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Evaluation Over Time of the Detection Capability of a Video-tracking System Through Daily Exposure of *Danio rerio* to Sodium Hypochlorite, Ethanol or Bisphenol A.

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DISSERTAÇÃO DE MESTRADO APRESENTADA

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Evaluation Over Time of the Detection Capability of a Video-tracking System Through Daily Exposure of *Danio rerio* to Sodium Hypochlorite, Ethanol or Bisphenol A.

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Abstract

Due to the increase of contamination sources worldwide the protection of the natural ecosystems has become a necessity and simultaneously an enormous challenge. It is necessary to develop new, faster and more efficient methods of detecting contamination. In several studies, behavior has proven to be a sensible endpoint which could be used to detect sub-lethal exposures. In a previous work was developed a video-tracking system using zebrafish locomotors behavioral analysis, to detect a sub-lethal concentration (9% 96h LC_{50}) of sodium hypochlorite (SH). The aim of this work was to use this video-tracking system to determine whether the detection capability does not deteriorate after successive exposures of the zebrafish to ethanol, sodium hypochlorite (SH) or bisphenol A (BPA). Three similar video-tracking systems were conceived to record the movement of the zebrafish. In each system four experimental conditions (control, exposure to ethanol, BPA, or SH) were tested at the same time. Fish were exposed once a day for 9 consecutive days to these toxicants for 1h30m, but only the second half hour of each day was used in the analysis. One assay was performed and later was repeated a second time with new fish. In the end the zebrafish locomotor behavior was transformed into XY coordinates and 9 movement descriptors were calculated. A cluster analysis was conducted using Artificial Neural Networks (ANNs), of the type Kohonen to define different behavior categories of the fish submitted to the different experimental conditions, with the information about the movement descriptors. Several correspondence analysis were performed to obtain a measure of the effect caused by the toxicants, that then was analyzed in each day, by linear and orthogonal multiple regression models.

The Presence/Absence model analyzed if the behavior of the fish was related with the presence/absence of the respective toxicant in the water. The Moment/Toxicant model allowed analyzing the progress of the behavior response, before and after adding the toxicants in the toxicant experimental units. This model was used to analyze the progress of the behavior response, in the moments before and after for the control experimental units. The Moment/Control model indicated that the behavior of the control fish was not influenced by the practical procedure, which means that the behavior changes

detected were only related with the toxicants. The Presence/Absence model indicated that the system was able to successfully detect the three toxicants. With ethanol the detection capability was maintained, but in the case of the SH and BPA a deterioration of the detection capability over the days occurred. The Moment/Toxicant model revealed that all of the toxicants influenced the behavior, but for SH, and BPA a decrease in the amplitude between the Moments Before and After of the behavior effect over the days was detected. This response may be due to the induction of detoxification mechanisms, and biochemical changes that lead to a decreased effect of the toxicants in behavior, or due to the accumulation of adverse effects caused by the repeated exposure to the toxicants. In order to prevent the loss of detection capability some procedures can be adopted such as the regular exchange of fish. In the case of ethanol, the system was resistant to the repeated exposures.

Through the ANNs, the correspondence analysis as well as the linear and orthogonal regressions it was possible to use the zebrafish behavior changes induced by the toxicants as a way to detect them, and it was also possible to evaluate the exposure conditions to which the fish were subjected. This study shows that the system was capable of detecting changes in fish behavior exposed to small concentrations, which indicates that it can be an important tool for early warning detections of contamination, it can help understand ecological consequences of exposure and have the potential to be integrated in ecotoxicological studies.

Resumo

Devido ao aumento das fontes de contaminação um pouco por todo o mundo, a proteção dos ecossistemas naturais tornou-se uma necessidade e ao mesmo tempo um enorme desafio. É necessário desenvolver novos métodos, mais rápidos e eficientes de deteção de contaminação. Em diversos artigos, o comportamento provou ser um parâmetro sensível, e que poderia ser usado para detetar exposições sub-letais. Num trabalho anterior foi desenvolvido um sistema de vídeo-rastreio utilizando a análise do comportamento locomotor do peixe-zebra, para detetar uma concentração sub-letal de hipoclorito de sódio (9% 96h LC_{50}). O objetivo deste trabalho foi utilizar este sistema de vídeo-rastreio para determinar se a capacidade de deteção não se deteriora após exposições sucessivas do peixe-zebra a etanol, hipoclorito de sódio ou bisfenol A. Conceberam-se três sistemas de vídeo rastreio iguais, para registar a movimentação dos peixes-zebra, e testaram-se em cada sistema quatro condições experimentais ao mesmo tempo (controlo, exposição ao etanol, bisfenol A, ou hipoclorito de sódio). Os peixes foram expostos uma vez por dia durante 9 dias consecutivos a estes tóxicos durante 1h30m, mas apenas a segunda meia hora de cada dia foi utilizada na análise. Realizou-se um ensaio, que depois foi repetido uma segunda vez com peixes novos. No final as movimentações dos peixes-zebra foram transformadas em coordenadas XY e 9 componentes de comportamento foram determinados. Foi realizada uma *cluster analysis*, utilizando uma Rede Neuronal Artificial do tipo *Kohonen*, para definir diferentes categorias de comportamento dos peixes submetidos às diferentes condições experimentais, com as informações sobre os movimentos descritores. Foram realizadas várias Análises de Correspondência, para se obter uma medida do efeito causado pelas substâncias tóxicas, que, em seguida, foi analisada em cada dia, por modelos de regressão linear múltipla e ortogonal.

