means in the same row lacking a common superscript letter (**a, b**) differ significantly (p<0.05). CP – ceruloplasmin, FRAP – reducing ability of plasma, GR – glutathione reductase, GPx – glutathione peroxidase, GST – glutathione-*S*-transferase, TBARS – thiobarbituric acid-substances.

Parameter [unit]	**Control**	**AHTN1**	**AHTN2**
Plasma			
CP [ΔA/min×10000]	113.3 ± 3.3[a]	120.1 ± 5.1[a]	114.1 ± 4.2[a]
FRAP [µmol/l]	748.7 ± 42.6[a]	759.6 ± 22.0[a]	805.4 ± 22.7[a]
Liver			
GR [nmol NADPH/min/mg protein]	13.3 ± 5.9[a]	10.4 ± 3.9[a]	10.9 ± 5.6[a]
GPx [nmol NADPH/min/mg protein]	35.1 ± 10.1[a]	37.5 ± 10.5[a]	34.7 ± 9.0[a]
GST [nmol/min/mg protein]	683.2 ± 184.6[a]	785.4 ± 194.8[a]	681.6 ± 178.6[a]
TBARS [nmol/g of tissue]	22.8 ± 20.0[a]	19.0 ± 9.4[a]	22.9 ± 11.2[a]
Caudal kidney			
GR [nmol NADPH/min/mg protein]	11.0 ± 3.7[a]	9.4 ± 2.3[a]	10.3 ± 3.1[a]
GPx [nmol NADPH/min/mg protein]	65.6 ± 16.8[a]	58.4 ± 14.6[a]	57.2 ± 18.2[a]
GST [nmol/min/mg protein]	393.4 ± 206.8[a]	440.2 ± 170.9[a]	411.0 ± 242.6[a]
TBARS [nmol/g of tissue]	10.0 ± 10.6[a,b]	8.4 ± 5.0[b]	12.9 ± 7.8[a]
Gill			
GR [nmol NADPH/min/mg protein]	13.5 ± 4.3[a]	11.8 ± 3.6[a]	11.5 ± 2.3[a]
GPx [nmol NADPH/min/mg protein]	62.0 ± 13.4[a]	65.7 ± 12.0[a]	64.6 ± 10.3[a]
GST [nmol/min/mg protein]	280.6 ± 98.1[a]	238.4 ± 65.4[a]	214.7 ± 72.0[a]
TBARS [nmol/g of tissue]	20.2 ± 7.9[a]	22.7 ± 11.7[a]	24.0 ± 15.0[a]
Gonads			
GR [nmol NADPH/min/mg protein]	11.0 ± 3.5[a]	11.1 ± 1.6[a]	10.6 ± 4.6[a]
GPx [nmol NADPH/min/mg protein]	30.9 ± 12.5[a]	28.7 ± 5.0[a]	28.0 ± 7.8[a]
GST [nmol/min/mg protein]	115.3 ± 57.0[a]	93.9 ± 27.6[a]	102.4 ± 39.0[a]

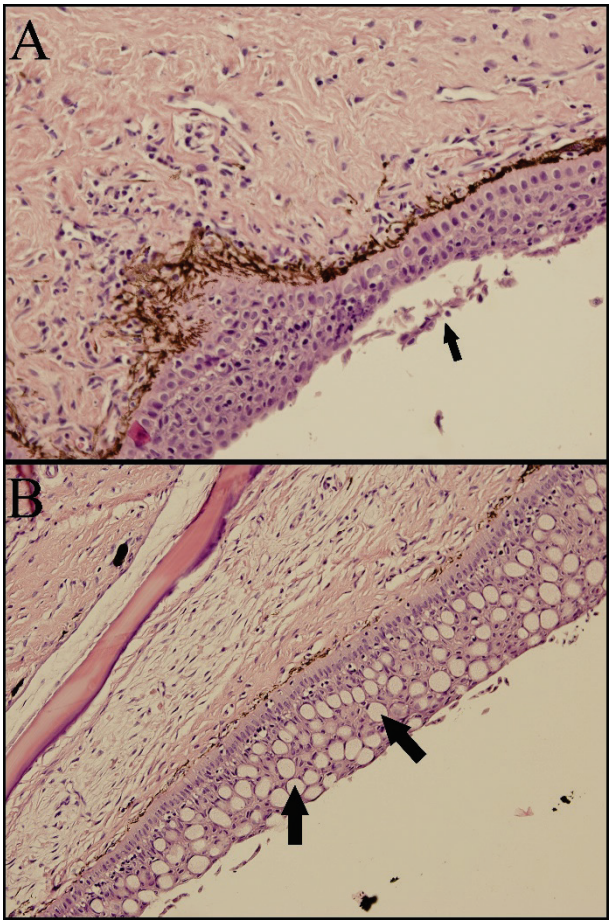

Fig. 2. The histopathological findings in skin tissue after oral exposure to two different concentrations of tonalide (AHTN): AHTN1 (854 µg/kg) and AHTN2 (8699 µg/kg). The control group (**A**) with artificial superficial erosion (black arrow), otherwise without pathological findings. The AHTN2 group (**B**) with numerous mucinous cells (black arrows). H-E staining, magnification 400×.

