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A New Developmental Toxicity Test for Pelagic Fish Using Anchoveta (*Engraulis ringens* J.)

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Abstract A series of six 96-h static bioassays were performed to validate the use of anchoveta (*Engraulis ringens*) embryos as test organisms for ecotoxicological studies. The standardization protocol utilized potassium dichromate ($K_2Cr_2O_7$) as a reference toxicant and egg mortality as the endpoint. The results indicated that the mean sensitivity of anchoveta embryos to potassium dichromate was 156.1 mg L^{-1} (range: 131–185 mg L^{-1}). The statistical data analysis showed high homogeneity in LC50 values among bioassays (variation coefficient = 11.02%). These results demonstrated that the protocol and handling procedures implemented for the anchoveta embryo bioassays comply with international standards for intra-laboratory precision. After secondary treatment, an effluent from a modern Kraft pulp mill was tested for *E. ringens* embryo toxicity, finding no significant differences from the controls.

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Waste water processing has evolved, along with the development of nations, as a feature of urban infrastructure designed to reduce the risk of pollution from sewage discharged into the environment. As a concomitant alternative to discharging waste directly into the sea at the shoreline or through a tributary emptying into the sea, sewage pipes have been extended far offshore, thereby avoiding the sanitary deterioration of costal waters. Based on the dilution paradigm, underwater marine emissaries may carry partially treated or raw waste water into the ocean. Studies of the impact that potential pollutants in the effluents have on local pelagic species are scarce.

The need to implement toxicity bioassays to protect early life stages of aquatic organisms has encouraged the development of bioassays using gametes, embryos, and larval stages as test organisms. For instance, various invertebrates in their early stages have been considered in routine bioassays with sediment and water samples (da Cruz et al. 2007; Losso et al. 2007). However, the use of marine fish species in bioassays remains scarce, especially in countries where ecotoxicology is still a young science. In Chile, incipient attempts to rear marine fish species in captivity have focused on high-priced big fishes, not precisely a desirable feature for laboratory animals; to date, only flatfish have given definitive results (Silva 2001). Only one marine fish, the newly hatched larvae of *Odontesthes regia laticlavia* (Valenciennes), has been proposed as a test organism in this country. Its eggs are collected from alga prairies between December and April (Silva et al. 2001) and, during the rest of the year, no fish bioassay is available. For practical purposes, test species for

ecotoxicological studies should be abundant, widely distributed, ecologically relevant, and experimentally manageable under laboratory conditions (Raisuddin et al. 2007). The anchoveta, *Engraulis ringens* Jenyns (Clupeiformes, Engraulidae), is a coastal pelagic species found from northern Peru (4°30′ S) to southern Chile (42°30′ S) (Serra et al. 1979). During the peak spawning season in its austral distribution zone, the mean egg volume is 0.31 mm^3 and embryonic development concludes with the hatching of labile larvae that are 2.7 mm long (Llanos-Rivera and Castro 2006). An important anchoveta spawning area is located in south-central Chile, with high egg concentrations (11–100 eggs 0.05 m^{-2}, Cubillos et al. 2007) in winter and early spring, precisely when *O. regia* eggs are not found. A new offshore Kraft wood pulp mill emissary is under construction in this area, raising considerable social concern as to the fate of the anchoveta spawning ground. Accordingly, the aim of this study is to validate and standardize the use of anchoveta embryos as a test organism for ecotoxicological evaluations in the future monitoring of the quality of the receiving waters.

Materials and Methods

In 2006–2007, we carried out a series of short cruises to the coastal area off Talcahuano (36°32′ S, 72°56′ W) in late winter-early spring, the peak spawning season for anchoveta. Gentle oblique tows were performed at 1.5 knots (conical net, 300 μm mesh) to collect zooplankton. The organisms were quickly deposited in black plastic buckets (30 L) with seawater from the same sampling area and kept in closed coolers to maintain the field temperature until arriving at the coastal laboratory of Universidad de Concepción. The anchoveta eggs were separated from the plankton under a stereomicroscope less than 2 h after collection time and the staging followed Moser and Ahlstrom's (1985) descriptions for *Engraulis mordax*. The eggs in early developmental stages (phase III, Fig. 1) were utilized as test organisms in bioassays with a reference toxicant (potassium dichromate).