O modelo Presença/Ausência analisou se o comportamento do peixe estava relacionado com a presença/ausência da respetiva substância tóxica na água. O modelo Momento/Substância tóxica permitiu analisar a evolução da resposta comportamental, antes e depois da adição das substâncias tóxicas nas unidades experimentais substâncias tóxicas. O modelo Momento/Controlo foi utilizado para analisar o progresso da resposta comportamental, nos momentos antes e depois para as unidades experimentais controlo. Este modelo indicou que o comportamento dos peixes controlo não foi influenciado pelo procedimento

prático, o que significa que as mudanças de comportamento detetadas estavam apenas relacionadas com as substâncias tóxicas. O modelo Presença/Ausência indicou que o sistema foi capaz de detetar com sucesso as três substâncias tóxicas. Com o etanol a capacidade de deteção manteve-se, mas no caso do hipoclorito de sódio e do bisfenol A, ocorreu uma deterioração da capacidade de deteção ao longo dos dias. O modelo Momento/Tóxico revelou que todas as substâncias tóxicas influenciaram o comportamento, mas para o hipoclorito de sódio, e o BPA, foi detetada uma diminuição na amplitude entre o tempo Antes e Depois do efeito comportamental ao longo dos dias. Esta resposta pode ser devida à indução de mecanismos de desintoxicação e alterações bioquímicas, que levam a um efeito reduzido do tóxico no comportamento, ou devido à acumulação de efeitos adversos causados pela exposição repetida às substâncias tóxicas. De modo a impedir a perda de capacidade de deteção, alguns procedimentos podem ser adotados, tais como a troca periódica dos peixes. No caso do etanol, o sistema mostrou-se resistente às exposições repetidas.

Através das Redes Neuronais Artificias, da Análise de Correspondência e da regressão linear múltipla e ortogonal, foi possível usar as mudanças de comportamento do peixe-zebra induzidas pelas substâncias tóxicas como uma forma de detetá-las, e também foi possível avaliar as condições de exposição a que os peixes foram submetidos. Este estudo demostra que o sistema foi capaz de detetar alterações comportamentais nos peixes expostos a pequenas concentrações o que indica que pode ser uma ferramenta importante para deteções de alerta precoce de contaminação, pode ajudar a compreender as consequências ecológicas da exposição e tem o potencial para ser integrado em estudos ecotoxicológicos.

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List of abbreviations

AChE - Acetylcholinesterase

ADH - Alcohol dehydrogenase

ALDH - Aldehyde dehydrogenase

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANNs - Artificial Neural Networks

BEWSs - Biological early warning systems

BPA – Bisphenol A

DNA - Deoxyribonucleic acid

GABA - Gamma-AminoButyric acid

GST - Glutathion S-transferase

LOEC - Lowest Observed Effect Concentration

NMDA - N-Methyl-D-aspartate

NOEC - No Observed Effect Concentration

KARs - Kainate receptors

ROS - Reactive Oxygen Species

SOM - Self-organizing map

SH – Sodium Hypochlorite

1. Introduction

Water is a vital resource for all organisms, including humans. With the increase of human population, the pressure on the water resources has also increased due to industrial development, agriculture, and domestic uses thousands of chemical substances are continuously reaching and contaminating many water resources all over the world (Houtman, 2010). Degradation of water quality poses serious ecological problems and to protect not only human health but also natural ecosystems its necessary to determine the toxicity of chemical substances, and detect quickly situations of environmental contamination (Storey *et al.,* 2011).

Risk assessment evaluates the likelihood of adverse effects occurring in ecosystems and relates the disturbance with the magnitude of the impact (Wright and Welbourn, 2002). It has 4 steps: the first is the hazard identification, which aims to determine whether exposure to a chemical substance can cause adverse effects and characterize the strength of the evidence that can have this effect, the second is the dose-response evaluation which aims to determine the relation between the dose of exposure to a contaminant and the following effect, the third is the exposure assessment that analyzes the magnitude and duration of the exposure to the agent and the fourth is risk characterization that summarizes the information of the 3 previous steps and analyzes the relation between the dose and the probability of occurring the adverse effect. Risk characterization puts the assessment of risk in a form that is useful for the competent authorities responsible for the decisions (Wright and Welbourn, 2002). Risk management occurs after risk assessment, and the objective is to take action and minimize the risk and the costs (Wright and Welbourn, 2002).

Toxicology is the study of adverse effects of chemical substances on organisms (Chapman, 2002). Toxicity tests are used to determine the concentrations of a substance and the exposure time required to produce critical effects such as mortality, alterations in growth or reproduction (Wright and Welbourn, 2002). These tests can help to understand the mode of action and the physiological or other type of effects of the chemical substances on the organisms (Chapman, 2002).The majority of such tests are conducted under controlled conditions (temperature, water quality, pH) in the laboratory

(Chapman, 2002). Institutions such as the Organization for Economic Cooperation and Development, the International Standardization Organization, the United States Environmental Protection Agency and the American Society for Testing and Materials) described several standard toxicity tests (Befyaeva *et al.*, 2010). In this standard tests the exposure of organisms to the test solution can be accomplished by an static regime, where there isn't renewal of the solution, semi-static regime where the frequency of medium renewal normally depend on the stability of the test substance, or flow-through regime, which continually dispenses and dilutes a stock solution of the test substance (Wright and Welbourn, 2002). Toxicity tests can be acute or chronic. Acute tests are accomplished for relative short periods of time normally between 48h to 96h, and the acute toxicity testing is usually determined by the concentration that is lethal to 50% of the test organisms i.e. the median lethal concentration (LC_{50}) (Wright and Welbourn, 2002). These tests are more used because they are simple to execute and produce fast results (Magalhaes Dde *et al.*, 2007). Chronic tests are executed for longer periods of time, and the chronic toxicity is determined by the lowest concentration that caused a statistically significant effect observed (LOEC) in the organisms and also by the highest concentration that has no statistically significant effect observed in the organisms (NOEC) (Wright and Welbourn, 2002). Chronic tests are designed to detect mostly sub-lethal effects on growth and reproduction (Wright and Welbourn, 2002). However these types of studies are expensive and not practical, because they require a lot of work and time.

The toxicological tests can also be executed on the field, in this case they are called ecotoxicological tests and they allow a better comprehension of the effects of the chemical substances on the organisms because they are performed under natural exposure conditions (Chapman, 2002), however ecotoxicological test may also be developed in laboratory. Ecology studies the interactions between organisms and their environment. Ecotoxicology comprises the toxicology and ecology and is the study of the effects of toxic substances on live organisms, populations and communities inside defined ecosystems. The objective of ecotoxicology is to be capable of predicting the effects of toxic substances on natural communities under natural exposure conditions (Chapman, 2002).

