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A sensitive biomarker for the detection of aquatic contamination based on behavioral assays using zebrafish larvae

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ABSTRACT

An effective biological early warning system for the detection of water contamination should employ undemanding species that rapidly react to the presence of contaminants in their environment. The demonstrated reaction should be comprehensible and unambiguously evidential of the contamination event. This study utilized 96 h post fertilization zebrafish larvae and tested their behavioral response to acute exposure to low concentrations of cadmium chloride ($CdCl_2$) (5.0, 2.5, 1.25, 0.625 mg/L) and permethrin (0.05, 0.029, 0.017, 0.01 μg/L). We hypothesize that the number of larvae that show advanced trajectories in a group corresponds with water contamination, as the latter triggers avoidance behavior in the organisms. The proportion of advanced trajectories in the control and treated groups during the first minute of darkness was designated as a segregation parameter. It was parametrized and a threshold value was set using one $CdCl_2$ trial and then applied to the remaining $CdCl_2$ and permethrin replicates. For all cases, the method allowed distinguishing between the control and treated groups within two cycles of light: dark. The calculated parameter was statistically significantly different between the treated and control groups, except for the lowest $CdCl_2$ concentration (0.625 mg/L) in one replicate. This proof-of-concept study shows the potential of the proposed methodology for utilization as part of a multi-species biomonitoring system.

1. Introduction

Biological early warning systems (BEWS) detect the presence of toxic compounds by continuously tracking the physiologic reaction of organisms (Van der Schalie et al., 2001). They allow for the management of drinking water and environmental water quality by detecting sudden increases of the concentration of pollutants (Kramer and Botterweg, 1991) and have been implemented in water quality monitoring in many facilities all over the world. Biomonitoring can provide more reliable predictions about substance toxicity than the chemical concentration alone, especially in case of mixtures of substances (Netto, 2010). This allows the detection of potentially adverse biological effects in cases where traditional detection methods are failed or too costly (Mikol et al., 2007).

Though biological monitors have been employed for water quality assessment for many years, the shift toward prevalent practice has occurred just in recent years and sprouts out in utilization of a wide range of organisms, as well as significant technological advancement (Storey et al., 2011). Decreased costs and increased availability of high-precision video-tracking devices and compatible software for immediate data evaluation contribute to enhancing behavioral tests as the current benchmark in water quality biomonitoring. Behavioral responses are easily observable, perceptible by recording equipment, and faster in comparison to mortality studies. Furthermore, changes in behavior can occur through different modes of action (e.g., avoidance, repellency, neurotoxicity) and thus also cause a multitude of different reactions. Nowadays evaluation software can distinguish between several of these behavioral components. Hence, fingerprints of the impact on behavior could be derived, which would provide initial identification of the stressors. When standardized, criteria for behavioral changes are more sensitive and more rapid indicators of chemically induced stress than morphological criteria

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traditionally used in ecotoxicological tests (Michels et al., 2000).

As an example for existing technology, the Daphnia Toximeter™ is a highly sensitive biological system that utilizes the freshwater cladocera *Daphnia magna* for early detection of potentially dangerous unknown substances (Jeon et al., 2008). The known drawback of the system is its inability to be used for chlorinated water, as the species is sensitive to chlorine. Moreover, in some cases the system is prone to false positive alarms due to its high sensitivity. Another example is the ToxProtect™, a monitor that can be equipped with different species of fish and that detects toxins in water by online analysis of the swimming activity of up to 20 individuals across an array of 80 photoelectric light diode barriers. It is claimed to be qualified for rapid contaminant detection, being sensitive to low concentrations for some contaminants. Nevertheless, it is incapable of detecting considerably high levels of fluoroacetate (Storey et al., 2011). In addition, the limitations of the system are its maintenance time and size required to house fish stocks.

Recently, Bae and Park (2014) conducted a review of biological early warning systems that utilize aquatic organisms. They list wide variety of organisms from various trophic levels that have been used for BEWS applications, including bacteria, algae, daphnia, macroinvertebrates, and fish. No less sophisticated are the methods used to analyze the acquired data. However, non-linearity of behaviors, variation in individual behavior, and ambiguity in interpretation complicate the analysis of the large amounts of data obtained from continuous monitoring. The authors conclude that despite advantages of behavioral monitoring, challenges remain in quantifying and interpreting the data.

