Acute toxicity of three antifungal chemicals on silverside *Odontesthes bonariensis* (Valenciennes 1835) eggs

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Abstract: The effects of sodium chloride (NaCl) and formalin (F) on some toxicological parameters and the hatching rate of *Odontesthes bonariensis* eggs were examined under laboratory conditions, and the determined values were compared with those obtained for Malachite Green (MG), which was used as a reference toxic. The 96 hours nominal LC_{50} were 1.51 mg L^{-1}, 652.14 µl L^{-1} and 34.82 g L^{-1} for MG, F and NaCl, respectively. The No-Observed-Effect Concentration (NOEC) and the No-Effect Concentrations (NEC) were 1.5 mg L^{-1} and 0.507 mg L^{-1} (MG), 400 µl L^{-1} and 530 µl L^{-1} (F) and 30.0 g L^{-1} and 28.68 g L^{-1} (NaCl), respectively. Hatching rates of 60% or higher were determined for eggs after 168 hours incubation in the assayed solutions at concentrations of 0.75 mg L^{-1} (MG), 300 µ L^{-1} (F) and 20 g L^{-1} (NaCl); at higher concentrations the hatching rates were severely affected.

Keywords: *Odontesthes bonariensis*; fish eggs; fungicides; 96 h LC_{50}; NOEC; no-observed-effect concentration; NEC; no-effect concentrations; hatching rate.

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1 Introduction

Fish eggs are commonly infected by numerous bacteria, parasites and fungi that are ubiquitous in aquatic environments and are responsible for serious production losses in intensive aquaculture. It is accepted that immersion in media containing the fungicides is an appropriate treatment for diseases in the early development stages and for external infections in adult individuals.

The use of antifungal agents is essential for the maintenance of healthy stocks of fish and their eggs in aquaculture operations. Several chemicals have been frequently used in aquaculture to effectively control fungal infections in early development stages of fish. Trials are usually conducted by exposing the eggs to different chemicals at variable concentrations for relatively short periods of time. The limited exposure time requires a high concentration of the chemicals to achieve the antifungal effect. Hence, this kind of treatment can lead to the contamination of large volumes of water, which may affect non-target organisms if released to the environment. A reduction in the therapeutic concentrations can be achieved by the implementation of continuous treatments. For such treatments it is necessary to know some acute toxicity parameters, which are not available in the literature (Schreier et al., 1996).

Odontesthes bonariensis (Valenciennes 1835) is an atherinopsid euryhaline teleost, which is native to the Río de la Plata river basin. Several authors have studied different aspects of this fish biology, both in natural environments and in culture systems (Berasain et al., 2008). Intensive culture of this commercially important species has been conducted for approximately a century. This species has been introduced to other Latin American countries and to Japan (Gómez et al., 2007; Somoza et al., 2008). A thorough study of fungal infections which naturally affect eggs of *O. bonariensis* showed the presence of Saprolegnial fungi: *Aphanomyces*, *Pythium*, *Leptomitus*, *Saprolegnia* and *Achlya* (Pacheco-Marino et al., 2006).

The aim of the present study is to determine some toxicological parameters of antifungal therapeutic agents in order to know their safe doses for their application in cultures of eggs of *O. bonariensis* in fresh water. The data from our study can contribute to improve the hatching rate and management. We present the results of laboratory toxicity tests for malachite green, formalin and NaCl carried out on fertilised eggs of the silverside *O. bonariensis*. We also report the effect of these substances on the hatching rate expressed as percentage of success. Preliminary reports of these results were published elsewhere (Pacheco-Marino and Salibián, 2008).

2 Materials and methods

Freshwater silverside *O. bonariensis* non-eyed eggs were obtained from Chascomús Experimental Fisheries Station located in Chascomús (Buenos Aires Province, Argentina) and transported 24 hours after spawning to the laboratory in water from the breeding station, in glass flasks previously disinfected with benzalkonium chloride 1% solutions and sterilised in autoclave for 20 min at 1 psi and 121°C.

Each set of assays was conducted using 96–120 hours post-fertilisation eggs from the same spawning, without adhesive filaments, that corresponds to stages 17–18 of embrionary development of pejerrey (Chalde, 2009). In these stages, the heart pumps actively and the blood flow through the vitelline veins can be observed; the tail of the embryo shakes freely; the eyes begin to show pigmentation. The antero-posterior axis of the embryo begins to curve around the yolk sac as it grows in length. All the eggs were carefully selected under microscope to ensure their viability, uniformity in development stage and absence of fungal infection.