Two preliminary bioassays were done to determine the range of toxicant concentrations to be used and to define further experimental conditions. The highest concentration tested should result in an effect of 100% whereas the lowest should preferably have no observable effect or be close to the control value. The transparent anchoveta chorion facilitates non-invasive observations of the embryo throughout development. Given the daily egg development observations and preliminary results, we opted for a static bioassay to be conducted over 96 h; egg mortality was taken to be the endpoint. The duration of the bioassay (96 h) was fixed considering that, at 12°C, the entire embryonic development of the anchoveta occurs in 80 h (Tarifeño et al. 2007).

Test solutions of potassium dichromate ($K_2Cr_2O_7$, Riedel-de-Haën, p.a. 99.8%) at selected concentrations (300, 180, 108, 64.8, 38.9 mg L^{-1}) were prepared by dilution (factor 0.6) of a stock solution in seawater (500 mg L^{-1}). The test chambers utilized in the bioassays

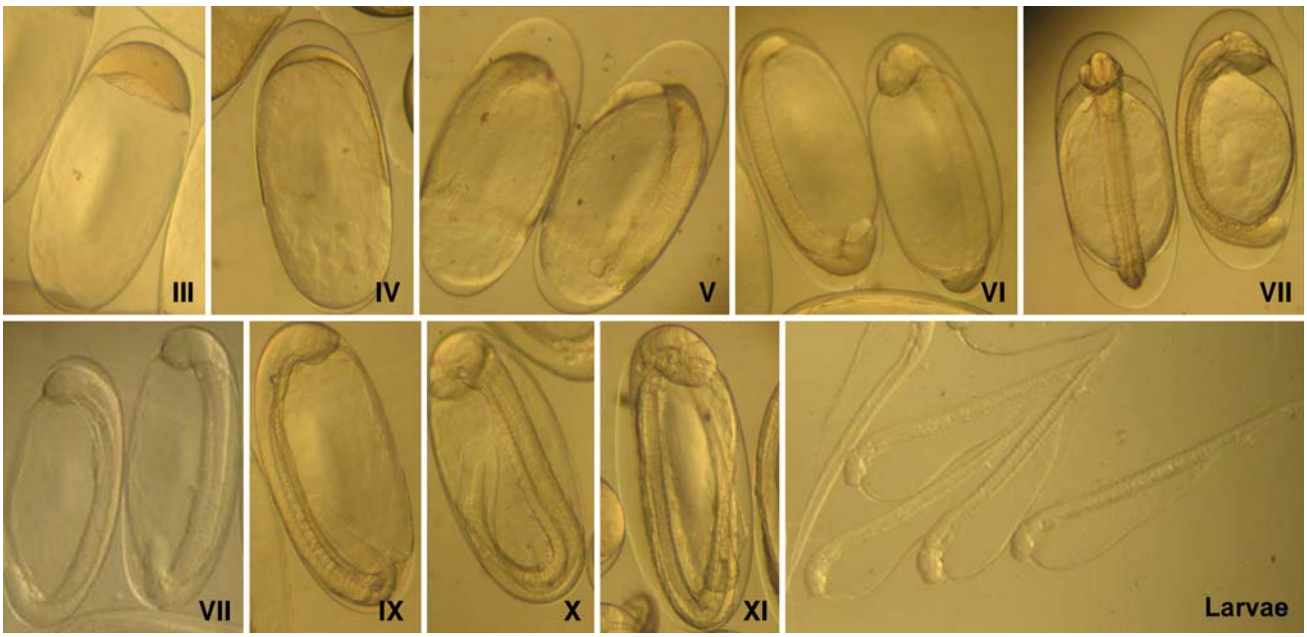

Fig. 1 *Engraulis ringens* normal embryonic development. The stages correspond to Moser and Ahlstrom's (1985) staging for *Engraulis mordax* embryos