In the environment it's often to occur constant exposure to sub-lethal concentrations (low concentrations) instead of acute toxicity to contaminants

(Houtman, 2010), so it's necessary to avoid the exposure of organisms and rapidly detect situations of contamination. Water monitoring is normally based on chemical analysis (direct identification of substances). These methods have some disadvantages including the discontinuity of sampling (e.g. 3 times per year) that may fail to identify intermittent discharges to the environment. In some cases the interval time between sampling and the results is also a disadvantage. The fact of not all chemical substances are included in the chemical analysis and because of that the detection is not always fast enough to prevent the occurrence of effects in organisms is another disadvantage (Gonzalez *et al*., 2009). The monitoring of water can also be achieved by physical-chemical parameters and biological methods that use for example physiologically, biochemical or behavior alterations of organisms and also changes in populations dynamics or in community structures to detect contamination (Gonzalez *et al*., 2009). The physical-chemical analysis can have lack of sensitivity but biomonitoring that uses the organisms to assess changes in the environment is typically sensitive to many chemical substances (Gonzalez *et al*., 2009). Biomonitoring is more relevant because it uses the mixture of chemical substances existing in the environment and can allow the organisms to integrate over time the potential toxic effects of different chemical substances throughout the life cycle also indicating the overall effects on aquatic ecosystems (Gerhardt, 1995). Bioindicators are organisms used to monitor environment and ecosystems (Gonzalez *et al*., 2009).

Behavior is becoming increasingly important for ecotoxicology being used in several studies including in biomonitoring in order to early detect water contamination (Magalhaes Dde *et al*., 2007). Behaviors reactions are vastly integrative responses, because behavior is related with biochemical and physiological processes (Brewer *et al*., 2001). Sub-lethal exposures to toxicants can trigger behavioral responses that allow quantitative measures of mechanisms modifications, which may have the potential to provide knowledge about individual and population effects of environmental contamination (Brewer *et al*., 2001).

The increasing integration of behavior analysis in toxicological and ecotoxicological protocols could help to better understand the impact and effects of chemical exposure in organisms. With the constant development of technology and statistical analysis, behavior could be valuable also for biomonitoring the

environment to avoid situations of pollution by detecting toxic substances, increasing the accuracy of risk assessment.

1.1. Behavior analysis

Behavioral toxicology in recent years has received an increased attention essentially because of the automation of the techniques to obtain and treat data (Bae and Park, 2014). Behavior is a selective response to internal and external stimulus (Gerhardt, 2007). It is an extremely structured order of actions and reactions designed to allow the best conditions possible in relation to fitness of the organism in the environment (Tierney, 2011).

Because higher concentrations are easier to test and analyze, a large number of toxicological assays use them to achieve results quicker (Magalhaes Dde *et al*., 2007). In most cases environmental contamination for the majority of contaminants only occur in natural aquatic systems at low concentrations that despite not be sufficient to cause mortality may lead to ecological functions losses (Houtman, 2010). These situations happen through behavioral alterations that may affect for example, predation and olfactory capacity (Scott *et al*., 2003). Behavioral changes induced in organisms by exposure to toxicants are usually subtle and may be detected at lower concentrations than those that cause permanent or irreparable damage with more serious consequences for the organisms, and therefore may be detected before the permanent effects (Scott and Sloman, 2004).

The majority of toxicological studies that use sub-lethal concentrations usually evaluate only the effects on chronic developmental or reproductive endpoints (Scott and Sloman, 2004) because they are typically easier to relate with the health of the organisms, although these may be more expensive and time consuming (Melvin and Wilson, 2013). However substantial technological improvements in computers, image analysis and video automation have made it easier and affordable to obtain interpret and apply behavioral endpoints for quantifying behavior in toxicity evaluation (Bae and Park, 2014).

Nowadays, one of the most used tools to motorize the activity of organisms is video-tracking. Through the use of video cameras to perform the recordings, the signal from the camera can be converted into a numerical video file and sent to a computer. Then it can be processed in real time by the software or it can be stored and examined later to avoid errors such as shadows (Delcourt *et al.*, 2013).

With video-tracking, not only locomotor activity (behavior responses to toxicants) common to several animal taxa can be analyze, but also more complex behaviors (e.g. social interactions, predation, feeding and mating), which might allow a greater understanding of the ecological impacts of environmental contamination (Scott and Sloman, 2004). Alterations in locomotor activity may affect the performance of different behavioral tasks such as the ability to prey (with consequences to growth and longevity), or foraging from predators (Little et al., 1990).

Software like LocoScan and EthoVision have been used in numerous studies (Blaser and Gerlai, 2006; Gerlai et al., 2006; Egan et al., 2009) to analyze different effects, on behavior of innumerous substances (e.g. pesticides, personal care products, pharmaceuticals, drugs, ethanol). These systems allow analyzing overall activity through endpoints like distance travelled, acceleration and angular velocity (Brewer *et al.*, 2001; Magalhaes Dde *et al.*, 2007; Liu *et al.*, 2011b), but also other types of behaviors for example social interactions, feeding, or predators avoidance of many different fish species.

A meta-analysis of the literature done by Melvin and Scott (2013) comparing studies that assess behavioral responses with acute lethality, developmental and reproductive procedures revealed that behavioral studies are in general more sensible and less time consuming. In fact concentrations ranging from 0.1 to 5.0 percent of the LC_{50}, were the lowest behaviorally effective toxicant concentrations that caused changes in fish swimming behavior (Little and Finger, 1990). In studies with multiple observations, changes in behavior occurred commonly 75% earlier than the onset of mortality (Little and Finger, 1990).

Behavioral toxicology as the advantage to be non-invasive, so the implementation of this type of analyses can be a powerful addiction in toxicological investigation in order to obtain more accurate consequences of

exposure, especially to environmental low-level exposures (Little and Finger, 1990).

With the purpose of monitoring water quality and protect aquatic ecosystems, biological early warning systems (BEWSs) have been developed to offer a rapid warning of contamination incidences (Gonzalez *et al*., 2009). These systems are capable of detecting different responses of organisms to disturbance, because organisms can detect a wide range of pollutants.

The Fish Toximeter, the ToxProtect, and the Aqua-Tox-Control are commercially available systems that use behavioral parameters. Some commercially available systems (Multispecies Freshwater Biomonitor, Biological Early Warning System, bbe Fish and Daphnia Toximeter), use more than one specie because different species have different sensitivity and reaction time to the same contaminants (Bae and Park, 2014).