To take into account seasonal variations in the biological conditions of the eggs (Marking et al., 1994) tests were conducted during the spawning periods (spring and summer) (Gómez et al., 2007). In the laboratory, eggs were kept under constant aeration, in reconstituted hard water (HW; US EPA, 2002). The chemical characteristics of the HW were monitored regularly. The recorded values ranged as follows: hardness (160–180 mg L^{-1} $CaCO_3$); total alkalinity (110–120 mg L^{-1} $CaCO_3$) and pH (7.6–8).

The assays were semi-static, with media renewal every 24 hours, and conducted following the protocol for Fish Early-Life Stage Toxicity Test of the Ecological Effects Test Guidelines (US EPA, 1996). Ten eggs were randomly placed in glass flasks containing 150 ml of antifungal solutions. Tests were carried out at constant photoperiod (12 D: 12 L) and temperature (17 ± 2°C). This temperature range has been indicated as the optimum for the incubation of *O. bonariensis* eggs (Gómez et al., 2007; Berasain et al., 2008). The endpoint was the mortality (absence of heartbeat, blood circulation and absence of reaction to external stimuli); it was checked daily during 96 hours. An additional check was performed at 168 hours.

The toxicity assays were conducted using malachite green, formalin and NaCl dissolved in HW; formalin was 37% aqueous solution of formaldehyde containing 12% methanol. After preliminary explorative tests, the selected concentration ranges for tests were the following: malachite green, 0.375–3.0 mg L^{-1}; formalin, 150–1600 µl L^{-1} and NaCl, 7.5–40.0 g L^{-1}. All chemicals were analytical grade. Malachite green was used as a reference toxic. The number of the assayed concentrations of each substance oscillated between six and seven, plus a control (in HW) without chemicals. All the concentrations

were assayed in triplicate, using 210–240 eggs in each assay. Four assays were conducted for each substance and were combined to determine the toxicity parameters. Mortality in controls ranged between 8% and 10%; during the assays eggs did not show signs of fungal infection.

Mean lethal concentration (96 h LC_{50}) values were calculated using the Trimmed Spearman-Karber (TSK) Program Version 1.5 (US EPA, 2002). The No-Effect Concentration (NEC) (Péry et al., 2002; Jager et al., 2006) was estimated using the DEBtox Program. The No-Observed-Effect-Concentration (NOEC) was calculated using the Dunnett's test, and when the data did not fit a normal distribution, Steel's Many-one Rank Test was used. The LC_{50} and NOEC values were expressed as mg L^{-1} or µl L^{-1} ± 95% confidence intervals. Hatching rates were determined in eggs that survived toxicity tests, relative to the initial number of individuals; eggs were kept in each medium and under the same experimental conditions for an additional 72-hour period. At that time (168 hours), the number of living hatched larvae was recorded. The hatching rate in each medium was expressed as percentage with respect to the total initial number of individuals, i.e.

$$\text{Hatching rate (\%)} = [N_e/N_i] \times 100,$$

where N_i = initial number of eggs and N_e = number of living hatched larvae after 168 hours of incubation.

3 Results and discussion

Table 1 shows the toxicological parameters (LC_{50}, NOEC and NEC) determined for each tested substance. The results revealed that malachite green was the chemical with the highest toxicity, while NaCl the one with the lowest.

Table 1 Median lethal concentrations (96 h LC_{50}), No-Observed-Effect Concentrations (NOEC) and No-Effect Concentrations (NEC) of three fungicides to eggs of *Odontesthes bonariensis*. Data are means ±95% confidence limits (between brackets). The F/MG ratio was calculated considering the concentration of formaldehyde (mg L^{-1}) in the formalin solutions used in the assays

Chemical	*96 h LC_{50}*	*NOEC*	*NEC*	*LC_{50}/NOEC*
Malachite green (MG) mg L^{-1}	1.51 [1.41–1.62]	1.50	0.507 [0.4–0.6]	1.01
Formalin (F) µl L^{-1}	652.14 [613.66–693.05]	400.00	530.00 [486.10–555.90]	1.63
Sodium chloride (NaCl) mg L^{-1}	34,820 [36,260–33,450]	30,000.00	28,680 [27,100–29,700]	1.16
F/MG	140.62	266.67		
NaCl/MG	23,059.60	20,000.00		

Table 2 shows the effects of fungicides on the hatching rate as percentage relative to the number of eggs at the beginning of treatments, after 168 hours of exposure to them.