were sets of 6-well plates, each well having an individual capacity of 10 mL. Each toxicant concentration and the sea water control had three replicates (one 6-well plate per replicate). For control and dilution water, we used seawater (0.5 μm filtered, UV-sterilized, 34 psu, pH 8.05, previously oxygen-saturated by air bubbling) obtained from the experimental hatchery of the Universidad de Concepción at Coliumo Bay (36°32′ S, 72°56′ W), an area free of industrial discharges. The water quality for the bioassay was checked by running a parallel test for dichromate lethal toxicity to *Artemia franciscana* nauplius in a 24 h exposure, comparing LC50 values from the natural sea water (56.1 mg L^{-1}) and a synthetic sea water (Instant Ocean) prepared at the same salinity (62.1 mg L^{-1}). The difference (9.7%) was smaller than the variation obtained from successive tests of potassium dichromate toxicity for *Artemia* in natural sea water (35%). Five anchoveta eggs were transferred to each well with freshly prepared test solutions (5 eggs × 6 wells = 30 eggs per replicate, 90 eggs per concentration). The test plates were placed in temperature-controlled baths (12°C) and held under a 12 h light:12 h dark photoperiod. The placement of the eggs in the different test chambers and their position in the bath were randomly assigned without regard to concentration or replicate sequences. Six trials were conducted from October 2006 to January 2007 following this protocol.

At hatching time, all rearing containers were checked and the number of dead embryos registered (non-translucent, compressed, and with agglutinated yolk). The data were expressed as egg mortality and were analyzed using the PROBIT parametrical statistic (US EPA 2002a). This method provides LC50 values and their 95% confidence intervals. When the data did not conform to the assumptions of the PROBIT model, the Spearman–Karber method was used. Intra-laboratory precision was evaluated through the coefficient of variation (CV %) according to the US EPA (2002a). A control chart was prepared to evaluate the cumulative trend of bioassay results from six trials (US EPA 2002a).

For additional test validation, on 11 December 2007, a bioassay was performed with an effluent from a Kraft wood pulp mill (CFI-Nueva Aldea). The secondary treatment effluent was sampled the day before and was kept in the dark at 7°C. As with many industrial and sewage treatment effluents, this was a freshwater discharge requiring a salinity adjustment. The effluent stock solution salinity was set at 34 psu using hypersaline brine made of evaporated natural seawater (US EPA 2002b). After this procedure, the bioassay was implemented by preparing a dilution set (0.5 dilution factor; 77, 38.5, 19.2, 9.6, and 4.8%) using seawater of the same source and characteristics as mentioned before.

The experimental design was completed with two control groups. The first consisted of pure seawater and the second of freshwater taken upriver of the intake pipe for the wood pulp plant, with the salinity adjusted as described above. Additionally, two extreme potassium dichromate

