Reproduction impact of mancozeb on rainbow trout (*Oncorhynchus mykiss* W.) and accumulation of its carcinogen metabolite, ethylene thiourea in fish products

Vlianie na mancozeba vurhu reprodukciata na dugova pusturva (*Oncorhynchus mykiss* W.) i natrupvane na negovia kancerogenen metabolit etilen tiourea v ribnite produkti

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Abstract

Pesticides can be taken up from the water and accumulated in tissues of hydrobionts, often becoming multiplied thousands of times higher in the organism than in the surrounding water. The dithiocarbamate mancozeb is applied in plant protection as fungicide. In recent years the amount of mancozeb used in Europe significantly increased. It is carcinogen due to its metabolite - ethylene thiourea (ETU), which causes thyroid and pituitary tumors. The purpose of this study is to determinate the quantity of ethylene thiourea in products of rainbow trout (*Oncorhynchus mykiss* W.), reared in environment containing permissible, according to the European law, amount of mancozeb. Seeking an answer to the question: is this concentration limit really safe for the reproduction of rainbow trout and can the more toxic metabolite - ETU, be accumulated in the fish eggs and fillet and afterwards make them harmful to the consumers? The study included 3 stages: feeding, analysis of ethylene thiourea in fish eggs and fillet by a new developed and validated HPLC (high performance liquid chromatography) method and study of the reproductive indicators. The assays of ETU in all analyzed samples (fish and water) were below the limit of quantification of the method, 0.05 $mg*l^{-1}$, so fish do not accumulate the carcinogen degradation product of mancozeb and the maximum residue level of mancozeb is really safe for the humans as consumers. But these environmental conditions caused reproductive disorders. They can be partly compensated by using sperm activation medium for artificial insemination of trout eggs, but successful fertilization does not guarantee successful hatching, especially of eggs in trout farms with presence of mancozeb in water, even in allowable

concentration. The presented results confirm previous investigation, that Salmonidae are very sensitive fish species, react to the lowest deviations in concentration levels of xenobiotics and are used for indicator of non-polluted water.

Keywords: ethylene thiourea, HPLC, mancozeb, rainbow trout, reproduction, sperm activation medium

Rezume

Pesticidite, popadnali vuv vodoemite, mogat da se poemat ot obitavashtite gi hidrobionti. Te sa v systoqnie da gi natrupat v svoite tukani i tiahnata koncetracia da se uvelichi, dori hiliadi puti, sravnena s tazi vuv vodata. Ditiokarbamatut mancozeb se prilaga v rastitelnata zashtita kato fungicide. Prez poslednite godini negovoto izpolzvano v Evropa kolichestvo znachitelno narasna. Kancerogennostta na mancozeba se dulji na metabolita mu, etilen tiourea (ETU), koiato prichiniava tumori na shtitovidnata jleza i hipofizata. Celta na tova izsledvane e da se opredeli sudurjanieto na etilen tiourea v produkti ot dugova pusturva (*Oncorhynchus mykiss* W.), otglejdana v sreda, sudurjashta dopustimo, suglasno evropejskoto zakonodatelstvo, kolichestvo mancozeb. Tursen e otgovor na vuprosa: tazi dopustima koncentracia naistina li e bezopasna za reprodukciata na dugovata pusturva i moje li da bude natrupan po-toksichniat metabolit - ETU, v haivera i fileto, koeto da gi napravi vredni za potrebitelite? Tova prouchvane vklyuchva 3 etapa: hranene, analiz na etilen tiourea v haiver i ribno file chrez novo razraboten i validiran HPLC (visokoefektivna techna khromatografia) metod i izsledvane na reproduktivnite indikatori. Kolichestvoto na ETU vuv vsichki analizirani probi (riba i voda) pokazaha sudurvanie pod granicata na kolichestveno opredeliane na metoda, 0.05 $mg*l^{-1}$; ribata ne natrupva kancerogennia product na razgrajdane na mancozeba i maksimalno dopustimata mu koncentracia e naistina bezopasna za potrebitelite. Tezi uslovia na okolnata sreda obache prichiniavat reproduktivni razstroistva. Te mogat da budat chastichno kompensirani chrez izpolzvane na spermo-aktivacionna sreda za iskustveno osemeniavane na haiver ot pusturva. No uspeshnoto oplojdane ne garantira uspeshnoto izlyupvane, osobeno na haiver v pusturvovi stopanstva s nalichie na macozeb vuv vodata, dori v dopustimata koncentracia.

