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Acute ammonia toxicity during early ontogeny of chub, *Leuciscus cephalus* (Cyprinidae)

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Abstract – Acute toxicity of ammonia was investigated in four life stages of juvenile chub, *Leuciscus cephalus* (cyprinid fish): 1, 10, 20 and 30 days after the first feeding. The fish used for the toxicity test were reared intensively in a closed recirculation system. Each acute toxicity test duration was 96 h and lethal concentration LC_1, LC_{50} and LC_{99} values were calculated for 24, 48, 72 and 96 h. The susceptibility of chub to acute ammonia toxicity decreased linearly with age and stage of development. The LC_{50} (48 h) values ranged from 0.62 mg L^{-1} of unionized ammonia nitrogen for one day after first feeding larvae to 1.73 mg L^{-1} for 30 days after first feeding ones. A significant linear relationship between chub larvae susceptibility to ammonia toxicity and both body weight and length was found. The critical level of unionized ammonia nitrogen for chub larvae was suggested as 0.49 mg L^{-1}.

Key words: Ammonia / Acute toxicity / Larval development / Chub / *Leuciscus cephalus*

1 Introduction

Freshwater aquaculture production is conducted on different intensification levels: from earthen-pond-based extensive culture through semi-intensive cage culture to highly intensive controlled rearing in recirculation systems. The latter method allows intensive rearing of larvae and juveniles at high temperatures and high stocking densities, together with high-protein feed application (van Rijn 1996; Remen et al. 2008). However, there is a high risk of waste accumulation in the recirculation system, especially nitrogen compounds. Even small amounts of these compounds have a great impact on fish growth; their health and survival rates (Biswas et al. 2006; Remen et al. 2008; Martins et al. 2009).

Ammonia is a product of protein catabolism in animals and is one of the best known waste products with a direct and negative influence on fish during intensive culture. Most fish do not produce urea due to the high energy costs as compared to excretion of ammonia via gills (Wood 1993). The toxicity of the total ammonia (expressed as the sum of NH_3 and NH_4^+) depends on the water pH. The portion of the un-ionized form (much more toxic) increases as the pH increases (Randall and Tsui 2002). Increased levels of un-ionized ammonia (NH_3) in water causes an increase of its content in the blood of fish through diffusion and poisoning of the central nervous system (Randall and Ip 2006; Svobodova et al. 2007). According to Wood (2004), a low ammonia concentration can positively influence fish growth. However, Brinkman (2009) reported that chronic exposure of rainbow trout in early life stages to ammonia concentrations as low as 0.19 mg L^{-1} of unionized ammonia nitrogen (UIAN) caused significant reductions in survival, growth and biomass of swim-up fry.

Chub *Leuciscus cephalus* (L.), is a rheophilic cyprinid, a very popular sport fish in Poland and many other European countries. However, some chub populations have recently become vulnerable (Bolland et al. 2008). This is probably due to the high sensitivity of this species to industrial pollutants, enabling the chub to be used as a bio-indicator of pollution (Hajkova et al. 2007). Research into the aquaculture of this species to date has been based on artificial propagation and rearing larvae under controlled conditions (Shiri Harzevili et al. 2003). However, an effective protocol for the production of stocking material is still being researched (Krejszeff et al. 2010). Stocked chub and other rheophilic cyprinids for many different purposes, including conservation, has become a prospective, profitable and therefore important part of aquaculture (Shiri Harzevili et al. 2003). In order to succeed, it is particularly important to overcome the obstacles of start-feeding during the early (3–4 weeks) larval period (Wolnicki et al. 2009). After this period, fish are able to ingest and digest most available types of food (Dąbrowski 1984), a fact which is crucial for effective restocking (Cowx 1994). Due to the preservation of genetic biodiversity of restocked populations

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(or supported by restocking), the breeders from the natural environment are used for controlled reproduction. This controlled reproduction is usually done in small local hatcheries, so high effectiveness of reproduction is required. It was found that cyprinids may be successfully reared in very high stocking densities (up to 600 individuals per litre) (Żarski et al. 2011). In such high densities, the risk of periodic occurrence of elevated ammonia levels and, in consequence, acute ammonia toxicity is very possible.

Acute toxicity of ammonia to fish was investigated in numerous works (Thurston et al. 1983; Tomasso and Carmichael 1986), but most of those papers referred to the fish after the completed larval metamorphosis. There is no information about changes in the sensitivity of cyprinid larvae during the short, but key, period of larval development. The aim of this study was to determine the acute toxicity of ammonia to four different stages of larval chub intensively reared under controlled conditions.

