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Analysis of Individual Versus Group Behavior of Zebrafish: a Model Using pH Sublethal Effects

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Abstract An image analysis biomonitoring system was used to evaluate sublethal effects of pH on the mean swimming velocity of zebrafish. Responses to stress were tested comparing individual and group responses. Group analysis indicated no effect for all acid pH and for pH 9.0–9.5. Individual analysis indicated behavioral differences for most acid pH and higher than 9.5. Sensitivity to sublethal pH was best assessed when using individual analysis. Zebrafish decreased hyperactivity and increased hypoactivity with more acid or alkaline pH. Individual approach allowed to determine hyperactivity or hypoactivity and the species' thresholds of exposure, which is critical for the management of impairments.

Keywords Swimming activity · Biosensor · Hypoactivity · Hyperactivity

Automated biomonitoring systems have been developed worldwide because they provide rapid detection of impairment when compared to acute tests (lethal concentrations after a 24–96 h exposure), chronic tests (e.g. long-term responses in growth and reproduction) or biomarkers (which is invasive and require long-time exposure) (Little and Finger 1990). Such systems are powerful tools to analyze behavior alterations of organisms as indicators of toxicity. Among the many behavioral responses of fish used as an indicator of impacts, the use of locomotion is based on its objectivity and quantitative nature, and because it can be measured in many fish species to characterize the consequences of sublethal exposure. Swimming activity is possibly the most suitable measure of swimming behavior for routine use in such studies because it is highly sensitive, appropriate for numerous aquatic species, relevant to survival and easy to measure (Little and Finger 1990). Therefore, it is a good indicator of fish general health. Swimming responses have been used in automated biomonitoring systems because of their consistent sensitivity to numerous contaminants (Kane et al. 2004; Smith and Bailey 1988). Most studies that deal with fish swimming activity use group means before and after exposure to stressor as endpoints, even if swimming activity was recorded individually (Hopkins et al. 2003; Wicks et al. 2002).

In this study we tested two approaches for analyzing data of swimming activity of zebrafish *Danio rerio* using an images analysis biomonitoring system (IABS)—one based on individual responses, on a fish-by-fish basis, before and after the exposure; and other using mean values of exposed and unexposed groups—and we discuss their power to detect behavior changes. To test these approaches we altered water pH to sublethal concentrations of Sulfuric acid (H_2SO_4) and sodium hydroxide (NaOH). We chose pH as a stressor because its effects on fish and on the environment are widely known. Acid waters affect fish by reducing oxygen transport capacity (Randall and Brauner 1991) and may damage chemosensory epithelia (Daye and Garside 1980), suppressing or modifying the orientation to chemosensory cues (Smith et al. 2008). Chronic exposal to

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alkaline water may affect both gas transport and muscle contractility (Randall and Brauner 1991) and may cause immediate and drastic inhibition of ammonia excretion with a subsequent increase in ammonia in the plasma which can be lethal (Wicks et al. 2002).

Materials and Methods

Adult male and female Zebrafish 120 days old were maintained in laboratory for 1 week for acclimation prior the experiments. Fish were fed with Spirulina Flakes 200 (Alcon Gold) once a day and kept in filtered dechlorinated tap water (water temperature 23–26°C, room temperature 22–24°C, hardness 170 a 240 mg/L $CaCO_3$, pH 6.5–7.0) in a closed flow-through system (water flow to filtering tank and return to tanks). Fish were not fed for 24 h before and during the tests. Zebrafish responses were evaluated two-fold: first, acute toxicity tests were performed to determine 24 h-LC_{50} (median lethal concentration for 24 h exposure) of NaOH (sodium hydroxide P.A. Vetec brand) and H_2SO_4 (Sulfuric Acid P.A. 98 % pro-analysis brand) then, behavioral responses of *D. rerio* were used to evaluate sublethal effects. Acute toxicity tests followed Brazilian regulations for *D. rerio* (ABNT NBR 15088 2004). A range-finding test was performed and the 24 h acute toxicity test was performed to determine the LC_{50} to NaOH and H_2SO_4 using the following exposure concentrations: 16.3×10^{-4}, 17.5×10^{-4}, 18.8×10^{-4}, 20.0×10^{-4} and 21.3×10^{-4} mol/L for NaOH and 3.57×10^{-4}, 3.94×10^{-4}, 4.32×10^{-4}, 4.69×10^{-4} and 5.63×10^{-4} mol/L for H_2SO_4. The pH for each concentration was 10.8, 11.0, 11.2, 11.3, 11.4 for NaOH and 4.3, 3.5, 3.2, 3.1, 3.0 for H_2SO_4. Filtered dechlorinated tap water was used as dilution water and for control group. Three replicates of ten individuals were used at each concentration and control. The exposures were conducted in 5,000 mL beakers. A Trimmed-Spearman-Karber test was used to determine the 24 h-LC_{50} values. During the tests, room temperature remained constant (25°C) with photoperiod 12/12 h light/dark.