Kluchovi dumi: dagova pastarva, etilen tiourea, HPLC, mancozeb, reprodukcia, spermo-activationna sreda

Introduction

Pollution of environment with pesticides is a serious global problem. They may remain in the environment much longer than expected or claimed, and the breakdown products may be also toxic to organisms. These xenobiotics enter in the organisms via absorption and cause metabolic changes. Pesticides can be taken up from the water and accumulated in tissues of hydrobionts, often becoming multiplied thousands of times higher in the organism than in the surrounding water. Pesticides can indirectly affect fish by interfering with their food supply or altering the aquatic habitat, even when the concentrations are too low to affect the fish directly (Ewing, 1999). Chronic exposure to certain pesticides can increase stress in juvenile salmonids and thereby render them more susceptible to predation. Pesticides can suppress the normal functioning of the immune system, resulting in a higher incidence of disease. According to Finn (2007) xenobiotics including pesticides are very harmful to the early life cycle of salmonids. Their appearance reduces hatching (Ankley et al., 1991; Mac and Schwartz, 1992; Matta et al., 1997); causes yolk-sac edema and early mortality syndromes, well-documented and summarized as Syndrom M74 (Atanasov et al., 2015); decreased disease resistance and long-term suppression of humoral immune competence (Milston et al., 2003; Ekman et al., 2004).

According to Directive 2005/72/EC, the dithiocarbamate mancozeb, a polymeric mixture of Zn- and Mn-ethylene dithiocarbamates, is applied in plant protection as fungicide. In recent years its amount used in Europe, including Bulgaria, significantly increased. Because of their chelating properties, the dithiocarbamates (DTCs) modify cellular metabolism by their direct interactions with different molecules such as signaling proteins, peptides and enzymes, and influence the oxido-reductive metabolism of the cells (Vohr, 2012). Carcinogenicity of DTCs, including mancozeb, is due to their metabolite - 2-imidazolidene thion (ethylene thiourea, ETU), which causes thyroid and pituitary tumors (Houeto et al., 1995; Panganiban et al., 2004; Axelstad et al., 2011).

Oncorhynchus mykiss W. is representative of Salmonidae, which are much sought on the fish market and the aquaculture has also been promoted in view of the fact that it provides consumers with safe, nutritious and high quality food products. Also these fish species are very sensitive to the environment, react to the lowest deviations in concentration levels of xenobiotics and are used for indicator of non-polluted water (Atanasov et al., 1999a, 1999b). The maximum residue level of several xenobiotics, allowed by the international law norms, can be accumulated in tissues of hydrobionts and often become magnified thousands of times higher in the organism than in the surrounding water. Often the effects of the metabolites impact is larger and do not occur immediately.

The aim of the present study is to determine the quantity of ethylene thiourea, carcinogenic metabolite of the pesticide mancozeb, in products of rainbow trout (*Oncorhynchus mykiss* W.), reared in environment containing permissible, according to the European law, amounts of mancozeb and to evaluate its impact on fish reproduction. An answer to the following questions is to be found: is this concentration limit really safe for the reproduction of rainbow trout and can the more

toxic metabolite - ETU be accumulated in the fish eggs and meat and subsequently make them harmful for the humans as consumers?

Materials and methods

Trial design and biological material

The study was conducted during 2015 - 2016 year and included 3 stages: feeding, analysis of ethylene thiourea in fish eggs and fillets and study of the reproductive indicators.

Experimental fish species was rainbow trout (*Oncorhynchus mykiss* W.), ca. 30 months old, first year spawners with body weight of 915 ± 23 g which were divided into three batches. The fish were bred in a Bulgarian fish farm near the Tundzha River and Sredna Gora Mountain. This farm is an aquaculture facility for intensive fish breeding and the qualitative and quantitative parameters of water are consistent with the Regulation 44/20.04.2006. The supplying water is taken from Tundzha River and includes rain and groundwater. It has temperature from 4.2 °C to 19.8 °C and dissolved oxygen from 9.1 $mg*l^{-1}$ to 10.8 $mg*l^{-1}$ depending on the season and daytime.

The fish were fed with specialized extruded feed AQUA ECO (Austria). Mancozeb was added daily in the ponds during eating, for 60 days, in quantities which lead to the final concentration of 0.5 $\mu g*l^{-1}$, meeting the requirements of Regulation 9/16.03.2001 of the Bulgarian Ministry of Environment and Water and Directive 2000/60/EC of the European Parliament and of the Council. Broodstock nutrition was finished in the end of November 2015.

The biological material for analysis of ETU - fish eggs and meat (fillet along the body) was obtained from all tree batches at the end of November 2015, and after collection was immediately frozen, transported in refrigerator, and stored for a maximum of 14 days at -12 °C prior to the analyses.

For studying the fish reproductivity were formed three experimental groups, each of them in 3 batches: 1st (control) group – 10 female broodstock farmed without mancozeb and its eggs inseminated with fresh water; 2nd group – 10 female broodstock farmed with mancozeb and its eggs inseminated with fresh water and 3rd group – 10 female broodstock farmed with mancozeb and its eggs inseminated with sperm activating medium № 49397 (Atanasov et al., 1991a). All 15 male broodstock (sperm donors) were farmed without mancozeb. Additionally in the reproductive period January – March 2016 were investigated commonly used biological parameters for assessment of the reproductive disorders - eggs fertilization rate, hatchability rate and survival rate of the rainbow trout fry (Dimitrov et al., 2000; Atanasov et al., 2006).

