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Video-tracking of zebrafish (*Danio rerio*) as a biological early warning system using two distinct artificial neural networks: Probabilistic neural network (PNN) and self-organizing map (SOM)☆

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ABSTRACT

Biological early warning systems (BEWS) are becoming very important tools in ecotoxicological studies because they can detect changes in the behavior of organisms exposed to toxic substances. In this work, a video tracking system was fully developed to detect the presence of commercial bleach (NaOCl) in water in three different concentrations (0.0005%; 0.0010% and 0.0020% (v/v)) during one hour of exposure. Zebrafish was selected as the test organism because it is widely used in many different areas and studies. Two distinct statistical models were developed, using probabilistic neural network (PNN) and correspondence analysis associated with self-organizing map (SOM–CA). The diagnosis was based only in the analysis of a few behavioral components of the fish, namely: mean angular velocity, mean linear velocity, spatial dispersion, mean value of the *X* coordinate and mean value of the *Y* coordinate. Both models showed good results in their diagnosis capabilities. However, the overall performance (accuracy) was always superior in the PNN model. The worst result was with the SOM–CA model, at the lowest concentration (0.0005% v/v), achieving only 65% of correct diagnosis. The best result was with the PNN model, at the highest concentration (0.0020% v/v), achieving 94% of correct diagnosis.

1. Introduction

Digital image processing has been effectively used for behavioral tracking in ecotoxicological risk assessment since the 1990s (Liu et al., 2011b). Recently, several computational analyses have been proposed, including artificial neural networks such as multilayer perceptron (Kwak et al., 2002) and self-organizing map (SOM) (Chon et al., 2004; Park et al., 2005).

Artificial neural networks (ANNs) have been widely applied in interpreting complex and nonlinear phenomena in many areas. There are several features of ANNs that make them valuable and attractive for prediction. Firstly, they are nonparametric data-driven self-adaptive methods and, as a result, incorporate few a priori assumptions. They are able to learn from examples and respond to subtle functional relationships within the data, even when the underlying relationships are unknown or difficult to describe (Zhang et al., 1998). Another important feature of ANNs is that they can generalize. After "learning" the data presented to them, they can often infer or predict an event or occurrence, even if the sample input data is "noisy" (Zhang et al., 1998). Moreover, they are usually more flexible than traditional statistical methods and are able to improve their performance gradually with the aforementioned learning processes (Zhang et al., 1998).

Nevertheless, the high potential of ANNs has not been fully explored. To date, there is no known works that directly use them to detect the presence of toxicants in the water through fish behavior analysis. Some works use them to identify (Kim et al., 2006; Kwak et al., 2002) or classify (Park et al., 2005) movement patterns based on variables that describe the movement of fish, or to define these same variables (Liu et al., 2011a). None of the previous works tried to apply the system in the detection of toxic substances in the water.

Currently, many commercial biological early warning systems (BEWS) are available for evaluating chemical toxicity using laboratory-based tests, portable devices and on-line biomonitors. These systems can use different types of organisms, including bacteria, algae, invertebrates and fish (Bae and Park, 2014). Fish were the sentinel organisms originally selected for BEWS, and they continue to be a popular choice (van der Schalie et al., 2001), mainly

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because they have ecologically important behaviors that are easily observed and quantified in controlled environment (Scott and Sloman, 2004).

Among the many fish species already tested, the zebrafish (*Danio rerio*; Hamilton, 1822), a tropical freshwater fish belonging to the minnow family (Cyprinidae) of order Cypriniformes, native to the streams of the southeastern Himalayan region (Mayden et al., 2007), and a standard species in aquatic toxicology (Chen et al., 2011), presents several advantages. Behavior analysis of zebrafish through video tracking-based automated quantification methods has shown, in several studies, that it can be appropriate for the use in the detection of disturbances in aquatic environment and in the triage of toxicants and drugs (Berghmans et al., 2007; Gerlai et al., 2008, 2006; Spieser et al., 1996). Zebrafish can be effectively used for video tracking because they are very active and move almost continuously during the light period (Grillitsch et al., 1999). Also, comparing to other fish species used in BEWS, such as trout or minnows (Kokkali and van Delft, 2014), they are smaller and easily maintained in laboratory conditions and manipulated during the experimental procedures. From an experimental point of view they represent important savings, both in terms of time and monetary resources, since they have a rapid reproductive cycle with a large number of offspring and require less water to maintain and test than, e.g., rainbow trout.

Sodium hypochlorite (NaOCl) has been used since the 17th century. Nowadays, NaOCl is one of the most widely used chemicals. It is primarily used for water disinfection and as a bleaching agent for paper and clothes (Nimkerdphol and Nakagawa, 2008). NaOCl is not harmful to humans when used at low concentrations (<10%). At higher concentrations (>10%) it can cause respiratory disorders, skin irritation or skin sensitization, abdominal pain, burning sensation, cough, sore throat, diarrhea, and vomiting (Nimkerdphol and Nakagawa, 2008). It has a high potential risk to the aquatic environment because it is used indiscriminately and its discharges are scarcely controlled (Magalhaes et al., 2007).