To evaluate the behavioral responses in swimming, an image analysis biomonitoring system (IABS) was used (Magalhães et al. 2007). A digital camera captured images and they were analyzed with a Videomex-V (columbus instruments) as white pixels over a black background using the software traveled distance of multiple objects (TDMO) to register the mean swimming velocity of each fish. A microcomputer with data storage software was used to record data in Excel spreadsheets. The recording cabin was made of acrylate (36 × 36 × 45 cm) and held the analogical video camera with an exhaustion system to remove volatile chemicals during the experiments. Attached to the chamber, two funnels connected to silicon tubes were used for introduction of chemicals or water to the treated- and control-chambers. An illuminating box above the holding boxes provided a shadowless diffuse soft lightning. An opaque glass aquarium (35 × 35 × 25 cm, 30 L capacity) was divided in two independent compartments, one for the control and one for the treated group, and inside each compartment, an opaque acrylate box with 3 mm holes of was divided into four holding boxes (9.5 × 5 × 2 cm), submerged 2.5 cm, where fish were kept individually. Both compartments were equipped with submerged water pumps for water mixing.

Two groups (control and treatment) with four fish each were monitored for swimming mean velocity with the IABS, simultaneously. Concentrations of 10 %, 30 %, 50 % and 70 % of 24 h-LC_{50} of NaOH and H_2SO_4 were chosen based on extent of sublethal pH alteration. Therefore, these were ideal concentrations for our study. Three tests for each concentration were performed, totaling 24 fish (12 control, 12 treatment) per concentration. Tests were performed for 5 h, with 1 h acclimatization (no data was recorded), 2 h with fish exposed only to water (tap filtered water, pH 6.5–7.0, hardness 30–40 mg/L $CaCO_3$) and 2 h after introduction of chemical in the treatment compartment. In the control compartment the same volume of water was added to simulate stress. The duration of experiment was based on Van Der Schalie et al. (2001) which stated that fish responses frequently occur within an hour to toxicants concentrations near the 96 h-LC_{50} for several chemicals. Ten minutes after introduction of the chemical, time necessary to mix, water samples were taken from each compartment and analyzed for Dissolved Oxygen and pH. The same was done after the experiment end (Table 1). Swimming mean velocity was registered every 5 min, totaling 48 intervals. All tests were performed at approximately the same time of the day, during the morning period.

Behavioral responses were evaluated in two ways: (1) group analysis—each group was used as its own control, a method commonly used and recommended by ASTM (2003) for automatic systems. We evaluated the differences of the mean of each 12 fish swimming mean velocity before and after the introduction of the chemical (*t* test for dependent samples). The control group was used to validate the experiment and to evaluate the stress caused by the introduction of water. (2) Individual analysis—each fish was used as its own control, analyzing the 24 data intervals before and after exposure (*t* test for independent samples). We considered a response when >50 % of the fish altered behavior significantly (seven or more fish out of the 12). Each fish was then classified as hyperactive (increased swimming velocity), hypoactive (decreased swimming velocity) or no-response (*t* test, $p > 0.05$). We classified