2 Materials and methods

2.1 Broodstock and hatched larvae management

Controlled reproduction of pond-reared chub spawners was conducted following Krejszeff et al. (2010). Prior to spawning, spawners were kept in 1000 L tanks with controlled photoperiod, temperature and dissolved oxygen (Kujawa et al. 1999). Ovopel (Unic-trade, Hungary) (mammalian analogue of GnRH with dopamine antagonist – metoclopramide) (Horvath et al. 1997) was used for the induction of maturation and ovulation. Eggs from 7 females were stripped manually and mixed in a plastic vessel and then fertilized with pooled semen obtained from 4 males. Egg adherence was removed using a one-hour bath in a modified Woynarovich solution (35 g of NaCl, 30 g of urine per 10 L of water) and a short 20 s bath in a tannin solution (7 g of tannin per 10 L of water). Egg incubation was conducted in Weiss jars at 20 °C, the optimal temperature for chub embryonic development (Kupren et al. 2008b). Shortly before hatching, embryos were transferred to a 150 L tank where a constant temperature (22 °C), water exchange and gentle aeration were provided. The stocking density of larvae in the tank was c.a. 20 ind. L^{-1}. The temperature was gradually raised to 25 °C (over a period of about 18 h) after the hatching of all the larvae. The larvae were then gently removed from the tank, counted manually and randomly stocked to 16 rearing tanks.

2.2 Rearing procedure

Rearing was conducted in 1 L glass rearing units placed in a small recirculating system which was equipped with a temperature control, photoperiod and biological filtration. Rearing was conducted from the beginning of exogenous feeding. The determination of that moment was based on the procedure performed every morning after the larvae started to swim. For this purpose, 30 randomly chosen larvae were placed into a separate 1 L chamber where gentle aeration was provided and freshly hatched Artemia nauplii were supplied (in excess). After one hour, these larvae were anaesthetized in 2-phenoxyethanol (0.4 ml L^{-1}) and the presence of the food in the digestive tract was checked under a stereoscopic microscope (these larvae were excluded from further experimental procedures). The day when over 90% of larvae had a digestive tract filled with food was designated as the moment of the beginning of exogenous feeding. That day food was supplied to the remaining rearing tanks where larvae for further experimental procedures were reared. Before second feeding, 100 randomly chosen specimens were removed to a separate tank and they were starved for the next 24 h. These larvae were then used for the toxicity test. The stocking density for the first 20 days was 150 ind. L^{-1}. Larvae were then taken out and randomly restocked for further rearing at 50 ind. L^{-1} in the rearing unit. The temperature (25 °C ± 0.1) and photoperiod (12L:12D) were constant throughout the rearing period. Fish were fed three times daily with hatched Artemia nauplii. After the larvae started to eat, food was offered ad libitum. Tanks were cleaned twice daily. Dead larvae were removed, counted and the survival rate was calculated. When the toxicity test began, 30 randomly chosen larvae (control group) were removed, anaesthetized in a solution of 2-fenoxyethanol (0.4 ml L^{-1}) and photographed under a stereoscopic microscope (Leica MZ 12.5, Germany). Additionally, the wet body weight (WBW) of larvae was determined (±0.1 mg). These fish were excluded from further experimental procedures. The photographs were then used for larvae measurements (±0.01 mm, with ProgRes® Capture Pro 2.5 software, Jenoptic, Germany) and determination of the developmental stage, using the ontogeny schemes proposed by Kucharczyk et al. (1997) for common bream *Abramis brama* (L.). Every 3 days, the concentrations of ammonia (HI 83214, Hanna Instruments, Italy) and nitrites (using an LF205 photometer, Slandi, Poland) were checked. Oxygen was also checked using an oxygen probe (HI 91410, Hanna Instruments, Italy).

2.3 Toxicity tests

A series of four tests were carried out on fish of different ontogeny stages. The first experimental group (D1) was sampled the day after first feeding (day 1st) and the next three groups D10, D20 and D30 were sampled on days 10, 20 and 30 after they started exogenous feeding, respectively.