HPLC analysis

Mancozeb was purchased from Agria Ltd., Bulgaria, 80% WP grade (water dispersible powder). The reference standard - ethylene thiourea (98.5 ± 0.5%, for HPLC) was purchased from Dr. Ehrenstorfer (LGC Standards, UK). The solvents

(methanol, CHROMASOLV®HPLC grade and n-hexane, p.a), potassium fluoride p.a., ammonia p.a. and aluminum oxide p.a. were provided by Sigma-Aldrich. Deionized water ($\sigma \leq 0.4$ µS*cm^{-1}) was used in the experiment.

Analytical HPLC was performed by a C18 column Hypersil Gold (5 µm; 150 mm * 4.6 mm) on a Thermo system composed of a Surveyor LC Pump Plus, Surveyor Autosampler Plus, and Surveyor photodiode array detector PDA Plus. The mobile phase methanol-water (5:95, v/v) was filtered through a 0.45 µm membrane and degassed before use. Isocratic elution was carried out at a flow rate of 0.8 ml*min^{-1} at room temperature. Chromatograms were recorded at 240 nm.

For calibration five standard solutions were prepared from one stock solution of ethylene thiourea (10 mg of the referent material, weighed to the nearest ± 0.1 mg dissolved in 100 ml methanol, HPLC grade by diluting with methanol yielding final concentrations of 0.045, 0.1, 0.25, 2.5, 5 and 10 mg*l^{-1}).

This experimental setup for chromatographic separation allowed the ethylene thiourea to be assayed in a 6 min single run. Typical chromatogram of solution of the referent material is shown in Figure 3 (Panel A); the retention time was ca 3.7 min.

Single-laboratory validation (SLV) of the method of ETU quantification was carried out in terms of linearity, accuracy, stability of the analytes in solution and recovery via matrix effect assessment according to the International Conference on Harmonization (ICH) guidelines and IUPAC Harmonized Guidelines by Thompson et al., 2002.

The linearity was studied in the concentration range of 0.045 – 10 mg*l^{-1} (75% of the expected lowest concentration of 0.06 mg*l^{-1} and 200% of the expected highest concentration of 5 mg*l^{-1}, according to Regulation (EC) No 396/2005 the allowable maximum residue level of ETU in food products is 50 µg*kg^{-1}). Each of the calibration standards in this study were run in triplicates. RSD, intercept and slope were calculated (Figure 1).

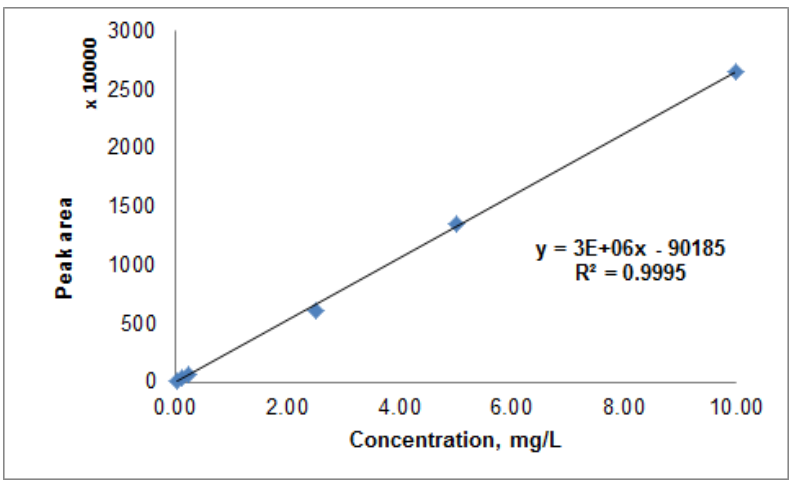


Figure 1. Linearity plot of ethylene thiourea (ETU)

Figura 1. Linejnost na metoda za opredeliane na etilen tiourea (ETU)

Limit of detection (LOD) and limit of quantification (LOQ) are defined as the lowest amount of analyte that can be detected above baseline noise: typically, three and ten times, respectively, the noise level. LOD and LOQ were 0.02 $mg*l^{-1}$ and 0.05 $mg*l^{-1}$, respectively.

To evaluate the accuracy in the present study, four different solutions with known concentration within the linearity range of ETU were prepared and injected in triplicates (Quality Check). Percent recovery of the response factor (area/concentration) was calculated (Table 1).

Table 1. Accuracy of ETU in samples with known concentration (n=3)

Tablica 1. Tocnost na ETU v probi s izvestna koncentracia (n=3)

Sample no	QC* $mg*l^{-1}$	Mean peak area	RSD** %	Recovery %
1	0.1	250,015	0.44	113.4
2	1	2,918,953	0.61	100.3
3	2	5,896,593	0.37	99.8
4	4	12,444,210	0.46	104.5

*quality check standard solution; **relative standard deviation of area response factor

*QC raztvor; ** otnositelno standartno otklonenie na saotvetnia koeficient

Precision/ repeatability was assessed in the study by the repeatability of the method on the basis of RSD of one standard solution representing 100% of the expected concentration level by assaying ten replicate injections during the same day and under the same experimental conditions. The RSD values of the retention time and peak area were shown in Table 2.