Table 1 Water physical and chemical parameters collected at control and treated groups, at the end of each experiment

Water samples	10 % 24 h-LC_{50}		30 % 24 h-LC_{50}		50 % 24 h-LC_{50}		70 % 24 h-LC_{50}	
	DO	pH	DO	pH	DO	pH	DO	pH
Sulfuric Acid								
Dilution	6.6–6.8	6.5–7.1	6.1–6.5	6.8–6.9	6.1–6.3	6.6–7.1	5.6–6.6	6.2–7.0
Control (final)	5.4–5.8	6.6–6.9	5.8–6.8	6.6–6.8	5.3–6.0	6.5–6.9	5.1–5.9	6.4–6.6
Treatment (final)	5.4–5.7	6.3–6.5	5.3–6.0	5.4–5.8	5.4–5.8	3.3–3.4	5.3–6.1	2.9–3.0
Sodium hydroxide								
Dilution	6.0–6.4	7.0–7.2	5.2–6.3	6.8–6.9	5.1–6.7	6.5–7.2	5.5–6.7	6.0–7.3
Control (final)	5.7–6.1	7.0–7.1	5.2–6.7	6.6–6.6	5.1–6.3	6.6–7.8	5.2–6.4	5.6–7.4
Treatment (final)	5.4–6.2	8.6–8.7	5.1–7.4	9.2–9.4	5.2–5.8	9.5–10.0	5.3–5.8	10.0–10.5

DO dissolved oxygen (mg/L)

zebrafish swimming behavior as toxic response if ≥50 % of the fish were hypoactive; as low observed effect level (LOEL) if ≥50 % altered behavior but <50 % were hypoactive, and as no observed effect level (NOEL) if <50 % of the fish showed some response.

Results and Discussion

Acute toxicity tests indicated *Danio rerio* 24 h-LC_{50} of 4.46×10^{-4} (pH 3.0–3.2; CI 95 % = 4.39–4.54 $\times 10^{-4}$) H_2SO_4 mol/L and 16.5×10^{-4} (pH 10.5–11.0; CI 95 % = 15.95–17.13 $\times 10^{-4}$) NaOH mol/L. These concentrations were used to calculate 10 %, 30 %, 50 % and 70 % of the LC_{50} (0.45×10^{-4}; 1.34×10^{-4}; 2.23×10^{-4} and 3.12×10^{-4} mol/L for H_2SO_4 (pH of 6.5, 5.5, 3.5 and 3, respectively), and 1.6×10^{-4}; 5.0×10^{-4}; 8.3×10^{-4}; 11.55×10^{-4} mol/L for NaOH (pH of 9, 9.5, 10 and 10.5, respectively). No record was found in the literature on the 24 h-LC_{50} for *D. rerio* exposed to these chemicals. All concentrations used in the behavior study were sublethal (lower than the lowest concentration which caused mortality in the acute test). For all tests, no significant differences ($p > 0.05$) were detected in control groups before and after introduction of water in the system.

The two approaches for analyzing the swimming activity indicated contrasting results. Sensitivity of *D. rerio* to sublethal pH values was best assessed when using individual analysis, on a fish-by-fish basis, than using group analysis, based on mean values of the group before and after exposure. When using group analysis no significant difference in swimming activity of *D. rerio* was found for all acid pH (Fig. 1). On the other hand, individual analysis indicated behavioral differences for most acid pH (30 %, 50 % and 70 % of H_2SO_4 24 h-LC_{50}) revealing a clear tendency of *D. rerio* to decrease hyperactivity (33 %, 33 %, 17 % and 9 % of the fish), and increase hypoactivity (9 %, 42 %, 58 %, 58 % of the fish) with increasing concentrations of H^+ ions (10 %, 30 %, 50 % and 70 % H_2SO_4 24 h-LC_{50} respectively). Fig. 2 show example of 30 % H_2SO_4 24 h-LC_{50}. Similar tests were performed for other concentrations. For alkaline pH, group analysis indicated no significant difference at pH 9.0–9.5 (10 and 30 % of NaOH 24 h-LC_{50}), and significant hypoactivity at higher pH values (Fig. 1). Individual analysis also indicated no significant response at pH 9.0 (10 % of NaOH 24 h-LC_{50}), but detected significant behavioral changes at pH 9.5 and higher (30 %, 50 % and 70 % of NaOH 24 h-LC_{50}, revealing a clear tendency of *D. rerio* to decrease hyperactivity (9 %, 9 %, 0 % and 0 % of the fish), and increase hypoactivity (25 %, 58 %, 83 %, 58 % of the fish) with increasing concentrations of OH^- ions (10 %, 30 %, 50 % and 70 % H_2SO_4 24 h-LC_{50} respectively.