Some matters still need to be resolved such as the problems related with data treatment (large quantity of data), advanced processing, the selection of appropriate bioindicators for each environment and the intrinsic variation of behaviors between organisms of the same specie (Bae and Park, 2014). However the development of BEWSs capable of detecting various types of pollutants with great sensitivity, with fast and reliable detection of adverse situations (faster than chemical detection) and with minimal cost of maintenance that are easy to use, could be a future worldwide solution, especially when integrating BEWSs with chemical monitoring. Chemical detection may be necessary to identify the toxicant because even though BEWS can detect a reaction to one substance in a toxicity test, in a natural environment where a mixture of toxicants is present most of the time, it's impossible for the BEWS to identify the toxicants (although identification is not the purpose of the BEWS). For example after an alarm of the BEWs, the substance that caused the biological effect can be identified by water chemical analysis (Gonzalez *et al*., 2009). In fact in Europe, specifically in Rivers Elbe, Meuse, Rhine there are BEWSs working in programs of biomonitoring (Gonzalez *et al*., 2009). In the river Rhine, the results of BEWSs, are always complemented and confirmed with physicochemical water analysis (Diehl *et al*., 2006).

The majority of organisms are sensitive to more toxic substances than some conventional analytical methods that are part of aquatic monitoring

(Gonzalez et al., 2009). Several organisms have been used in BEWSs including bacteria, algae, invertebrates and fish (Gonzalez et al., 2009). Fish have an important role in the trophic chain and significant commercial value, being therefore important to protect these organisms from contamination (Viarengo et al., 2007). In this sense fish have been used for the monitoring of drinking water, wastewater effluents, surface water and aquaculture (Bae and Park, 2014).

1.2. Zebrafish

Different types of fish species are used in behavioral testing to toxicant exposures or other types of stimulus. Fish have some specific advantages, such as direct contact with the aquatic environment (body surface), ecological important behaviors that are easily observed and quantified in controlled environment, the well documented life cycle that some species have (Scott and Sloman, 2004) and because the early stages of its life cycle are extremely sensitive to contaminants. One of the species most used is the zebrafish (*Danio rerio*).

The zebrafish is a small (up to 4.5 cm), tropical freshwater teleost fish belonging to the family Cyprinidae of the order Cypriniformes that it is native of the Himalayan region (South Asia) (Befyaeva *et al.,* 2010). A great number of Zebrafish can be easily maintained in the laboratory with a relatively low cost, a female can produce up to 200 transparent eggs every other day (Blaser and Gerlai, 2006; Gerlai *et al*., 2006), it has a rapid reproductive cycle (Befyaeva *et al*., 2010), its genome has been completely sequenced and several genes of high mammalian homology also have been discovered (Tierney, 2011). Zebrafish has been identified as an excellent model for pharmacology, toxicology and pharmacogenomics studies (Gerlai *et al*., 2006). Exposure to novelty evokes robust anxiety responses in zebrafish, as with rodents (Blaser and Gerlai, 2006). In the last few years, zebrafish have been used in several paradigms adapted from rodents with video-tracking systems such as the Novel tank test (exposure to a novel arena where vertical behavior is analyzed), open field test (exposure to a novel arena where horizontal behavior is analyzed), the light-dark box as a measurement of scototaxis (dark/light preference), shoaling (measures the

effects of anxiety on social behavior) after pre-treatment with for example anxiolytic substances (ethanol and fluoxetine), or potential anxiogenic substances (caffeine) (Egan *et al*., 2009; Maximino *et al*., 2011). The effect of predators in behavior has also been studied (Gerlai *et al*., 2000; Gerlai *et al*., 2009). Zebrafish behavioral analysis through toxicant induced modifications can be an important process to study the function of the brain (Blaser and Gerlai, 2006).

Consequently it's possible to use not only the behavior of this species in toxicological studies including studies that help to uncover the mechanisms of action of certain substances, and their effects on the behavior, but also in biomonitoring studies as an early warning signal. Several statistical analyses can be used in the behavior evaluation, to enable a better understanding of the behavior and to increase the detection sensitivity.

1.3. Characterization of ANNs

In the last few years several computational analyses were developed to handle data of behavior tracking. Some techniques include ANNs (Artificial Neural Networks) such as Multi-layer Perceptron (Kwak *et al*. 2002) and Self-organizing map (SOM) (Park et al., 2005; Liu et al., 2011b).

The ANNs have been applied in several areas, because they have numerous characteristics that make them interesting and attractive for prediction (Teles *et al*., 2006). In contrast to traditional model-based methods the ANNs are nonparametric data-driven self-adaptive methods and because of that incorporate few apriori assumptions (Zhang *et al.,* 1998). They are able to learn from samples and respond to subtle undetected functional relations between the data (Zhang *et al.,* 1998). ANNs can also generalize after ''learning'' the data, and can frequently predict an occurrence even with noisy information (Zhang *et al*., 1998). They are capable of approximate nonlinear multivariate functions to any desired accuracy (Zhang *et al*., 1998). These characteristics make them ideal to decipher problems that are too complex for conventional statistical methods (Zhang *et al.,* 1998).

In several studies, ANNs are used to identify (Kwak *et al.*, 2002) or classify (Park *et al.*, 2005) standard movements previous established by the authors or to set themselves these movements patterns based on variables that describe the fish movement (Liu et al., 2011b). However, the potential of the ANNs has not been fully explored. Only in one previous work (for publication), the ANNs together with the correspondence analysis (SOM-CA) were directly and successfully applied in the detection of toxic substances in the water through the zebrafish behavior. So the ANNs have the potential to evaluate the different behavioral responses that may occur when organisms are exposed to toxicants along time.

1.4. Tolerance

The development of tolerance is a response that may occur when organisms are exposed continuously to toxicants, and this response may affect the detection capability of video-tracking systems. It's becoming often the presence of different types of contaminants in the environment, especially in aquatic ecosystems (Wirgin and Waldman, 2004). Normally they exist at low concentrations with the exception of punctual discharges that introduce or greatly increase the concentration of some pollutants. These compounds may cause lethal or sub-lethal effects that can impair behavior, morphological or biochemical processes depending on the mode of action of the contaminant. However organisms have the capacity in some cases to tolerate toxicants in contaminated situations and for example fish populations frequently survive and prosper in highly polluted sites (Wirgin and Waldman, 2004).