The experimental design was based on the Organisation for Economic Cooperation and Development (OECD) standard No. 203 “Fish acute toxicity test” for semi-static tests. In each of four tests, fish were randomly divided into 10 groups (1 control and 9 experimental forming a set of increasing concentrations. The range of concentrations was different for each age group. Each experiment was conducted in duplicate (n = 10 for the D1, D10 and D20 tests and n = 7 for the D30 test). Specimens randomly chosen for the toxicity test were not fed for 24 h before the test. The fish were then placed in 1 L test glass vessels which were placed in a water bath. The test water temperature was 25 ± 0.1 °C. Gentle aeration was provided for each vessel.

A set of 9 concentrations of NH_4Cl was used in each experiment. Each solution and the water in the control group were

renewed every 24 h. The test duration was 96 h. The water temperature, pH and total ammonia nitrogen (TAN) concentration were measured twice a day. The TAN concentration was measured with a multiparametric analyzer (HI 83214, Hanna Instruments, Italy). The number of dead fish was recorded and dead fish were removed twice a day.

2.4 Ammonia solutions

A new stock solution of 10 g L^{-1} of NH_4Cl (ammonium chloride pure p.a.; supplier Chempur, Poland) in redistilled water was prepared every day. Appropriate stock solution volumes were added to clean glass vessels according to the developed set of concentrations. Each vessel was then filled with laboratory tap water up to 1 L. The tap water was aerated and heated to 25 ± 0.1 °C for 24 h before use. The pH was not adjusted. The tap water parameters were as follows (mean ± SD): hardness 262 ± 12 ppm $CaCO_3$ and conductivity 550 ± 26 μS cm^{-1}.

2.5 Data analysis

The mean pH and mean temperature were calculated for each test vessel. The mean UIAN concentration was then calculated according to the algorithm given in "Update of Ambient Water Quality Criteria for Ammonia" (USA Environmental Protection Agency publication number EPA-822-R-99-014; 1999).

Lethal concentrations that kills 50% of the fish, LC_{50} values for unionized ammonia were calculated with Probit software (EPA, version 1.5) for 12, 24, 36, 48, 60, 72, 84 and 96 h, if possible.

The relationships between measured parameters were analyzed using the linear regression method. The significance level of 0.05 was used.

3 Results

3.1 Controlled rearing

Throughout the rearing period, a low chub larvae mortality rate was observed (4.7% cumulative mortality at day 30). The growth rate was very satisfactory. The mean (±SD) total length (TL) of larvae was 8.0 (±0.3), 13.5 (±0.7), 20.3 (±0.9) and 25.9 (±1.5) mm at D1, D10, D20 and D30, respectively. The mean (±SD) wet weigh of larvae was 1.5 (±0.5), 17.6 (±3.3), 71.3 (±17.9) and 113.8 (±26.6) mg at D1, D10, D20 and D30, respectively. Throughout the rearing period, the concentration of ammonia or nitrites did not exceed 0.05 and 0.05 mg L^{-1}, respectively. The oxygen level in rearing tanks did not drop below 80% saturation during 30 days of rearing.

3.2 Developmental stages

The fish used in the following tests were characterized by a more advanced level of ontogeny. The first three tests were conducted on larvae (groups D1, D10 and D20). The last one (D30) was done on juvenile chub. Larvae from the first experimental group (D1) had already started active movement and exclusive exogenous feeding. The operculum covered the whole gill, which had taken over the main respiratory function by that time. A cluster of mezenchymes also appeared in the region of the future skeleton of the caudal fin. After the next 10 days of rearing, the larvae were better swimmers. All the tested fish had almost formed fins and the back chamber of the swim-bladder was filled. Twenty days after beginning exogenous feeding, some fish completed the larval period (the finfold had disappeared), but most of them still had reduced finfolds in the preanal part of the body (between the pelvic and the anal fin). In addition, all fish had ossified rays in their fins and the first scales were also found. Individuals from the last experimental group (D30) had finished their metamorphosis and most of them had full scale-cover and had attained a body shape similar to that of adults.

3.3 Ammonia toxicity

The results of four tests are summarized in Table 1. No mortality was observed in the control groups. Due to an insufficient number of mortality points, it was not possible to determine the LC_{50} value for 12 h in the D20 group and for hours 12, 24, 72, 84 and 96 for group D30. The exponential function model shows the best fit to the ammonia toxicity results (Fig. 1). The susceptibility of chub larval stages to ammonia toxicity decreased with the larvae development, by approximately 2.9 times between day 1 and day 30 after the first feeding. The susceptibility of chub to ammonia toxicity decreased linearly with age and the developmental stage (correlation coefficient $r = 0.996$), 48 h LC_{50} values ranged from 0.59 UIAN mg L^{-1} for the youngest fish to 1.73 UIAN mg L^{-1} for the oldest ones. A significant linear relationship was found between chub susceptibility and body weight together with body length (correlation coefficients were $r = 0.985$ and $r = 0.998$, respectively, Fig. 2).