We found that using individuals as their own control before and after exposure was the best way to consider individual behavior variability in a population of fish. Group analysis may be less powerful to detect impairment, because mean numbers may mask the response to impairment if hyper- and hypoactivity are found in individuals of the same group. In Fig. 2 we show a clear example on how variable the swimming behavior of individual fish can be (comparing fish on control phase, before the experiment) and how individuals may respond differently to stress—some increasing swimming activity while others decreasing, and thus masking group response. Other approaches were tested to assess individual analysis—e.g. using swimming repeatability performance (Kolok et al. 1998; Kolok 2001) or using individual fish as its own control (Kane et al. 2004)—but they did not consider hypo- and hyper-activity as a response to the stress. In our study using individual analysis we were able to determine the "direction" of the response (if hyper- or hypoactivity) and that provided information on species' thresholds of exposure.

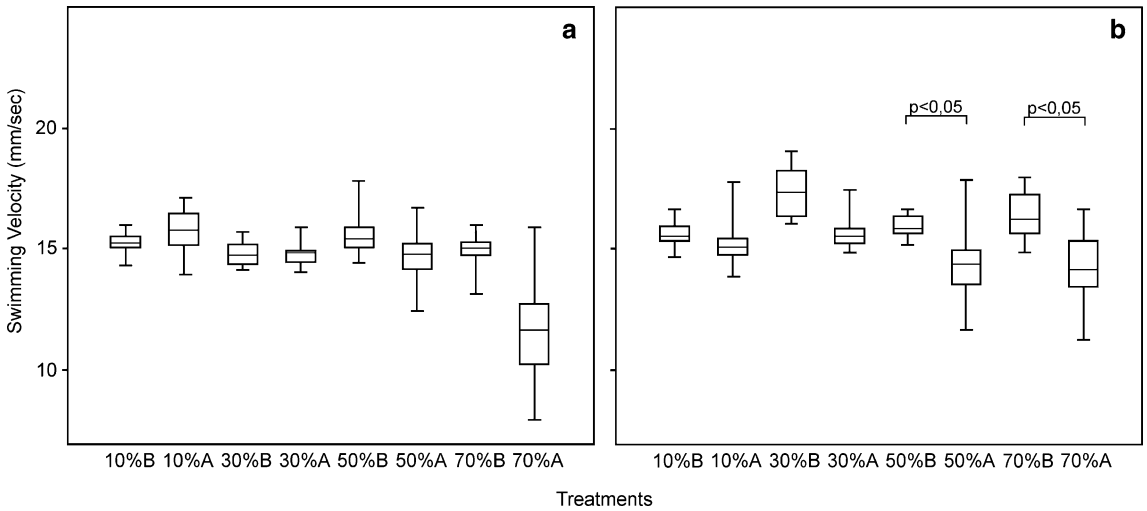

Fig. 1 Mean swimming velocity of zebrafish group at 10 %, 30 %, 50 % and 70 % of 24 h-LC_{50} of H_2SO_4 (**a**) NaOH (**b**) before (B) and after (A) the introduction of the chemical. In the Box-and-Whisker plots, *center lines* represent the median, *boxes* represent the 25–75 % quartiles, *bars* represent the maximum and minimum numbers. Significant *p* levels of *t* tests are indicated