This tolerance may be due to adaptation, this is genetically based resistance. Normally, occurs in relatively long time scales (several generations) at the population level, and represents the plasticity of the genotype (Meyer and Di Giulio, 2003). In a population, where normally exists variability, and in the constant presence of contaminants, the individuals more resistant are selected, resulting in more resistant generations (Ownby *et al.*, 2002). If this selective pressure disappears, it's possible that the adaptations also disappear, although not instantly (Wirgin and Waldman, 2004). Adaptation may cause reduction of

fitness, for example greater sensitivity to other chemical substances and abiotic factors (e.g. salinity), but it can also increase tolerance to other substances (Wirgin and Waldman, 2004). Embryos and larvae Killifish (*Fundulus heteroclitus)* of adults collected from New Bedford Harbor (USA) that is contaminated with polychlorinated biphenyls (PCBs) were more resistant than embryos and larvae of fish from reference sites after the exposure to PCBs, being the concentrations necessary to produce sub-lethal and lethal effects of two orders of magnitude higher than the ones producing effects in reference site embryos (Nacci *et al*., 1999). Ownby *et al* (2002) also proved that wild populations of *Fundulus heteroclitus (*collected form the Elizabeth River), had inherited tolerance to polycyclic aromatic hydrocarbon (PAH). Alteration of the biological functions can also occur at the individual level, when an organism pre-exposed to a particular contaminant become less sensible to his effects when another exposer occurs (acclimation) (Klerks, 1999). This process does not include alterations in the genes of the organism, so it's not transmitted and it should disappear in remediated environments (Wirgin and Waldman, 2004).

The tolerance to the toxicants probably arises from, the induction of mechanism, such as metallothioneins (proteins), that have the capacity to bind to heavy metals, and act as defense mechanism to protect the organisms (Perez-Coll *et al*., 1999). Other mechanisms include reduced uptake, storage of toxicant in isolated structures, biotransformation of the toxicant into inactive metabolite, or elimination from the cell by excretion or secretion (Wright and Welbourn, 2002). These mechanisms probably require more energy which prevents energy storage, and may cause consequences on the organism (Holmstrup *et al*., 2011).

1.5. Toxicants

To test the detection capability of the video-tracking system over the days, three toxicants belonging to different chemical groups were selected. The toxicants selected were sodium hypochlorite, ethanol and bisphenol A.

1.5.1. Sodium hypochlorite (SH)

SH is one of the substance most used in the world, and has been used since the 17th century (Nimkerdphol and Nakagawa, 2008). Is main uses include water disinfection of drinking water, swimming pools and wastewater, but is also used in industrial activities, power plants, pharmaceuticals, hospitals, being used extensively in household (Nimkerdphol and Nakagawa, 2008). SH is a strong oxidizing agent and because of that is highly effective in killing most bacteria fungi and viruses (Emmanuel *et al.*, 2004). This substance is corrosive and considered dangerous for humans at concentrations superior to 10%, because it can cause respiratory disorders, skin irritations, abdominal pain, burning sensation, cough, sore throat, vomiting and diarrhea (Nimkerdphol and Nakagawa, 2008).

SH can reach out environment accidentally and intentionally. SH solutions are very unstable and are rapidly degraded by temperature and light (Elia *et al.*, 2006). López-Galindo *et al.*, (2010a) showed that in natural seawater a concentration of 0.3 mg/L, decreased 50% after 1h and 90% after 8h. Concentration between 0.1 - 0.2 mg/L of SH were detected in discharge water of water plants in France (Manduzio *et al.*, 2004). SH reacts with water originating hypochlorous acid, which in turn originates hypochloride ions (figure 1) (Emmanuel *et al.*, 2004).

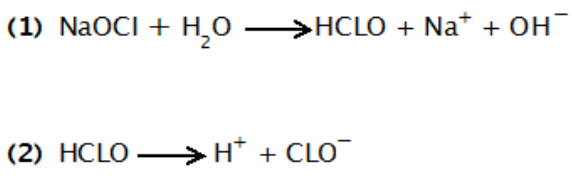

Figure 1: (1) Formation of hypochlorous acid through SH. NaOCl = Sodium hypochlorite. HClO =Hypochlorous acid. (2) Formation of hypochlorite ion. CLO^- = Hypochloride ions.

These substances also have disinfectant properties, being hypochlorous acid more powerful than hypochloride ions. They can react with organic compounds through addition, substitution and oxidation, originating for example halogenated products (Emmanuel *et al.*, 2004). Reactions with organic compounds, can also create chlorination products such as free halogens trihalomethanes and haloamines (Jenner *et al.*, 1997), that are more powerful

oxidants and have biocide action by for example disturbing enzymes activities (López-Galindo *et al.*, 2010b).

Chlorine is dangerous to aquatic organisms and in concentrations most of the times lower than 1 mg/L (Emmanuel *et al.*, 2004). A limit was calculated for total residual chlorine in water discharge of 631 ug/L (maximum daily limit), and 456 ug/L (average monthly limit) by the USEPA (2012). For fish species the capacity to induce physiological responses and gill damages (hypertrophy, lamellar fusion) that can lead to hypoxia and death has been proved (López-Galindo *et al.*, 2010b). Several studies revealed that the exposure to SH can trigger the detoxification mechanisms of organisms. Juveniles *Solea senegalensis* (flatfish) exposed to SH 0.1 mg/L for 7 days showed an increase in the first day of catalase (enzyme that protect the cell from oxidative damage by reactive oxygen species (ROS)), lipid peroxidation (oxidative degradation of lipids), and GST in liver, demonstrating an enzymatic defense response however after 7 days these antioxidant defenses decreased, according to the authors probably because of an increase in ROS production or due to enzyme inactivation from SH (López-Galindo *et al.*, 2010b). ROS are molecules capable of causing oxidative stress, such as damage of Deoxyribonucleic acid (DNA), lipid peroxidation and oxidations of proteins including enzymes. In gills occurred an increase in GST and an inhibition of AChE (enzyme that hydrolyzes the neurotransmitter acetylcholine) activities over time. Lipid peroxidation after 7 days wasn't observed, but several gills pathologies such as hypertrophy and lamellar fusion were detected (López-Galindo et al., 2010b). Specimens of carp *Cyprinus carpio* exposed to SH (0.7 mg/L) for 10 and 20 days also showed a response in hepatic antioxidant enzymes such as GST (increase), catalase (depletion) or total glutathione (increase) (Elia *et al.*, 2006). *Cyprinus carpio* exposed for 20 days to SH (1.24 ± 0.19 mg/L) presented only after 10 days a 15-fold increase in the activity of CYP2B1-linked penthoxyresorufin O-dealkylation (Canistro *et al.*, 2012). SH can also be dangerous to several aquatic organisms, vertebrates and invertebrates. Mussels of the specie *Mytilus galloprovincialis* exposed to SH (0.1, 0.2, 0.5 mg/L) for 14 days also presented affected levels of GST, catalase and AChE in gills. Gills exhibited a pattern typical of acute toxicity after being in direct contact with chemical substances (e.g. ciliar alterations) (López-Galindo *et al.,* 2010c).