To verify the stability of the test solutions was prepared a solution of ETU (1 $mg*ml^{-1}$), as mentioned above, analyzed immediately after preparation, and the stability evaluation continued over a period of 24 and 48 hours. The solutions are stored in capped volumetric flasks at 4 °C (Table 3).

The most common approach for assessment of matrix effect is the standard addition method: the response of a sample is measured and recorded, afterwards a small volume of standard solution (as small as possible) is added and the response is measured again. In this study the target analyte was measured in four samples: matrix sample (muscle tissue) and 3 matrix samples plus 0.05, 0.1 and 1 $mg*l^{-1}$ ETU standard solution added before the extraction step of the assay (Figure 2 and Table 4).

Table 2. Precision/repeatability of the assay for ETU evaluated on the basis of 10 replicate injections of one and the same solution at 100% of the expected concentration level (1 mg*l^{-1})

Tablica 2. Preciznost/povtariaemost na nalaiza za ETU, ocenen vz isnova na 10 povtorenia na edin I sasht raztvor s koncentracia 100% ot ochakvanata (1 mg*l^{-1})

Inj. no	Retention time	Peak area	Inj. no	Retention time	Peak area
1	3.732	2,912,772	6	3.732	2,887,872
2	3.724	2,905,218	7	3.724	2,909,765
3	3.741	2,938,868	8	3.741	2,902,429
4	3.752	2,921,951	9	3.752	2,911,105
5	3.711	2,891,587	10	3.711	2,878,390
Retention time mean		3.727	Peak area mean		2,878,390
Retention time RSD %		0.60	Peak area RSD %		0.61

*relative standard deviation

*otnositelno standartno otklonenie

Table 3. Stability of ETU solutions (n=3)

Tablica 3. Stabilnost na raztvora na ETU (n=3)

Time hours	Retention time min	Peak area	Peak area RSD %	Percent of the initial
0	3.732	2,918,953	0.61	-
24	3.724	2,891,587	0.62	99.1
48	3.741	2,887,872	0.7	98.9

*relative standard deviation

*otnositelno standartno otklonenie

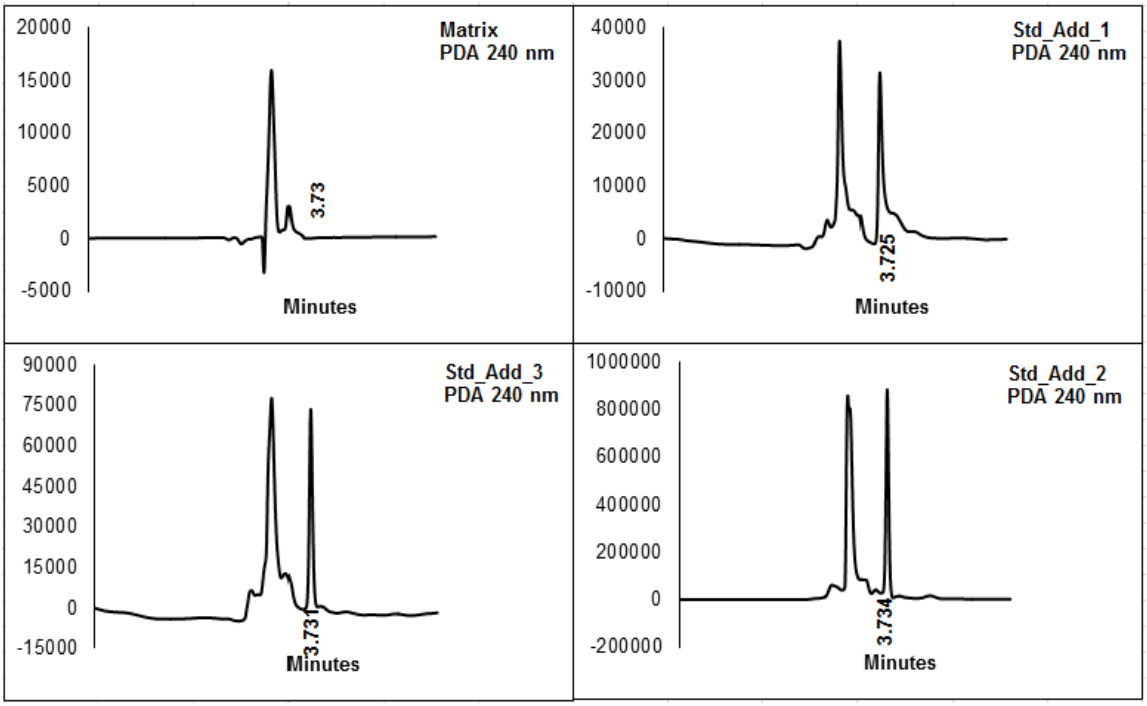


Figure 2. Chromatograms of quantification of ETU in matrix (fish fillets) and matrix plus standard addition

Figura 2. Hromatogrami ot kolichestveno opredeliane na ETU v matrica (ribno file) I matrica sas standartna dobavka

Table 4. Matrix effect of the assay for ethylene thiourea evaluated by the standard addition method