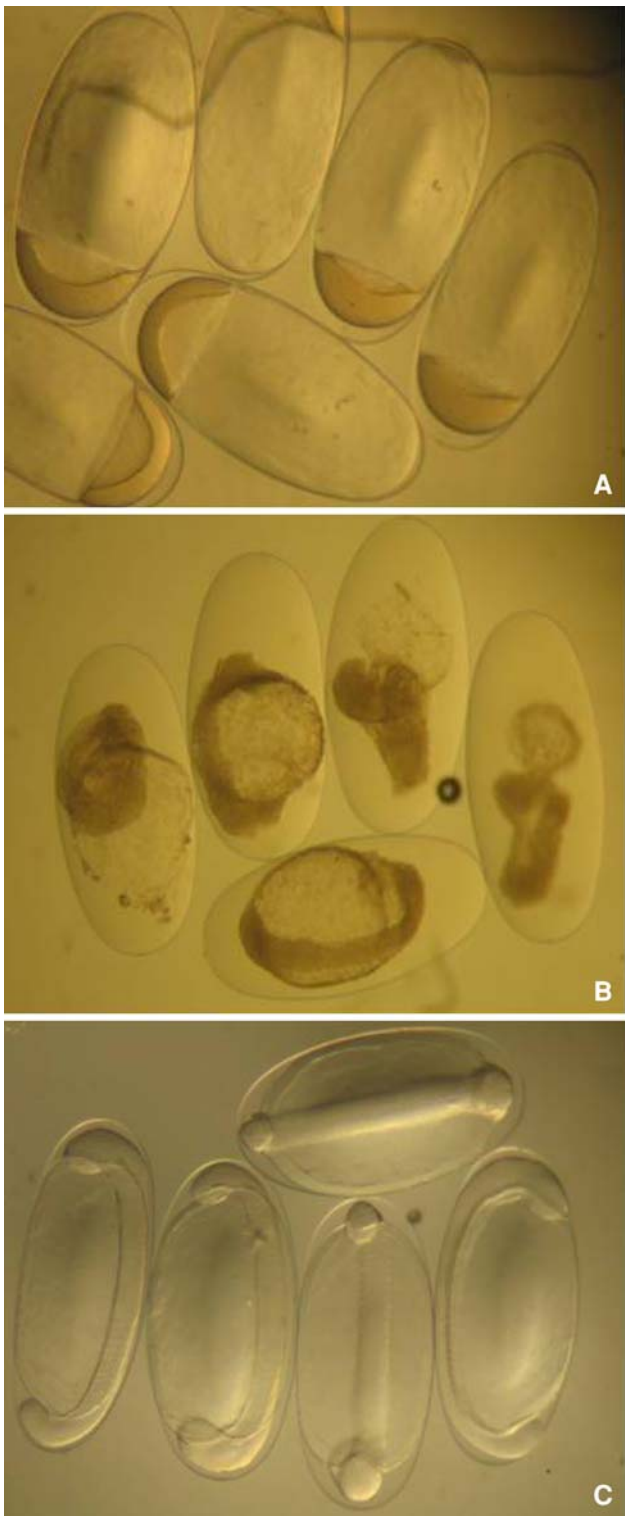

Fig. 2 *Engraulis ringens* embryos in static 96 h exposure standardization bioassays with potassium dichromate ($K_2Cr_2O_7$) as a reference toxicant. **a** Embryo at the beginning of the test. **b** Dead embryo at stage VII. **c** Live embryo at stage VII in control plates

concentrations (300 mg L^{-1} and 38.9 mg L^{-1}) were included to check embryonic sensitivity to the reference toxicant. The data were expressed as egg mortality and analyzed with the software Toxstat V3.4 (West Inc. and Gulley 1994) after checking them for a normal distribution (Chi square test) and homogeneity of variance (Bartlett test). ANOVA was used to compare egg mortality between the effluent and controls.

Results and Discussion

The experiments were initiated with stage III anchoveta eggs according to the Moser and Ahlstrom (1985) staging key for *Engraulis mordax* eggs (Fig. 1). This stage depicts the beginning of gastrulation and is characterized by the appearance of the blastodisc as tissue and the yolk mass divided into translucent granules (Fig. 2a). In the plates with higher concentrations of potassium dichromate, development stopped at stages VI or VII and egg coagulation was observed (Fig. 2b). This developmental stage was characterized by the closure of the blastopore and the clear differentiation of the embryonic axis (Fig. 2c).

In the chambers with the lower concentrations or without the reference toxicant (control), egg development progressed normally until hatching. In the different bioassays, the hatching success of anchoveta eggs in the controls was close to 80% (Table 1), higher than the minimum control value (60%) suggested for bioassays with the European species *Clupea harengus* and *Gadus morhua*. Thus, the controls in the bioassays exceeded the overall survival acceptability standard for other recommended species used in embryo or sac-fry toxicity tests (OECD 1998).