Magalhaes *et al.* (2007) used a video-tracking system, to detect and evaluate the effects in zebrafish behavior (exposed during 5h) of different

concentrations of SH, 4.8 mg/L (10% of the 24h LC_{50}), 9.6 mg/L (20%), 14.4 mg/L (30%) and 19.2 mg/L (40%). The analysis was done by regression analysis of distance traveled, and two main responses were detected, an increased swimming activity between 1% and 10% called escape response to avoid the water contaminated, and a gradual decrease in swimming activity from the 20% to 40%, with avoidance response (hypoactivity) at higher concentrations. Hypoactivity is a behavior response that appears in some situations to increase the ventilation and to eliminate the toxicants form the gills (Gerhardt, 2007). Nimkerdphol and Nakagawa (2008) used a 3D video-tracking system to analyze the swimming velocity of zebrafish exposed (for 1h) to a maximum concentrations of 0.005% v/v, and discover that the swimming velocity tended to increase with the increase of the concentration.

1.5.2. Bisphenol A (BPA)

The synthesis of BPA by the condensation of phenol with acetone was first reported by Thomas Zincke in 1905 (Blankenship and Coady, 2005). BPA is an organic compound that has two phenol rings linked by a methyl bridge, with two methyl functional groups and it's used since the 1950s as a monomer to produce polycarbonate plastics, flame retardants and epoxy resins for coatings (Blankenship and Coady, 2005). Polycarbonates are used on food containers, such as water bottles and baby bottles, and epoxy resins are used inside of food cans and dental materials (Blankenship and Coady, 2005). BPA can also be found in electronic and automobiles equipment (Blankenship and Coady, 2005).

BPA is an endocrine disruptor (chemical substance that alters the function of the endocrine system and cause adverse effects), because it mimics the natural hormone 17β-estradiol (estrogen). BPA has the capacity to bind to estrogen receptors ERα and ERβ (although the affinity is about 1000 fold less than estradiol) (Kuiper *et al.*, 1998). It's possible that BPA can also suppress transcriptional activity by binding to thyroid hormone receptors, blocking the connection of thyroid hormone (although high concentrations are necessary) (Moriyama *et al.*, 2002). There are some evidences that indicate that BPA has others mechanism of action for example, it can act has an androgen receptor

antagonist (BPA, blocks the receptors, preventing the connection of androgens hormones like dihydroxytestosterone) (Lee *et al*., 2003).

BPA is associated with reproductive effects and development problems, and because of these possible affects the Directive 2011/8/EU of the European Union ban BPA from the plastic baby bottles. However BPA, is still one of the substances most produced in the world, and is present in the environment because of direct releases from manufacturing and handling or leaching from products and from treatment plants (Cousins *et al*., 2002).

BPA is rapidly degraded by aerobic biodegradation (Ying and Kookana, 2005). In water the half-live is about 2.5 to 4 days, and in soil the half-lives is 7 days (aerobic condition) but it can travel in a river several hundreds of kilometers (Dorn *et al*., 1987; Cousins *et al*., 2002; Ying and Kookana, 2005). Although some variation between studies exists, because of different conditions, for example in anaerobic circumstances BPA can resist in soils (Ying and Kookana, 2005). Normally, the concentrations of BPA detected in the environment are relatively low (<1µg/L), however in surface waters in Netherlands (rivers, lagoons), were detected concentrations up to 21 µg/L (Belfroid *et al*., 2002), in pre-treated waters in Japan leaching from landfills a maximum concentration of 17.200 µg/L was also detected (although the concentration of BPA in effluent was much lower because of treatment) (Yamamoto *et al*., 2001) and on sewage sludge a maximum concentration in Germany of 1363 µg/kg dry wet was detected (Fromme *et al.,* 2002).

BPA is capable of affect growth and development of numerous different types of organisms including invertebrates, fish, amphibians, reptiles, birds and even mammals (Flint *et al*., 2012). The effects on reproduction of fish such as the zebrafish *(Danio rerio)*, Carp (*Cyprinus carpio),* and rainbow trout (*Oncorhynchus mykiss*) can occur at high concentrations. Vitellogenin induction and decreased estrogen to androgen ratios in blood are some of the effects reported at high concentrations (Lindholst *et al*., 2003; Mandich *et al.,* 2007). However male brown trout (*Salmo trutta f. fario)* exposed to environmental relevant concentrations of BPA (1.75–2.4µg/L) during the spawning period (3 and half months), exhibited reduced sperm density, motility rate, and swimming velocity initially at 1.75 µg/L, and reduced sperm density and motility rate at 2.4 µg/L in the middle of the spawning period. Females exposed at 5 µg/L did not ovulate, and females exposed to concentration of 1.75 µg/L and 2.4 µg/L only ovulated 2

and 3 weeks later than controls (Lahnsteiner *et al.*, 2005). Carp (*Cyprinus carpio*) exposed for 14 days, to 1 µg/L BPA exhibited gonad structural changes (existence of eosinophilic granulocytes and amorphous extra-cellular in the interlobular space, or decrease of spermatogenic cyst and lobule diameter), and 10% of females exposed at 1 to 10 µg/L BPA presented oocyte atresia (follicle death). Exposed organisms (*Cyprinus carpio*) to 1000 µg/L BPA, presented increase estrogen to androgen blood ratios (17β-estradiol/11-ketotestosterone), with a significantly decrease in testosterone concentrations and a significant induction of plasma vitellogenin (Mandich *et al.*, 2007).