Tablica 4. Matrichen efekt pri opredeliane kolichestvoto na etilen tiourea chrez metoda na stndartnata dobavka

Sample	Conc. added, $mg*l^{-1}$	Spiked conc., $mg*l^{-1}$	Mean peak area	RSD* %	Recovery %	Standard addition plot data
Matrix	-	-	0	-	-	Squared correlation coefficient $r^2 = 0.9998$ equation: $y = 3.10^6x – 62,511$ (n=3) C_0: 0.021 $mg*l^{-1}$
Std Add** 1	0.05	0.054	71,466	0.41	108	
Std Add** 2	0.1	0.111	241,719	0.24	111	
Std Add** 3	1	0.932	2,707,067	0.01	93.2	

*relative standard deviation; **standard addition

*otnositelno standartno otklonenie; **standartna dobavka

Ethylene thiourea extraction from fish

The extraction was following the technical steps described in AOAC 992.31 Official method of analysis (2000) with minor modifications. The biological material (fillet and fish eggs) was thawed at room temperature, 2 – 2.5 g were weighed to the nearest ± 0.0001 g, 0.2 g KF was added and homogenized with mechanical tissue homogenizer in 10 ml mixture of methanol and deionized water (3:1 v/v) for 30 min at room temperature. The homogenized samples were centrifuged for 5 min at 800 g. The pellets were vortexed twice for 30 s with the solvent mixed and centrifuged at the same conditions. The supernatant layers were collected and washed in a separatory funnel triplicate with 15 ml n-hexane. The water layer was taken; pH was adjusted with 2.5% ammonia to 8 and filtered through 5 g Al_2O_3. The alumina filter was washed up with 10 ml methanol, the solvents were removed from the collected filtrates by rotary evaporator at 40 °C and the dry residue was dissolved in 2 ml of methanol (HPLC grade). The methanol extracts were stored overnight at 4 °C prior to the HPLC-analysis. A small quantity of each extract was transferred into a cupped vial and placed in the HPLC system autosampler.

Water samples

Two water samples, each of 2 l, were collected daily from the ponds for growing fish immediately before and 30 min after feed throwing. pH was adjusted with 2.5% ammonia to 8 and a small quantity of each water sample was filtered through 0.45 µm syringe filter and transferred into a cupped vial and placed in the HPLC system autosampler.

Reproductivity

The reproductivity of rainbow trout, reared in environmental water containing mancozeb in allowable maximum concentrations according to European low, was assessed by commonly used biological parameters - eggs fertilization rate, hatchability rate and survival rate of the rainbow trout fry (Dimitrov et al., 2000; Atanasov et al., 2006).

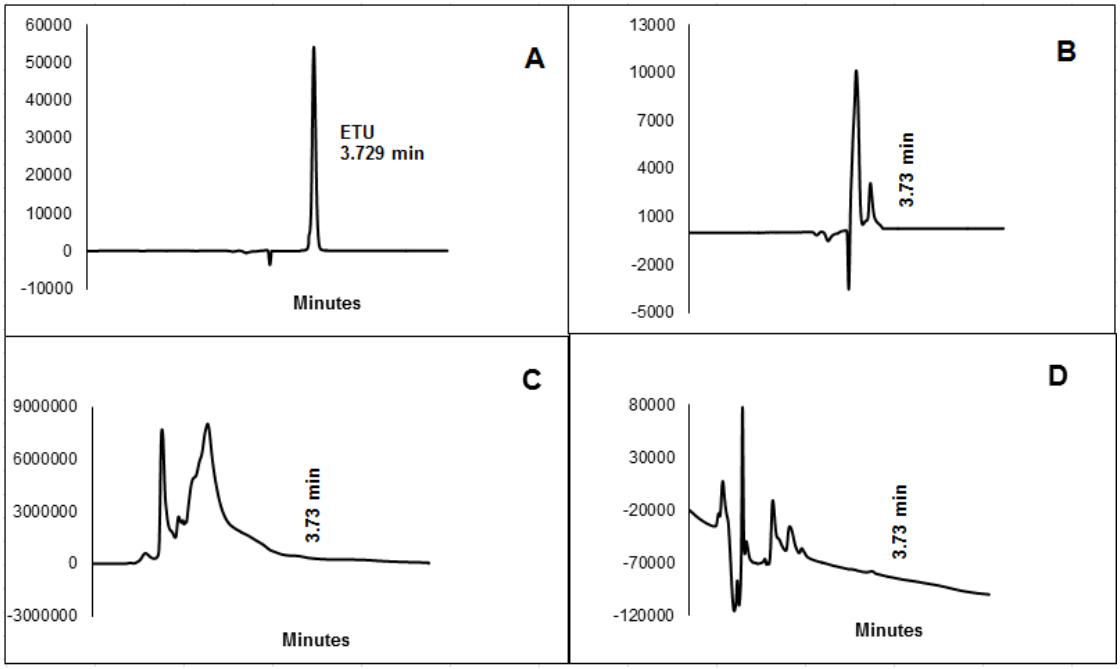
Statistical analysis

The statistical analyses were performed by Statistica 6.1 (StatSoft Inc., 2004) using ANOVA test.