In ecotoxicology, genetics, developmental biology, neurophysiology, and biomedicine the zebrafish is one of the most important vertebrate model organisms, and the species gain increasing popularity in behavioral studies (Miklósi and Andrew, 2006; Spence et al., 2008; Lovato et al., 2016). During recent years, automated imaging techniques allow for an advanced analysis of behavioral responses and for the detection of very subtle changes in behavior (Creton, 2009). The approach enables the detection and quantification of more than 20 behavioral endpoints, from traveled distance and top duration to erratic movements and not moving frequency (Stewart et al., 2013). de Paiva Magalhães et al. (2007) showed that behavioral responses of adult zebrafish to exposure to sodium hypochlorite could be detected in an image analysis biosensor system based on swimming activity change. The authors report that swimming activity was readily perceptible and efficient to identify toxic effects of the tested compound and thus recommend incorporation of the method in biomonitoring protocols in Brazil. The study of Huang et al. (2014) showed that zebrafish activity allowed the detection of 1% of the $LC_{50-24\ h}$ (the concentration at which 50% of the population dies within 24 h) for deltamethrin within five hours and thereby proving behavioral changes of zebrafish to be a sensitive method for the detection of certain contaminants. Consequently, zebrafish seem to have a set of qualities necessary to be employed in BEWS. As maintenance of adult zebrafish may cause additional tangible costs and is restricted by the Animal Protection Law, researchers show a special interest in studying zebrafish embryos and larvae. Up to 120 h post fertilization these developmental stages are not protected as vertebrate animals according to EU legislation (European Commission, 1986; European Commission, 2010). In ecotoxicology, the embryos and larvae of zebrafish are frequently used for the study of teratogenic effects of single substances and environmental samples (Strähle et al., 2012; Wernersson et al., 2015). The fish embryo toxicity (FET) test using zebrafish embryos has already replaced the acute fish test in whole effluent testing in Germany since 2005 and is also proposed for the testing of chemicals (Lammer et al., 2009).

Lower values of the lowest observed effect concentrations (LOEC) and no observed effect concentration (NOEC) for behavioral responses have been reported for toxicants compared to those obtained in tests observing teratogenic effects (Legradi et al., 2014). Early developmental stages of zebrafish are sensitive towards toxic substances, and behavior is a sensitive bioindicator. Hence, the system is a promising tool to detect toxic substances rapidly and in low concentration ranges (Huang et al., 2010). Even during the first week of development, zebrafish larvae are capable of escape and avoidance behaviors induced by tactile, acoustic and visual stimuli, and in response to exposure to various chemical compounds (Colwill and Creton, 2011(aa). The zebrafish larvae behavioral assay was first introduced by Emran et al. (2007). They demonstrated a sudden spike in subjects' movement in response to change in illumination from light to infrared. In further studies, it has been shown that acute exposure of zebrafish larvae to toxicants in low concentration ranges is detectable via their locomotor activity (Kienle et al., 2008; MacPhail et al., 2009; Irons et al., 2010). Patterns in zebrafish larvae behavior in response to chemicals were even linked to certain compounds or groups of compounds (Rihel et al., 2010; Reif et al., 2015). Schnorr et al. (2012) used a sudden light-to-darkness transition to measure the level of anxiety, defined as the percentage of distance moved in the peripheral zone of the well. This parameter was significantly decreased by an anxiolytic drug, and significantly increased by caffeine. The results of these studies suggest that using zebrafish larvae behavior could be a promising system for implementation in BEWS. At the same time, it has to be noted that used test methodologies differ greatly depending on the scope of the corresponding study, and so far, no standardized approach exists for the evaluation and statistical analysis of zebrafish larvae behavior data (de Esch et al., 2012; Legradi et al., 2014). It is recommended to researchers in this field to share the obtained raw data to enable the development of a comprehensive analysis approach.

The present study focuses on behavioral responses as indicators of acute toxic stress in zebrafish larvae, in particular locomotor behaviors during light/dark transition test, and aims to designate an easily calculated determinative parameter that allows distinguishing between treated and non-treated subjects. Here, we utilized 4 dpf old larvae in order to determine whether the larvae's behavior in the light/dark transition test is stable enough to determine statistically significant differences between the treatment groups. Using 4 dpf larvae in a real world application would allow using an animal test free method as a sensitive sensor for vertebrate toxicity. This setup requires the replacement of test organisms in short intervals, which would prevent the adaption of test organisms to toxic substances in low concentrations. Avoidance behavior in larvae is already observable and well characterized (Miklósi and Andrew, 2006; Schnörr et al., 2012). In a well plate with circular wells, avoidance behavior results in the circular swimming activity of larvae close to the border of the well. The hypothesis is that the number of larvae that show these directed movements (i.e., advanced trajectories) in a group corresponds with water contamination, as the latter triggers avoidance behavior in the exposed organisms. Here, we present a parameter that allows for the distinction between larvae's overall movement and their directed movement that we propose as a more refined endpoint for the detection of organism stress after acute exposure to toxicants. The strategic goal of the current study is to advance toward a framework that utilizes zebrafish larvae in real-time biological early warning systems.