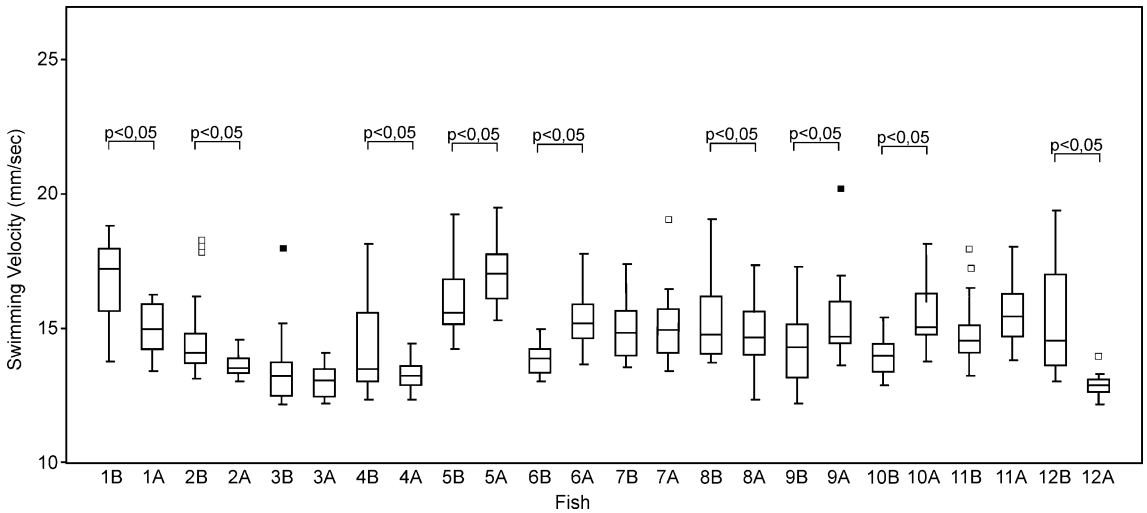

Fig. 2 Example of individual mean swimming velocity test. Twelve fish were exposed to 30 % 24 h-LC_{50} of H_2SO_4. "B" and "A" represent, respectively, before and after the introduction of the chemical. Based on significant *p* levels of *t* tests (indicated in *graph*), four fish were hyperactive (33 % of total), five fish were hypoactive (42 % of total) and three fish showed no statistical response (25 %; $p > 0.05$). *White squares* represent outlier values, and *black squares* represent extreme values

According to our classification system pH 6.5 e 9.0 were classified as NOEL (>50 % of the fish were not significantly responsive), pH 5.5 as LOEL (≥50 % were responsive but <50 % showed hypoactivity) and pH 3.0–3.5 and 9.5–10.5 were toxic (≥50 % of the fish showed hypoactivity). The rationale is that more resistant individuals are unresponsive to exposure, those with intermediate resistance respond hyperactively and more sensitive fish respond hypoactive when exposed to sublethal concentrations. Hyperactivity typically characterizes an escape response, where the organism tries to avoid the area impacted by the chemical (Smith and Bailey 1988). This behavior signal the threshold tolerance for a pollutant, i.e., from this point, higher concentrations would probably be toxic to the test organism (Ellgaard et al. 1978). The decrease in swimming velocity is an avoidance behavior, designed to lessen the probability of death or the metabolic costs incurred in maintaining physiological homeostasis (Schreck et al. 1997). Decreasing the physiological activities may be an efficient strategy if exposure is temporary, but reduced activity for a long period of exposure may impair performance of fish (e.g. reduction in feeding and mating) and/or lead to a metabolic collapse (Baganz 2005). Because swimming performance is central to many aspects

of fish biology, reduced performance following exposure to adverse situations may have important implications for inter- and intraspecific interactions, and even reduce fitness of affected individuals (Hopkins et al. 2003).

In conclusion, the individual analysis approach was more powerful to detect sublethal behavioral alterations on swimming activity of zebrafish than the group means comparison approach. Also, it allowed to determine thresholds of exposure for the species, which is critical information for the management of impairments.

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