Results

ETU content in the fish fillets and eggs

In the chromatograms of the samples of muscle tissue and eggs was not found any peak at the target retention times (Figure 4, Panels B and C). The ETU content in the samples is lower than the quantification limits of the method, 0.05 $mg*l^{-1}$.



Panel A – standard solution containing 1 mg*l^{-1} ETU; Panel B – sample solution of fish fillets; Panel C – sample solution of fish eggs; Panel D – sample solution of surrounding water

Panel A – 1 mg*l^{-1} standarten raztvor na ETU; Panel B – raztvor ot proba file; Panel C – raztvor ot proba hajver; Panel D – proba ot zaobikaliashtata voda

Figure 3. Typical chromatograms of standard and sample solutions

Figura 3. Tipichni hromatogrami na raztvori na standarti i probi

Water analysis

In the chromatograms of the water samples was not found any peak at the target retention times (Figure 3, Panel D). The ETU content in water samples is lower than the quantification limits of the method, 0.05 mg*l^{-1}.

Reproductivity

The data obtained from the all tree experimental groups are shown in Figures 4, 5 and 6.

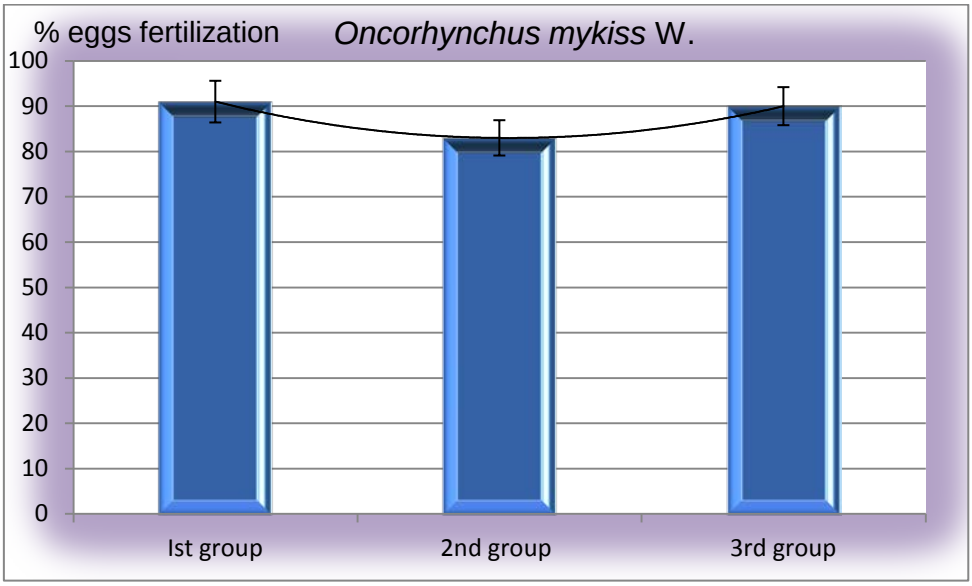


Figure 4. Rainbow trout eggs fertilization rate after artificial insemination

Figura 4. Stepen na oplozhdane na hajver na dagova pastarva sled izkuztveno osemeniavane

The fertilization rate of rainbow trout eggs (2nd group) after artificial insemination was significantly reduced. It could be seen that the sperm activating medium have excellent effect on trout eggs fertilization in the 3rd group.

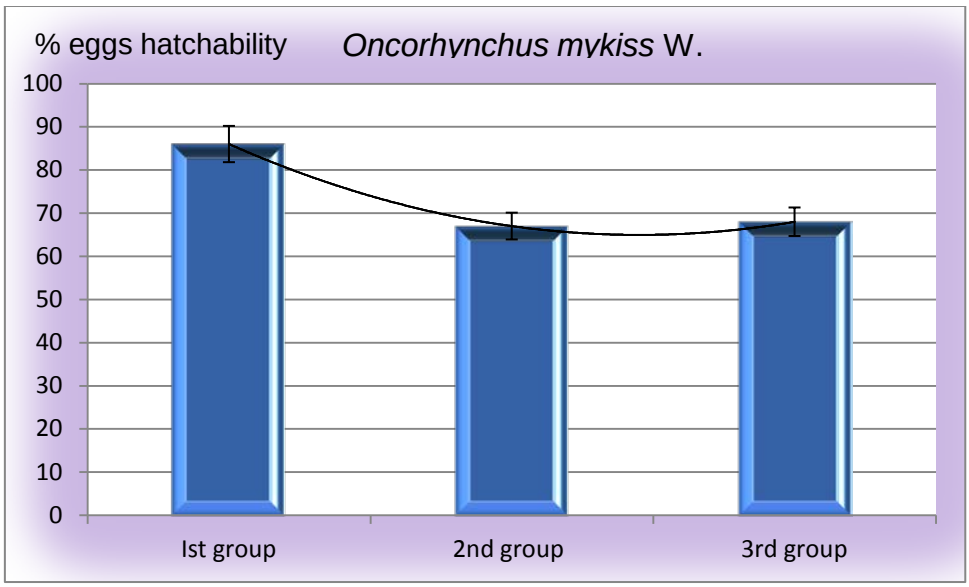


Figure 5. Rainbow trout eggs hatchability rate after artificial insemination

Figura 5. Lyupimost na hajvera na dagova pastarva sled izkustveno osemeniavane

The eggs hatchability rate mainly depends on the mancozeb presence in the water of fish farms (2nd and 3rd groups), not only from the artificial insemination, improved by

using sperm activating medium. It clearly shows that the methods of assisted reproduction cannot entirely compensate the negative influence of xenobiotics on embryonal survival.