4 Discussion

Chub larvae, similar to other cyprinids, undergo quick and dramatic changes in body proportions resulting from the development and refinement of internal organs such as gills, muscles and liver in the first few days after hatching (Van Snick et al. 1997; Kucharczyk et al. 1997; Osse and Van den Boogaart 1999). During the next days of metamorphosis, the process of cell proliferation and morphological differentiation decreases in intensity. The definitive digestive, respiratory, excretory and motor organs began functioning more effectively, simultaneously with growth during the larval period (Penaz et al. 1983; Fuiman 1983; Calta 2000). The first scales appear in the caudal part of the body when young chub reach approximately 16 mm (Economou et al. 1991). The finfold disappeared when they were 22 mm long and the fish were fully covered with scales when their body lengths reached 25 mm.

Table 1. Results of acute toxicity tests of ammonia to early developmental stages of chub at 25 °C. Lethal concentration (LC) values expressed as unionized ammonia nitrogen (UIAN). Groups D1, D10, D20, D30 represents 1, 10, 20 and 30 days after the moment when chub started exogenous feeding, respectively (for detailed description of each stage see "Developmental stage" in the Results section). Part A: 24 h and 48 h; Part B: 72 h and 96 h.

PART A

Group	pH		LC	24 h				48 h			
				UIAN	95% confidence limits		slope(sdt. error)	UIAN	95% confidence limits		slope(sdt. error)
	mean	range		mg L^{-1}	lower	upper		mg L^{-1}	lower	upper	
D1	8.6	8.46–8.67	LC_1	0.49	0.40	0.57	11.88 (2.33)	0.48	0.34	0.53	20.26 (5.40)
			LC_{50}	0.76	0.69	0.84		0.62	0.58	0.66	
			LC_{99}	1.20	1.04	1.61		0.81	0.74	1.04	
D10	8.5	8.49–8.58	LC_1	0.84	0.66	0.91	25.56 (6.74)	0.72	0.55	0.80	17.60 (3.95)
			LC_{50}	1.04	0.97	1.09		0.95	0.90	1.05	
			LC_{99}	1.27	1.18	1.60		1.32	1.19	1.69	
D20	8.5	8.46–8.52	LC_1	1.01	0.69	1.17	9.60 (3.05)	1.02	0.70	1.17	14.06 (5.79)
			LC_{50}	1.51	1.37	1.66		1.44	1.31	1.58	
			LC_{99}	2.23	1.92	3.32		2.02	1.77	2.99	
D30	8.5	8.45–8.54	*LC_{50}	<1.72 – >1.80				1.61	1.11	1.69	77.11 (32.06)
								1.73	1.60	1.77	
								1.86	1.80	2.37	

* 0% mortality at 1.72 UIAN mg L-1 and lower levels; 100% mortality at 1.80 UIAN mg L-1 and higher levels; too few mortality points to calculate LC values

Table 1. Continued.

PART B										
Group	pH		LC	72 h				96 h			
				UIAN	95% confidence limits		slope (sdt. error)	UIAN	95% confidence limits		slope (sdt. error)
	mean	range		mg L^{-1}	lower	upper		mg L^{-1}	lower	upper	
D1	8.6	8.46–8.67	LC_1	0.23	0.14	0.29	7.94 (1.53)	0.16	0,05	0.22	6.59 (1.75)
			LC_{50}	0.45	0.39	0.50		0.38	0.26	0.40	
			LC_{99}	0.90	0.73	1.32		0.79	0.63	1.56	
D10	8.5	8.49–8.58	LC_1	0.61	0.44	0.70	13.26 (2.78)	0.52	0.37	0.60	10.70 (2.01)
			LC_{50}	0.91	0.84	0.99		0.85	0.77	0.93	
			LC_{99}	1.36	1.20	1.82		1.41	1.21	1.89	
D20	8.5	8.46–8.52	LC_1	1,00	0.68	1,15	12.64 (3.35)	0.94	0.59	1.07	8.32 (2.85)
			LC_{50}	1.41	1.28	1.55		1.28	1.16	1.41	
			LC_{99}	1.98	1.73	2.95		1.77	1.55	2.74	
D30	8.5	8.45–8.54	*LC_{50}	<1.15 – >1.53				<1.15 – >1.53			

* 0% mortality at 1.15 UIAN mg L^{-1} and lower levels; 100% mortality at 1.53 UIAN mg L^{-1} and higher levels; too few mortality points to calculate LC values.