In natural environment, is difficult to prove that the effects detected in fish are due to BPA, because other estrogenic compounds are also normally present. BPA is normally considered to have a low potential for bioaccumulation (accumulation of substances in an organism), because the bioconcentration factors (ratio of the substance concentration in an organism to the concentration in water) calculated are quite small, for example three different wild fish species in a lake on China presented bioconcentration factors for BPA ranging between 29 to 49 (Liu *et al.*, 2011a). However in Netherlands in several locations, although the surface water had only 0.01 to 0.33µg/L BPA, the level in fish, Bream (*Abramis brama)* and flounder (*Platichthys flesus*) were between 2 and 75 µg/kg in liver and 1 to 11 µg/kg in muscle (Belfroid *et al.*, 2002). Interestingly, BPA was also detected in flounder liver and muscle (1–6µg/kg), in locations where no BPA (<0.18µg/L) was detected in the surface water. In fish, there are two phase II conjunction reactions responsible for the metabolism of BPA, sulphation and mainly glucuronidation. BPA sulfate and BPA glucuronide (metabolites) were identified from zebrafish exposed to 100 µg/L (Lindholst *et al.*, 2003). The metabolism of BPA was faster in zebrafish liver than in rainbow trout liver, which indicates that it can vary (Lindholst *et al.*, 2003).

Effects of BPA in social-sexual behaviors have been described in rats. Male rats whose mothers were administered with 40 µg/kg/day, demonstrated decreased male mating behavior, and females demonstrated increased sexual motivation and receptive behavior (Farabollini *et al.*, 2002). BPA early life exposures, seems to also cause anxiogenic effects in mice, because juvenile and adult male mice whose mothers were administered with 250 ng/kg/day from gestational day 10 to postnatal day 20 spent less time in the center area of the open field (anxiety behavior) than the controls (Matsuda *et al.*, 2012). Zebrafish,

larvae exposed to 23 µg/l for 48h presented larval hyperactivity (increased movement duration) and also adult learning deficits (delays) (Saili *et al.*, 2012). Zebrafish exposed during embryonic development (228–3424 µg/L), presented decreased swimming velocity in response to light stimulation, however at this concentrations, axial muscle damage (probably due to the increased ROS formation and oxidative DNA damage) was also identified which may explain the deficits in swimming performance (Wang *et al.*, 2013).

Although the environment values of BPA, are generally consider insufficient, to cause effects to wildlife, the truth is that BPA has become ubiquitous, because of his constant release. Even in small concentrations, exposure to BPA, has proven to cause several effects specially in development and reproduction, and even though environmental BPA limits do not exist, the risk that BPA and other estrogenic disruptors possesses to aquatic pollutions, specially to organism earlier stages, is too high, and therefore a fast detection of BPA and other endocrine disruptors may be essential to conserve wildlife.

1.5.3. Ethanol

Ethanol is a liquid substance that is volatile. It can be obtained by the fermentation of sugars (example corn or sugarcane), normally used in alcoholic beverages but also in fuel. It can also be obtained by ethylene hydration to be used has solvent, or in the production of plastics, polishes, plasticizers, and cosmetics, and it is also used as anti-infective (Strohm and Sweet, 2005). Ethanol fuel, when used as an additive (gasoline oxygenate) to gasoline, it contains denaturants (hydrocasrbons), which turns this additive unsuitable for human consumption (Freitas and Barker, 2013). In the environment, it has a half-life of less than 10 days in water, and of 5 days in air because it is extremely volatile and it also has a low bioaccumulation potential (Strohm and Sweet, 2005).

Ethanol is rapidly absorbed in the stomach and especially in the small intestine, reaching the blood very quickly, because it is water and lipid-soluble (Strohm and Sweet, 2005). Then it's metabolized in the liver mainly by the enzyme alcohol dehydrogenase (ADH). Initially the ADH transforms ethanol in acetaldehyde. After this process, the enzyme aldehyde dehydrogenase (ALDH)

metabolizes the acetaldehyde in to acetate (Swift, 2003). In the liver it can also be metabolized by the CYP2E1 and by the enzyme catalase located in the peroxisomes of hepatocytes (Swift, 2003). Acute effects in humans are muscular incoordination, visual impairment, decreased reaction time, behavior changes and severe intoxication can lead to vomiting, nausea and hypothermia and even eventually coma, hypertension, and death (Strohm and Sweet, 2005). Chronic consumption can lead to several types of liver damage (cirrhosis and alcoholic hepatitis), cancer, cardiac problems and during pregnancy can lead to congenital malformations (fetal alcohol syndrome) such as mental deficiency (Strohm and Sweet, 2005).

Ethanol is a substance that acts in the brain. Short-term alcohol exposure, normally enhance the action of GABA (Gamma-AminoButyric Acid) and glycine, (inhibitory neurotransmitters) by increasing the function (inhibitory effects) of their receptors $GABA_A$ (GABA receptor) and GlyR (glycine receptor) (Mihic *et al.*, 1997). This explains why alcohol in some circumstances decreases anxiety. On the other hand ethanol inhibit the action of glutamate (excitatory neurotransmitter) by inhibiting is receptors, NMDA (*N*-Methyl-D-aspartate), and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), and KARs (kainate receptors) although ethanol is considered a weak agonist of NMDA even at high concentrations (Dildy-Mayfield and Harris, 1995; Wright *et al.*, 1996). Ethanol also affects the $5\text{-}HT_3$ (5-hydroxytryptamine$_3$) receptor, where the neurotransmitter serotonin (modulator of physiological functions, including perception, aggressiveness, anxiety, sexual behavior) binds, and the nicotinic acetylcholine receptors (nAChRs), where the neurotransmitter acetylcholine binds (Cardoso *et al.*, 1999; Davies *et al.*, 2006). Chronic exposure to ethanol has the contrary effect, which is decreased inhibitory neurotransmission and increased excitatory neurotransmission, in an attempt to reach equilibrium (Faingold *et al.*, 1998). This process, may explain the development of tolerance, for example animals previously exposed to ethanol appear resistant to its effect when compared with controls. Studies with zebrafish reported that ethanol can increase the activity for example of AChE (Rico *et al.*, 2007), and chronic exposure can lead to different significant gene expression levels in brain, suggesting an adaptive response (Pan *et al.*, 2011).

Although ethanol is not a threat from an environmental perspective, many studies have shown that ethanol can affect many aspects of zebrafish behavior.