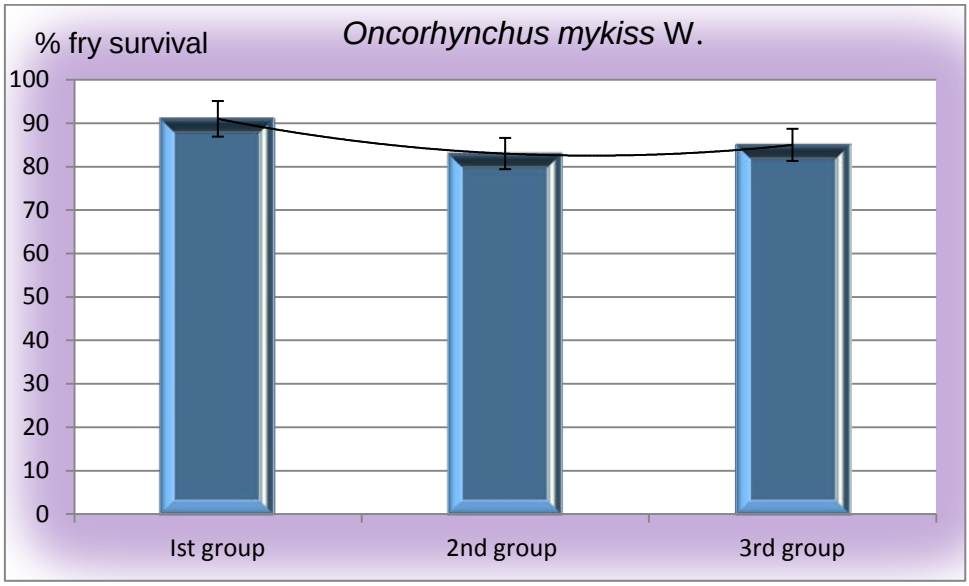


Figure 6. Rainbow trout yolk sac fry survival after hatching

Figura 6. Oceliaemost na lichinkite na dagova pastarva

The present date on Figure 6 proves that the survivability of the yolk-sac fry depends on the quality of eggs derived from different trout groups. In this aspect assisted reproduction cannot entirely compensate the negative influence of xenobiotics on fry survival after hatching.

Discussion

The influence of dithiocarbamates and its metabolites on fishes and their health is widely studied: Short-term toxicity of 26 DTCs and related compounds was determined in tests with different hydrobionts (Van Leeuwen et al., 1985) and the obtained results showed that DTCs and their degradation products are cytotoxic substances. The effect of mancozeb and its metabolite ETU on biochemical and enzymatic parameters in Clarius batrachus was studied by Srivastava and Singh (2013a, 2013b) and LC_{50}-values were estimated on different fish life stages and they were dose as well as time dependent. Mancozeb is able to induce genotoxic effect in catfish (Srivastava and Singh, 2013c). Enzymatic changes in liver and muscle tissues of catfish were tested by Srivastava et al. (2014) and their results proved that fingerlings were more sensitive to mancozeb than adults and 40% of LD_{50} was due to the secondary metabolites of mancozeb. Atanalp and Yanik (2003) studied the impact of 1/2 LC_{50} (1.1 $mg*l^{-1}$) of mancozeb on rainbow trout and reported that this dose caused important changes in the hematological properties.

There is not scientific data about the accumulation of carcinogenic metabolites of DTCs in fish organisms and the potential risk to the humans as fish products consumers.

Considering the importance of the safety of the fish products and the impact of the pesticides and their metabolites on fish reproduction, this study provides important information and data for the freshwater fish farmer and consumers of salmonids products.