Table 2 Hatching rate (HR) of eggs of *Odontesthes bonariensis* after 168 hours of incubation in MG, F and NaCl solutions at different concentrations (C). Data as percentages (means) of hatched eggs, relative to the initial number

Chemical		*Assay concentrations (C) and hatching rates (HR)*							
Malachite green (MG)	C (mg L^{-1})	0	0.375	0.5	0.75	1	2	2.5	3
	HR (%)	76.7	73.3	60.0	66.7	28.1	6.7	0	0
Formalin (F)	C (µl L^{-1})	0	150	300	400	600	800	1200	1600
	HR (%)	87.8	91.9	60.0	11.9	3.3	1.1	0	0
Sodium chloride (NaCl)	C (g L^{-1})	0	7.5	10	15	20	25	30	35
	HR (%)	76.7	96.7	83.3	89.7	83.9	3.3	0	0

The presence of fungus in freshwater may adversely affect fish and eggs, both in natural environments and culture systems. The use of chemical treatments may be necessary to improve hatching success, especially in intensive aquaculture operations; these have been used extensively in the control or prevention of parasites (Srivastava et al., 2004), bacterial diseases and fungal infections that may affect fish. Several agents have been tested to evaluate fungicide action on pathogenic aquatic species (Marking et al., 1994; Schreier et al., 1996; Rach et al., 1997a; de Pedro et al., 2007; Nakamura et al., 2008). Malachite green, formalin, hydrogen peroxide, potassium permanganate, acetic acid and iodine can be mentioned among the most commonly used; malachite green is considered the most effective but it is no longer used because of its adverse side effects.

Those chemicals were shown to be useful fungicides, but treatment protocols have not been well defined. In general, the tests proposed for determination of treatment efficiency during the initial developmental stages of fish are conducted by pulses, exposing the eggs to different chemicals, either in a unique or in several separated applications for short periods of time (15–60 minutes) and in relatively high concentrations that might be lethal if the duration of the treatments is longer (Barnes and Soupir, 2007). Thus, it is imperative to design a precise protocol to apply the chemicals both at safe concentrations and during specific exposure times.

To our knowledge no other studies have examined comparatively the effects of the assayed fungicides on eggs of *O. bonariensis*. Owing to the lack of accurate information regarding the safe concentrations of fungicides that should be used for embryonic and early larval stages of the pejerrey, the aim of this study was to determine comparatively the safety margin for standard use of malachite green, formalin and NaCl, based on acute (LC_{50}) and sub-lethal (NOEC and NEC) toxicity parameters determined in standardised experimental conditions. Note that the tested egg stage is the most sensitive to fungal infections and handling (Liu et al., 1995; Rach et al., 1997b).

The LC_{50} value reveals limited information because it usually refers to toxic effects after a number of hours of exposure. In addition, it is influenced by several factors (exposure time, some characteristics of the tested species, temperature, properties of the toxicant, etc.). The NOEC is an index referred to particular impacts of toxics being the highest tested concentration that is not statistically significant difference from the reference or control treatment. On the other hand, the NEC represents the highest concentration of the chemical that does not cause any harmful effects (relative to the control) on the test organism; it is a biology-based risk assessment parameter that represents the concentration of a chemical that if inside the organism and that will not harm it, independently of the exposure time.

The results of 96 h LC_{50} show that MG was the most toxic for *O. bonariensis* eggs, while NaCl was tolerated up to comparable concentrations to those of the sea water salinity; formalin showed an intermediate toxicity. Then, on the basis of 96 h LC_{50} values, the tested substances can be ordered according to the following toxicity scale: MG > F > NaCl. When NOEC and NECs are considered, the ordering is the same. The 96 h LC_{50}/NOEC ratio can be adopted as indicative of the safety margin of each particular chemical. This ratio was 1.0, 1.63 and 1.16 for MG, formalin and NaCl, respectively.

In the three cases, the hatching rate was severely affected at values close to the LC_{50}s. It was completely inhibited after exposure to NaCl and an acute effect was recorded for formalin, with hatching rate reduced to 3.3%; in eggs incubated in MG the hatching rate was also reduced down to 28%. Concentrations within the confidence interval of the estimated NEC values also showed an adverse effect on the hatching rate. The hatching rate of eggs treated with MG at a concentration within the confidence interval of the NEC was 66.7%; for NaCl it was 83.9%, while it was considerably lower for F (11.9%).

It is important to mention that the results of the hatching rate are reliable since quality and sanitary condition of the initial material was carefully verified at the beginning of bioassays, reducing variability which was pointed out as limitative of the value of this parameter (Rach et al., 1997b).

The use of malachite green is currently limited to the treatment of species that are not consumed by humans because it has been demonstrated having adverse effects on some fish and mammals (Srivastava et al., 2004; Sudova et al., 2007; Sapkota et al., 2008). The 96 h LC_{50}, NOEC and NEC values of 1.51, 1.50 and 0.5 mg L^{-1} were determined by acute toxicity assays for malachite green; the hatching rate remained high up to 0.75 mg L^{-1}. These values are below the recommended dose range (between 2 and 5 mg L^{-1}) for the treatment of eggs by pulses of 15 minutes (Marking et al., 1994; Treves-Brown, 2000). All the determined toxicological parameters were lower than the LC_{50} (2.45 mg L^{-1}) reported by Shafiezade et al. (2006) for Persian sturgeon eggs.