dystrophy was also observed together with small deposits of pigments; otherwise, the liver tissue in the control was without gross lesions. For the cranial kidney, congestion was observed along with hyaline degeneration of some tubules in AHTN1; in higher concentration, congestion was observed simultaneously with small deposits of pigments – the same finding as in the control group. In contrast, the caudal kidney showed multiple pigment deposits with intact parenchyma in AHTN1 and congestion with hyaline degeneration of some tubules in AHTN2. In the control, pigment deposits with hyaline droplets in some tubules of the caudal kidney were. The gill lamellas were hypertrophic and vacuolization together with surface alterations was observed in AHTN1. In AHTN2, total devastation of lamellas with inflammatory lesions was observed. The control group was intact. Spleen tissue in both AHTN1 and AHTN2 showed congestion with intact parenchyma; the same findings were observed in the control group. Skin tissue in the control was without gross lesions; the higher the AHTN concentration, the more mucinous cells were observed and, in AHTN2, myodystrophia was also revealed. The gonads were completely intact in the control and both experimental groups. The histopathological changes of skin tissue and caudal kidney compared to control group are presented in Figure 2 and 3, respectively.

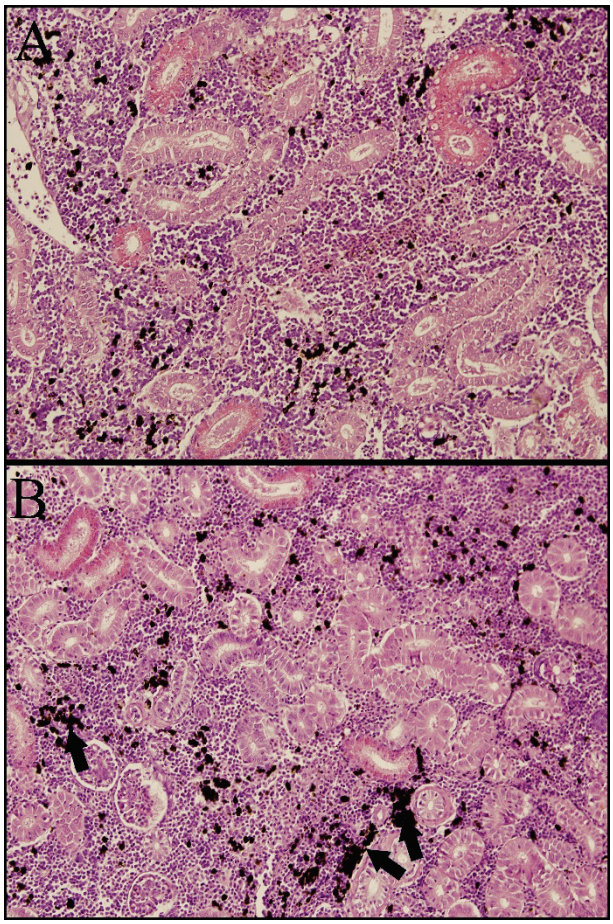

Fig. 3. The histopathological findings in caudal kidney tissue after oral exposure to two different concentrations of tonalide (AHTN): AHTN1 (854 µg/kg) and AHTN2 (8699 µg/kg). The control group (**A**) with normal tubular system and some pigmented deposits. The AHTN2 group (**B**) with droplet hyaline degeneration of some tubules (black arrows). H-E staining, magnification 200×.

Discussion

The presence of PCMs, and AHTN specifically, was observed in many environmental matrices, due to not 100 % effective water purification and high bioaccumulation capacity and stability (Li *et al.* 2020). Thus, AHTN presence was detected in surface water, drinking water, sediment or even in the air and biota, as

reviewed by Tumova *et al.* (2019). The aquatic biota is the main vulnerable group due to the reported adverse effects on the health of its organisms, as discussed above. As the model organism for our study, the rainbow trout was selected to be the typical fish in our country with a high bioaccumulation level and, to our knowledge, no PCMs toxicity testing has ever been performed in this organism.

According to biometrical analysis, hematological and biochemical indices, there were significant changes in PCV which increased in both AHTN1 and AHTN2. Additionally, the MCV also increased depending on AHTN concentration; in contrast, the MCH and MCHC were significantly decreased in both tested AHTN concentrations. These findings indicated that erythrocytes could become swollen by induction with AHTN, as it was previously documented in fish by sudden pH changes (Harter *et al.* 2018); the swelling could lead to irregularities in physiological functions of erythrocytes (Javed *et al.* 2016). The prolonged exposure to higher AHTN concentrations could thus be harmful for maintaining homeostasis.