The first task of the study was to determine the amount of the carcinogenic metabolite ethylene thiourea of the world-wide used pesticide, mancozeb, in fish eggs and fillets, produced in environment conditions containing permissible under European law amounts of mancozeb. For this purpose was developed and validated a new analytical method, which includes two steps: selective extraction of ETU and precise quantification by high performance liquid chromatography. The linearity of the method demonstrates a proportional relationship of instrument response versus analyte concentration over the working range of 80 - 120% of the expected target concentration, covered by minimum of five concentration levels. Linearity of the data is often assessed by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. The regression coefficient (r^2) > 0.998 is generally considered as evidence of the acceptable fit of the data to the regression line. The relative standard deviation (RSD) must be ≤ 2%. In the present study, the correlation coefficients (r^2) obtained for the regression lines is 0.9995 which demonstrated the excellent relationship between peak area and concentration in the concentration range 0.045 – 10 mg*l^{-1} (Figure 1). The results shown in Table 1 suggest that the method is accurate and since the recovery of ETU in the sample solutions was in the range of 99.8 – 113.4% it is evident that the method is accurate enough within the desired recovery range. Precision is the other parameter obtained by this single-laboratory validation. It is normally expressed by the relative standard deviation (RSD) in percent for a statistically significant number of samples. According to ICH requirements it should be calculated from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each), or from a minimum of six determinations at 100% of the test or target concentration. A precision criterion for an assay method is a value of RSD ≤ 1%. In this study the repeatability of the method was assessed on the basis of RSD of one standard solution representing 100% of the expected concentration level by assaying ten replicate injections during the same day and under the same experimental conditions. The precision study shows that the RSD criteria are satisfied, the RSD are less than 1% (Table 2). The limits of detection and quantification - 0.02 mg*l^{-1} and 0.05 mg*l^{-1} respectively, show the high sensibility of the applied method. Ethylene thiourea is a final metabolite of dithiocarbamate degradation and so is very stable to air and sunlight. Anyway, the stability of its solutions under storage conditions should be verified. Change in the analytical response of less than 2% of a sample or standard solution compared to a freshly prepared solution is considered as acceptable stability. Based on the experimental data (Table 3) can be concluded that ETU solutions can be assayed within 48 hours of the standard solution preparation or sample extraction. The data, obtained by the recovery test and assessment of matrix effect showed good recoveries: from 93.2% to 111% (Figure 2 and Table 4).

The accumulation of the ETU in fish products, eggs and fillets was determined by the aforementioned method. The results demonstrated the consumer's safety of the feed, produced from fish, reared in environmental water containing mancozeb in allowable maximum concentration, according to Regulation 9/16.03.2001 of the Bulgarian ministry of environment and water and Directive 2000/60/EC of the European Parliament and of the Council.

But the impact of mancozeb, even in allowable maximal concentration, on reproductivity is negative. The fertilization rate of rainbow trout eggs after artificial insemination was reduced by 10% (Figure 4). It could be seen that the sperm activating medium have excellent effect on trout eggs fertilization. This proves that the environmental conditions (including presence of mancozeb) which lead to reproductive disorders can be partly compensated using sperm activation medium for artificial insemination of trout eggs. In contrast, using fresh water as a natural sperm activator in the1st group, the fertilization rate depended on the physico-chemical parameters of water. The present studies confirm previous investigations on salmonids semen used for artificial insemination (Georgiev et al., 1991; Atanasov et al., 1991a, 1991b, 2004; Atanasov, 1995).

The eggs hatchability (Figure 5) rate mainly depends on the mancozeb presence in the water of fish farms (environmental conditions), not only from the artificial insemination, improved by using sperm activating medium. In this aspect the methods of assisted reproduction cannot entirely compensate the negative influence of xenobiotics on embryonal survival. In other words, successful fertilization does not guarantee successful hatching, especially for eggs in trout farms with mancozeb in the water. This is evident by the same slopes of the polynomial curves of Figures 5 and 6. The slope of the polynomial curve on Figure 6 proves that the survivability of the yolk-sac fry depends on the quality of eggs derived from different trout groups. The presented results are not surprising because newly hatched trout fry self-powered its energy and metabolic needs from yolk sac. This confirm previous investigation (Atanasov et al., 1999a, 1999b) that Salmonidae are very sensitive fish species that react to the lowest deviations in concentration levels of xenobiotics and therefore are used for indicator of non-polluted water.

Exactly the post-hatching development (generally early developmental stages of Salmonidae) is the most critical period due to thiamine deficiency and the accumulated xenobiotics in yolk sac. Moreover, survival of the larvae largely depends on the ambient conditions (Armstrong and Nislow, 2006; Viant et al., 2006; Finn, 2007; Keinanen et al., 2012). According to Vuori et al. (2004), Baltic salmon (*Salmo salar*) yolk-sac fry mortality is associated with disturbances in the function of hypoxia-inducible transcription factor (HIF-1alpha) and the consecutive gene expression.

Conclusion

The resulting conclusions of this research are: Fish do not accumulate its carcinogenic degradation product, ETU and after 60 day exposure to mancozeb in concentrations allowed by European law the maximum residue level of mancozeb is completely safe for human consumers. To understand the effects of long-term exposure of fish to mancozeb future studies are required. The applied method for

ETU quantification in fish products is validated. It is relatively easy to perform – it is quick, selective and very sensitive, and the used solvents, techniques and equipment are affordable for most laboratories.

Risky in regard to reproductive disorders are only the trout farms subjected to anthropogenic pressure including presence of mancozeb. The unsuitable environmental conditions, which lead to reproductive disorders, can be partly compensated by using sperm activation medium for artificial insemination of trout eggs, but successful fertilization does not guarantee successful hatching, especially of eggs in trout farms with presence of mancozeb in water, even in concentration level, permissible by European law.

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