Our results indicate that the 96-h LC50 of potassium dichromate to anchoveta embryos is 156.09 mg L^{-1} (range: 131–185 mg L^{-1}). This value is higher than that determined for the same reference toxicant in early larvae of other marine fishes such as *Odontesthes regia* (LC50 = 88.18 mg L^{-1}) (Silva et al. 2001). The current literature recognizes that the chorion protects the embryo from external conditions (Kunz 2004) but, as our results show, it is not an impermeable barrier. This statement agrees with our preliminary determination of potassium dichromate lethality on anchoveta yolk-sac larvae, which averaged 28.61 mg L^{-1} (range: 21.86–37.45 mg L^{-1}). Nonetheless, the yolk-sac stage of anchoveta is so labile that it is not recommended for ecotoxicological studies; the organism handling required for these studies causes considerable mortality. Therefore, we choose to work with embryo development, which is more reliable and sensitive in ecotoxicity bioassays monitoring the quality of effluents and sea water.

The statistical analysis of the data obtained from the bioassays showed high homogeneity in LC50 values among bioassays (CV = 11.02; Table 1). Another major step in the method for species standardization is the evaluation of intra-laboratory precision. This aspect was considered in the control chart of laboratory performance (Fig. 3) and consisted of estimating the upper and lower control limits (±2 SD) for the cumulative mean of successive LC50 values. The chart defines a range within which the results of future bioassays using the same reference toxicant (potassium dichromate) should fit. For anchoveta embryos, this range goes from 95.5 mg L^{-1} to 197.5 mg L^{-1}.

The toxicity assessment of the CFI-Nueva Aldea pulp mill effluent using the anchoveta embryo test did not reveal significant differences between the effluent and controls in the number of dead eggs after 96 h of exposure ($F_{(6,\ 14)} = 0.56$, $p > 0.05$, Fig. 4). Mean mortality was 16.7% (±10.4) for the controls and 18.9% (±3.8) for the salinity-adjusted effluent. The mortality response to two simultaneous reference toxicant concentrations was 100% at 300 mg L^{-1} and 11.7% at 38.9 mg L^{-1} of potassium dichromate in sea water. Thus, this effluent lacks lethal toxicity for anchoveta eggs given short-term exposure, even when this is long enough to cover the entire embryonic development.

Table 1 Date, lethal concentrations (96 h – LC50), confidence intervals (CI 95%), and control larval survival (mean ± SD) in the six bioassays carried out with *Engraulis ringens* embryos using potassium dichromate ($K_2Cr_2O_7$) as a reference toxicant

Bioassay number	Date dd–mm–yy	96 h – LC50	CI 95%	Survival of control larvae (%)
1	03–10–06	145.42	128.00–158.46	68.7 (±6.7)
2	24–10–06	150.30	120.31–163.79	76.7 (±12.0)
3	31–10–06	181.04	169.44–193.43	93.3 (±6.7)
4	21–11–06	135.05	120.34–147.73	78.9 (±5.1)
5	13–12–06	152.68	138.89–165.42	88.0 (±13.8)
6	10–01–07	172.07	160.85–184.07	87.8 (±10.2)
Mean		156.09		82.3
SD		17.20		9.07
CV		11.02		

SD standard deviation, *CV* coefficient of variation (%)