The main goal of our experiment was to assess the endocrine disruption effects of AHTN, while the production of VTG served as a marker of xenoestrogenic potential. VTG is a precursor of egg yolk and is synthetized in female liver. Even though the juveniles and males also have the gene for VTG synthesis present in their genetic equipment, gene expression does not occur. However, some chemicals with endocrine-disruptive potential can induce VTG synthesis and its production and this potential were also reported for AHTN (Sumpter and Jobling 1995, Jobling *et al.* 1996, Yamauchi *et al.* 2008). In our experiment, 25 % and 15 % of the samples were positive for VTG in the AHTN1 and AHTN2 groups, respectively. Even though 20 % of samples were positive in the control group, the concentration levels were just slightly above the detection limit. The differences in total VTG concentrations in plasma between the control and both tested AHTN groups were without statistical significance and were not higher than 1000 ng/ml. In contrast, the physiological VTG detection limit is 200 to million-fold higher in females than males depending on their sexual maturity (Copeland *et al.* 1986, Wozny *et al.* 2020). The positivity in some of our negative control samples was probably caused by the fish origin as they were taken from natural water sources where chemicals with endocrine-disrupting potential could be present. In summary, due to the detected VTG levels in male samples, endocrine disruption caused by AHTN was not confirmed for rainbow trout.

The induction of oxidative stress in tissues after AHTN exposure was previously reported in earthworm (Chen *et al.* 2011), zebra mussel (Parolini *et al.* 2015), shrimp larvae (Li *et al.* 2020) and zebrafish (Blahova *et al.* 2018). The multiplication of free radicals could lead to cellular or tissue damage because they are not completely disposed by antioxidant enzymes. In our study, the oxidative stress marker TBARS was significantly increased in caudal kidney tissue in AHTN2. The TBARS is a marker of lipid peroxidation and its analysis includes not only detection of the major formed substance, malondialdehyde (MDA), but also the minor related compounds developing in response to oxidative stress (Wang *et al.* 2014, Chen *et al.* 2012). When the goldfish *Carassius auratus* (Linnaeus 1758) was exposed to another PCM representative, HHCB, the activity of antioxidant enzymes (superoxide dismutase, catalase and peroxidase) was significantly elevated with a correlation to higher tested HHCB concentrations. Additionally, MDA in fish liver also increased. In the same study, the prolonged HHCB exposure led to modulation of antioxidant defense system and the enzyme levels returned to their initial levels after 21 days. Similarly, increased levels of superoxide dismutase, catalase, GST and MDA were observed in shrimp larvae after their exposure to AHTN by Li *et al.* (2020). In our study, the environmentally-relevant AHTN1 concentration was probably tolerable for fish with the origin in free nature and the antioxidant capacity was able to maintain a balance. However, with a 10-times higher concentration, the TBARS content significantly increased, thus confirming the disruption of the cell antioxidant defense system in caudal kidney.

The histopathological findings on the caudal kidney in AHTN2 revealed congestion and hyaline degeneration of some tubules (Fig. 3) which could have developed as a response to AHTN and formed reactive oxygen species causing oxidative stress in cells. Additionally, some pathological findings were discovered in the liver and skin histological images. The hepatic dystrophy in liver tissue is a response to toxic effects of AHTN, as previously reported in the case of other PCMs including AHTN for Chinese sturgeon *Acipenser sinensis* (Wan *et al.* 2007), medaka *Oryzias latipes* (Yamauchi *et al.* 2008) and goldfish *C. auratus* (Chen *et al.* 2012). In

the case of skin, hyperplasia of mucinous cells was found. These cells are responsible for production of the mucous layer on the fish body surface serving as the innate immune barrier (Dash *et al.* 2018). Multiplication of these cells is a response to disruption of the physiological barrier, which could, if severe, lead to a lower ability to defend the body against pathogens, while fish mucus includes many bactericidal substances for fish protection. Together with the observed hepatic dystrophy, the presence of AHTN in surface water could be potentially harmful to the fish organism and maintaining homeostasis.

Based on the data collected in this study, the presence of AHTN in surface waters can lead to disruptions in physiological parameters and have a potential to cause tissue damage and metabolic disorders as a result of lipid peroxidation in rainbow trout. However, no effect on mortality, behaviour, biochemical profile and endocrine disruption was observed in our experiment.

Conclusions

In conclusion, the result of our experiment showed that the musk compound AHTN has no effect on mortality, behaviour and hematological or biochemical indices, except for PCV, which increased after exposure to both tested concentrations. Formation of reactive oxygen species was not found in plasma samples, based on CP and FRAP results. However, the induction of lipid peroxidation was confirmed in caudal kidney. The xenoestrogenic potential of AHTN was not confirmed.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

Project was funded by the ERDF/ESF “PROFISH” [no. CZ.02.1.01/0.0/0.0/16_019/0000869]. Authors would like to thank Mrs. Ludmila Faldikova for manuscript improvement and English correction.

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