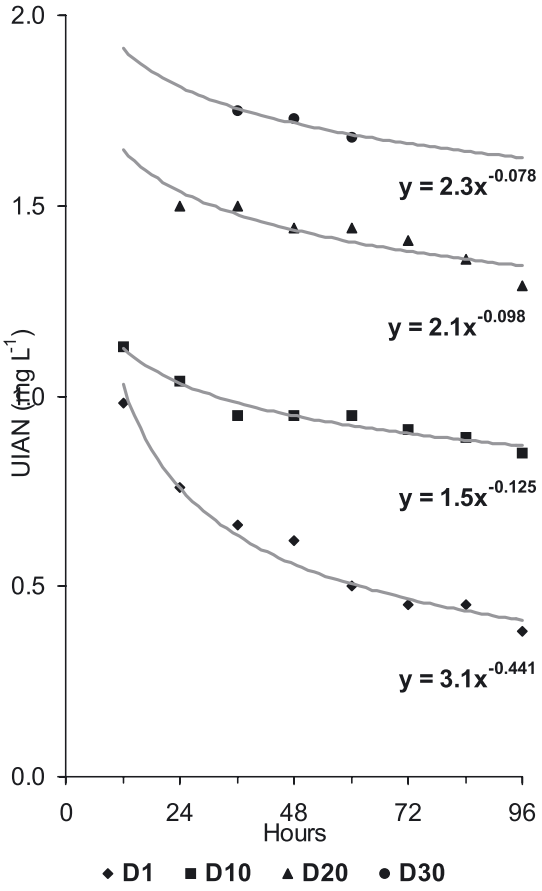

Fig. 1. Toxicity curves of ammonia to four developmental stages of chub.

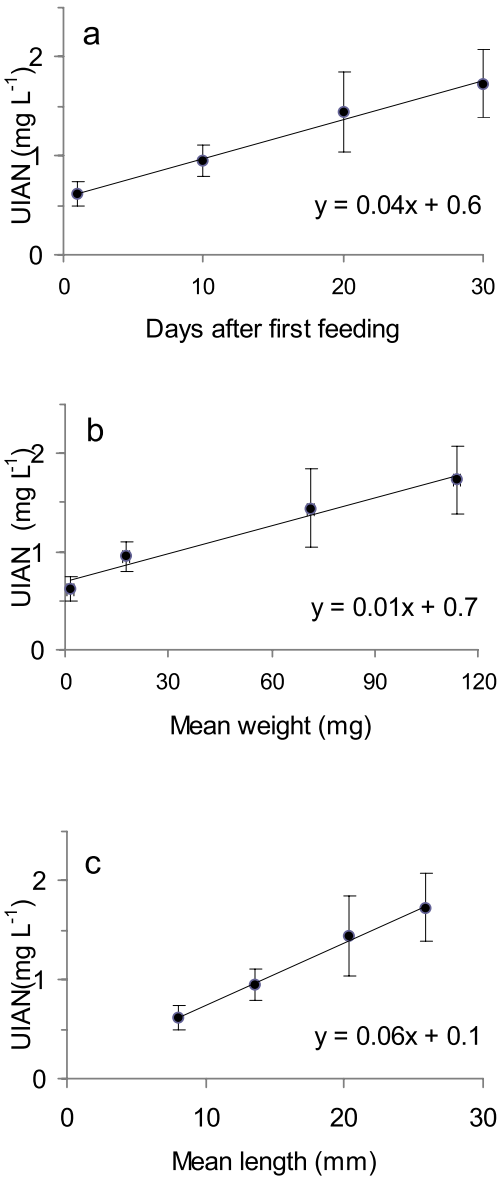

Fig. 2. The relationship between acute ammonia toxicity (48 h LC50) and (a) days after feeding (r = 0.996, p = 0.004); (b) mean wet body weight (r = 0.985, p = 0.015); (c) mean body length (r = 0.998, p = 0.002) of juvenile chub.