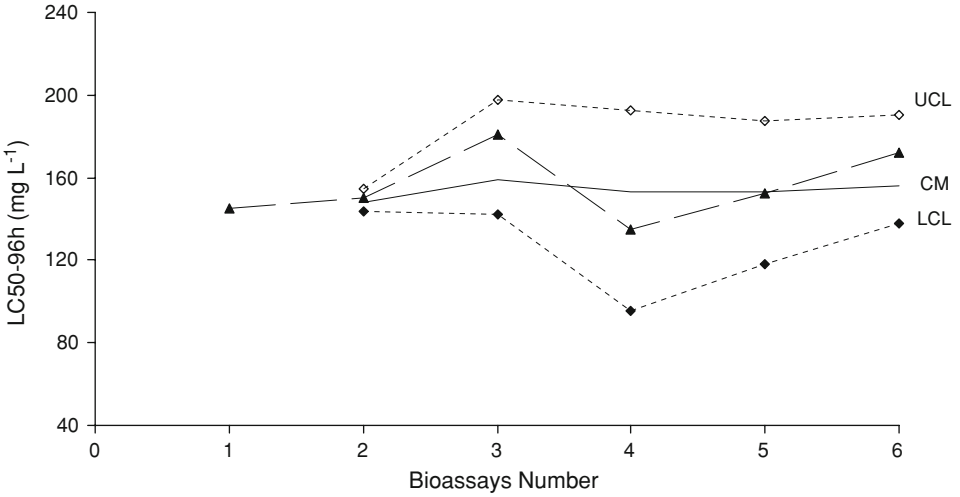

Fig. 3 Control chart for potassium dichromate ($K_2Cr_2O_7$) as a reference toxicant in acute toxicity bioassays with *Engraulis ringens* embryos. The solid line shows the LC50 cumulative mean (CM) with each successive test. The triangles represent specific LC50 values. The unfilled and filled diamonds correspond to the upper and lower control limits (UCL, LCL), respectively

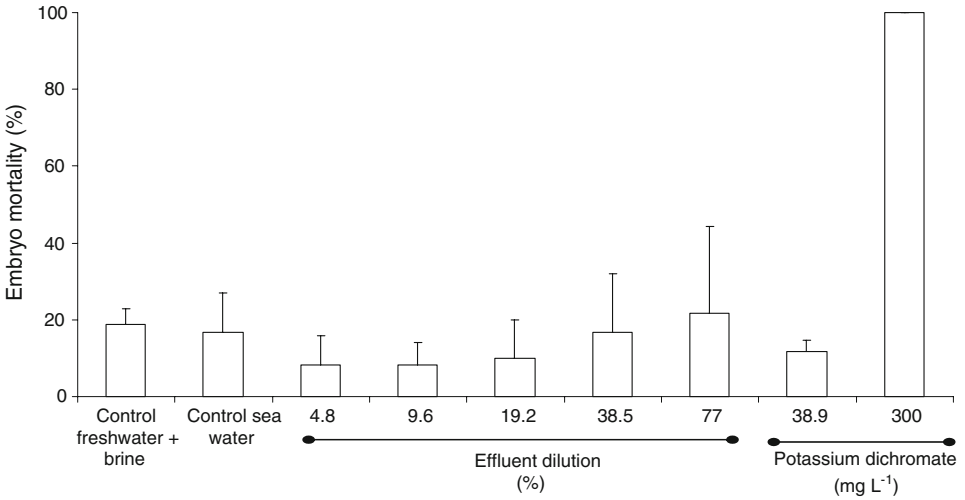

Fig. 4 *Engraulis ringens* embryo mortality in a 96 h static effluent dilution experiment. The bars show the means for the replicates and the lines indicate +1 SD

In summary, our results show that anchoveta *Engraulis ringens* eggs are appropriate for ecotoxicological studies. This species spawns during most of the year, peaking in winter and early spring when abundant eggs are found along the coast. The species is widely distributed, ecologically and commercially relevant, and its eggs are experimentally manageable under laboratory conditions. Another advantage is the transparent chorion that facilitates non-invasive observations of the embryo throughout development. Its high economic importance as a fishery resource makes anchoveta all the more interesting for these types of ecotoxicological studies since the capability to detect potential environmental constraints on the normal early development of the species provides environmental regulators with a tool to protect the resource when such risk is present in waste water discharges.

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