From the obtained toxicological parameters we conclude that even at low doses, MG is more toxic than formalin and NaCl. Based on these results, the application of MG in concentrations lower than 0.5 mg L^{-1} should be recommended for prophylactic purposes without toxicological risk for the eggs. These recommendations are in agreement with those by Willoughby and Roberts (1992) who proposed that MG at 0.25 mg L^{-1} was effective in the elimination of spores of fungi of the genus *Saprolegnia*. However, other authors (Edgell et al., 1993; Marking et al., 1994) recommended higher concentrations to prevent infections by Saprolegniales.

Formalin is currently a registered fungicide approved by the US FDA for use in aquaculture to control of fungal infections fish eggs of salmonids and esocids. Recommended doses for the treatment of salmonid eggs oscillate between 250 µL L^{-1} and 1000 µL L^{-1} with a 15-minute exposure period (Schreier et al., 1996; Treves-Brown, 2000; Rach et al., 2005; Gieseker et al., 2006). Formalin concentration of 1000 mg L^{-1} was fungicidal but a concentration of 250 mg L^{-1} might have a fungistatic effect, being the efficacy progressively increased after longer exposures (Marking et al., 1994). Our 96 h LC_{50} (652.14 µL L^{-1}), NEC (530 µL L^{-1}) and NOEC (400 µL L^{-1}) values are within the recommended range for their application as fungicide in other species (Soupir and Barnes, 2006); those concentrations of F are equivalent to 241.3 mg L^{-1} and 196.1 mg L^{-1} of formaldehyde, respectively. These results agree with those reported by Khodabandeh and Abtahi (2006) who found that optimal concentration to control fungal infection in *Cyprinus carpio* eggs was 400 mg L^{-1}.

However, we found that the hatching rate was considerably reduced in eggs exposed to concentrations higher than 300 µl L^{-1}; these results differ from those reported by Liu et al. (1995) in eggs of Chinese sucker *Myxocyprinus asiaticus*, in different developmental stages, treated with 50–500 µl L^{-1}; in their case the hatching rates were not affected. The LC_{50} of formalin to Persian sturgeon eggs reported by Shafiezade et al. (2006) was higher than the one obtained in our assays (1900 mg L^{-1}). Rach et al. (1997a) found a significant difference in species sensitivity to formalin.

NaCl is widely used in aquaculture as a therapeutic chemical to control fungal and bacterial diseases, protect the osmotic balance or mitigate the toxic effect of ammonia and nitrite in environment (Marking et al., 1994; Treves-Brown, 2000). The NaCl 96 h LC_{50} has been found to be 34.82 g L^{-1}. It is interesting to note that this value is similar to the salt concentration in sea water; since *O. bonariensis* is a euryhaline species the determined LC_{50} would indicate that an early development in media with salinities higher than fresh water might be tolerated with little impact on the egg survival, assuring their further development. The values obtained to NOEC and NEC (30.00 – 28.68 g L^{-1}) were very close to the 96 h LC_{50}, the hatching percentage decreased in concentrations higher than 20 g L^{-1}. The NEC, NOEC and the hatching rate were within the concentration range recommended by other authors for application in treatment by pulses (20.00–30.00 g L^{-1}) (Marking et al., 1994; Kitancharoen et al., 1997; Treves-Brown, 2000). Khodabandeh and Abtahi (2006) obtained a high hatching rate (85.4%) in eggs of *C. carpio* treated whit 35000 mg L^{-1}, this concentration also resulted effective to control fungal infection. However, contrasting opinions exist on NaCl efficiency in controlling fungal infections, since sensitivity of fungi to NaCl is variable (Schreier et al., 1996; Kitancharoen et al., 1997; Pacheco-Marino et al., 2008).

The obtained results showed that the availability of precise toxicity data is a useful tool, since the effects of chemical agents applied at the early stages of their development in certain species cannot always be safely extrapolated to other species. Moreover, toxicity and therapeutic efficacy of a waterborne chemical may change not only according to the egg species, stage and quality, but also to water-quality parameters (hardness, pH and temperature) which can, in turn, affect the toxicity or fungicide efficiency or of a particular chemical (Rach et al., 1997b; Barnes et al., 2004). In our case, the daily renewal of the incubation media contributes to maintain a constant chemical environment along the assays.

Our results also indicated that lower doses than those applied to salmonids should be used to control and/or treat fungal infections in silverside's eggs. As Marking et al. (1994) stated, the use of lower treatment concentrations in prophylactic treatments is more practical, it could reduce operational costs and decrease discharge levels of chemical substances capable of altering the environment (see Medina and Ramos-Jiliberto, 2009), and reduce the probability of producing sub-lethal effects in the development of the treated species.

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