The detection of early warning signals through behavioral changes of sentinel organisms is ecologically relevant, economic and faster than chemical detection (Gerhardt, 2000). In general, behavioral changes induced by exposure to toxicants are subtle, but can be detected at concentrations much lower than those required to cause permanent or irreversible damage to the organism. In the literature, several studies using zebrafish support this idea for different types of toxicants, such as ethanol (Carvan et al., 2004; Chen et al., 2011); methylmercury (Samson et al., 2001); dimethylsulfoxide (Chen et al., 2011); 2,2′,4,4′-tetrabromodiphenylether (Chou et al., 2010) or NaOCl (Magalhaes et al., 2007; Nimkerdphol and Nakagawa, 2008).

The lowest behaviorally effective toxicant concentration that induces changes in swimming behavior of fish ranges normally from 0.1 to 5.0% of the LC_{50} (Little and Finger, 1990). Behavioral changes commonly occur 75% earlier than the onset of mortality (Kane et al., 2005). Likewise, alterations in swimming speed have been detected at toxic compound concentrations as low as less than 0.1% of the LC_{50} values for *Artemia* sp. and *Brachionus plicatilis* (Garaventa et al., 2010). The objective of this study was to use ANNs, considered as a universal regressor, to detect disturbances in the aquatic environment. The presence of commercial bleach in highly dilute solutions was analysed through the study of zebrafish behavior in laboratory. The concentrations corresponded to 0.5%, 1.0% and 2.0% of the 24h-LC_{50} values for adult zebrafish (Magalhaes et al., 2007). Behavioral responses of zebrafish exposed to sublethal concentrations of sodium hypochlorite using an image analysis biomonitoring system were also evaluated in at least two other works (Magalhaes et al., 2007; Nimkerdphol and Nakagawa, 2008). However, they did not use neural networks, and only used analysis of variance and/or linear regression analysis. In one case (Magalhaes et al., 2007), the tested concentrations (10–40% of LC_{50}) were 5–20 times higher than those use in the present work (0.5–2.0% of LC_{50}).

2. Material and methods

2.1. Test organism: zebrafish – origin, age, size, maintenance

This work used wild-type adult *D. rerio* with about 3 months of age and 2.5–3.0 cm in length from the same batch (ORNI-EX, Lda, Arcozelo, Vila Nova de Gaia, Portugal).

The fish were acclimated for one month prior to any test. They were fed once a day with Tetra Min Bio Active Formula and kept in a photoperiod of 12 h of light/12 h of dark. Mortality was insignificant and the animals were used exclusively in the definitive trials. They were not fed for 24 h prior to the tests.

2.2. Recording areas

There were two separate recording areas (Fig. 1a). Each recording area had one water bath and one aquarium that was internally divided into four equal sections (the four arenas represented one

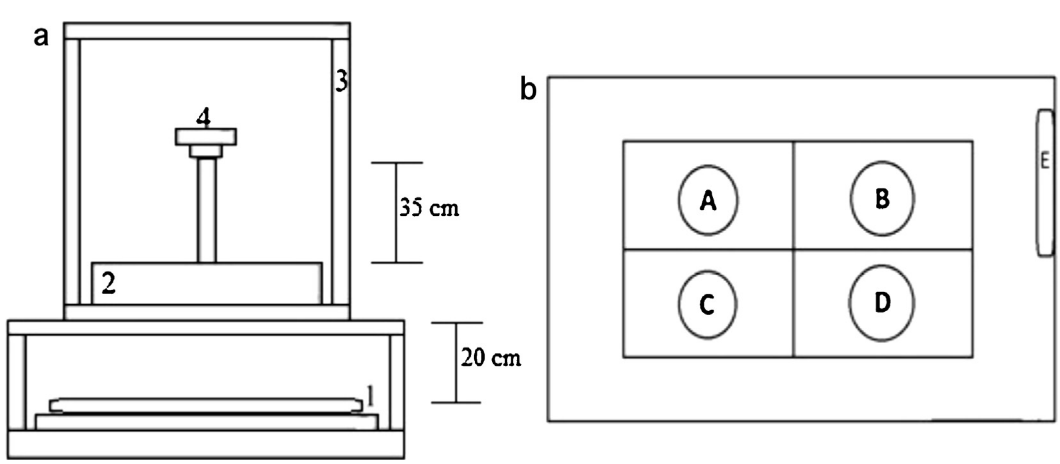

Fig. 1. (a) Schematic representation of the recording areas. 1 – Illumination (5 fluorescent lamps of 60 Watts (50 cm in length and 3 cm diameter)); 2 – Water bath and arenas (equipped with a Tetratec HT50 electrical resistance to maintain the water temperature of 28 °C); 3 – Sound insulation plates of 5 cm thick polystyrene foam, forming a compartment 41 cm wide and 39 cm high; 4 – Velleman Camset W7 camera. (b) Schematic representations of the water bath and arenas (top view). A–D – individual arenas; E – Tetratec HT50 electrical resistance. All the fish were recorded simultaneously.