In the present study, the susceptibility of chub larval stages to ammonia toxicity decreased with larval development. Similar results were reported by Karasu Benli and Köksal (2005) for young tilapia *Oreochromis niloticus*. They found 48 h LC_{50} as high as 0.82 ± 0.02 mg L^{-1} and 6.09 ± 0.01 mg L^{-1} of UIAN for larvae and fingerlings, respectively. Thurston and Russo (1983) studied acute ammonia toxicity to rainbow trout ranging <0.1 g to 2.6 kg of weight (from yolk sac larvae to 4-year-old adults). They found 96 h LC_{50} values ranging from 0.13 to 0.9 of UIAN mg L^{-1}. The susceptibility of trout to ammonia decreased with increasing fish size in the range from 0.6 to 2.0 g of body weight, however, it increased gradually as fish grew larger. However, Calamari et al. (1977, 1981) found larvae and fingerlings of trout more resistant than older stages. Thurston et al. (1983) did not find any dependence between fish size and susceptibility to ammonia in fathead minnows, *Pimephales promelas*. Abbas (2006) studied ammonia toxicity in young carp *Cyprinus carpio* of 5, 10 and 15 g; in the experiment conducted in pH 8.5 (8.49–8.55). In this study, 48 h LC_{50} were 1.74; 1.92 and 2.32 UIAN mg L^{-1} respectively. These results indicate that carp susceptibility to ammonia decreases slightly with increasing weight in the tested range of weights.

The process of ammonia excretion occurs mostly across the gills by diffusion. Only a few air-breathing species have been found capable of active ammonia excretion (Randall and Ip 2006). In most fish species, there are mechanisms determining the level of resistance to ammonia toxicity. A well-known mechanism is glutamine synthetase (GSase) activity (Vedel et al. 1998; Wicks and Randall 2002). This enzyme catalyzes ammonia conversion to glutamine through glutamate. Glutamine, as well as glutamate, are non-toxic to fish and play a very important role in the adaptation of fish organisms to elevated ammonia levels (Wicks and Randall 2002). Glutamine (via GNase) and glutamate (via glutamate dehydrogenase) synthesis are affected by increased internal ammonia concentrations which are up regulated by the brain and liver

(Vedel et al. 1998; Wicks and Randall 2002). The muscles were found to be very important glutamine "temporary storage" tissue (Wicks and Randall 2002).

The 96 h LC_{50} of ammonia to chub was 1.63 UIAN mg L^{-1}, at 30 days after the first feeding (after the formula given in Fig. 1). The comparison of this value to mean values, given for numerous genuses and species in an review of US Environmental Protection Agency publication "1999 Update of Ambient Water Quality Criteria for Ammonia" (EPA-822-R-99-014), indicates that the chub is relatively resistant to ammonia toxicity at the end of its larval development.

The decrease in susceptibility of chub larvae to ammonia toxicity was probably caused by a general increment in both physiological efficiency (brain and liver) and muscle capability for glutamine storage.

The results obtained in the present study show the dynamics of changes in susceptibility to ammonia during the larval period of chub. These are very important data for larviculturists who are engaged in controlled rearing of freshwater cyprinids.

It can be assumed that the first days of feeding are the most critical period for the larvae because of its sensibility to ammonia. Thus, during that period, special attention should be paid to ammonia concentration monitoring. We suggest to use the LC_1 for 96 h (0.16 UIAN mg L^{-1}) as a warning level and the LC_1 for 24 h (0.49 UIAN mg L^{-1}) (Table 1) as a critical unionized ammonia nitrogen concentration for chub larvae. In the case of elevated ammonia levels in rearing tanks, the feeding regime should be readjusted. According to our experience, increasing the water flow in rearing tanks or intensification of water aeration during the first 2 weeks of rearing is not recommended as it can damage the delicate larvae. Regarding the high larvae susceptibility to ammonia, special attention should be paid to the proper preparation of biological filters to achieve maximum efficiency before the start of rearing (Żarski et al. 2010). Meinelt et al. (2010) recommended the addition of commercial humic substances preparations to reduce ammonia toxicity. Treasurer (2010) found that addition of ClorAm-X^{TM}(Reed Mariculture)(the commercial preparation of sodium hydroxymethan sulfonate for removing ammonia, chlorine and chloramines) effectively reduced ammonia level during cod fry transportation. It seems that ClorAm-X^{TM}is also suitable in case of emergency of ammonia level increase in freshwater aquaculture systems.

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