Gerlai *et al*. (2000) exposed zebrafish for 1h in an acute treatment to 0.25% v/v, or 0.5% v/v (considered intermediate doses), and the fish presented increases in general activity and aggression, and diminished fear (distance to the image) in relation to an predator image and shoaling. This type of behavior is linked to the anxiolytic properties of ethanol. At higher concentration (1% v/v), decreased the activity, however the authors argued that this response, is probably due to sedative effects of ethanol. Egan *et al*. (2009) demonstrated that zebrafish exposed to ethanol (3% v/v) acute treatment (5 minutes), presented decreased erratic movements and increased exploration, and ethanol (3% v/v) chronic treatment (7 days), caused increased exploration, average velocity and total distance travelled, all considerate anxiolytic effects. Larvae zebrafish (6 days post fertilization) exposed to ethanol demonstrated hyperactivity at lower concentrations (0.5–2% v/v) and hypoactivity at higher concentration (4% v/v) (de Esch *et al*., 2012). Gerlai *et al*. (2006) also showed that zebrafish exposed chronically, for two week to ethanol (0.25% v/v) had developed a significant tolerance to this substance. Fish exposed to the highest (1.00% v/v) acute ethanol concentration (for 1h) that were exposed earlier to the chronic ethanol treatment, presented decreased distance from the predator, an anxiolytic effect that was not attenuated by the chronic exposure. All zebrafish exposed only to acute ethanol concentrations (0.25% v/v, 0.5% v/v, 1% v/v) presented an almost linear concentration response, and the higher ethanol concentration increased the total path length swum by the fish. One of the explanations for this particular result is that maybe the strain (long-fin wild type) was more resistant (than the one used in 2000), and thus a higher concentration was necessary to cause hypoactivity (Gerlai *et al*. 2006). Some behavior variation has been reported between zebrafish species exposed to similar ethanol concentrations, as the previously mentioned (Dlugos and Rabin, 2003). Gender differences were also detected in chronic ethanol (0.5% v/v) exposed wild-type zebrafish (10 weeks), because females had heightened sensitivity (increased nearest neighbor distances), but males didn't present this response (Dlugos *et al*., 2011). Tran and Gerlai (2013) also demonstrated in a 1h time-course experience (behavior monitoring during all period), that zebrafish exposed acutely to ethanol (1.00% v/v) exhibit an inverted U shaped trajectory in distance travelled. These authors argued that initially, the temporal trajectory increased due to an elevation of ethanol levels in the brain resulting in stimulation, and then the levels reached the maximal blood/brain ethanol levels which lead to a depression in the trajectory of the distance

travelled. However zebrafish in the same situation that had been previously exposed to chronic concentration (0.5% v/v) demonstrated a blunted response, suggesting tolerance.

1.6. Toxicant Selection

BPA and SH are capable of causing behavioral changes in zebrafish, (Magalhaes Dde *et al.*, 2007; Nimkerdphol and Nakagawa, 2008; Saili *et al.*, 2012; Wang *et al.*, 2013) and in this sense are good objects of study. Furthermore, because they are widely used, they are constantly released in the environment, and from this point of view are ecologically relevant. They also have other advantages, including all the three toxicants have known values of 96h LC_{50} for zebrafish and a high solubility in water, even BPA (moderately soluble) that has the lowest solubility in water 300 mg/L (Shareef *et al.*, 2006). Ethanol has a lower toxicity than the others two toxicants, but it causes different effects on behavior. Several studies such as Dlugos and Rabin, (2003), Gerlai *et al.*, (2006), Gerlai *et al.*, (2009), Dlugos *et al.*, (2011), Tran and Gerlai, (2013), Tran *et al.*, (2014), use ethanol in chronic exposures with zebrafish, and proved the development of behavior tolerance, which makes this toxicant ideal to serve as "positive control", to verify if all the experimental procedure and the subsequent statistical analysis are suited to detect this type of behavior, that eventuality may occur.

2. Objectives

After the development in previous works (for publication) of a video-tracking system with zebrafish, which proved to be very accurate and sensitive, the main objective of this work was to determine whether the detection capability of this system does not deteriorate after successive exposures of the zebrafish to the toxicants ethanol, SH and BPA.

Other aims included:

Use the zebrafish behavior changes induced by environmental disturbances (toxicants), as a way of detecting them, through the time series of the Kohonen ANNs.

Use the ability of the Kohonen ANNs, the correspondence analysis and the saturated orthogonal multiple linear regression analysis as a method of diagnosis of environmental conditions to which the test organisms were exposed.

3. Material and Methods

3.1. Organization of Experimental Material

Initially, a Tetra aquarium was assembled with a capacity of 50 liters (64x34x29 cm; length x width x height), equipped with a Trixie aquarium heater (50 Watts; 30-60 L), and one Trixie water pump filter (7 Watts; 40-60 L), capable of chemical cleaning, through activated carbon for chlorine, and biological cleaning by sponge. Four Aqua Cyan 15 aquariums (34x17x24 cm; length x width x height), with a maximum capacity of fifteen liters equipped with aquarium heaters Aquapor 25 and water pumps filters Rena H20 Max 35 were also assembled. Each of these four aquariums was divided into three equal parts, through the use of fine a fin net, in order to isolate simultaneously and separately twelve fish (three per aquarium).

3.2. Arenas

To assemble three arenas sets (the tanks where the fish were filmed) with the dimensions of 35x20x15 cm (length x width x height), glass plates 2mm thick were glued together, and they were internally divided by glass plates in four equal divisions (each division represents a recording arena) in order to isolate one fish per arena. The bases of the arenas were also glass plates 2mm thick. The external glazing of the recording arenas was frosted glass that allowed greater contrast of footage, however to the internal glass plates (that divided the arenas) white plastic boards were glued to eliminate visual contact and completely isolate the fish from each other.

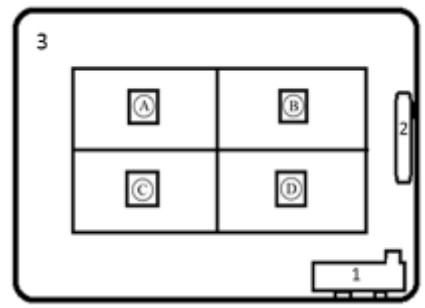

Figure 2: Scheme of the arenas. A-Control, B-SH, C-Ethanol, D- BPA. 1- Aqua Pro internal filter M200 water pump, 2- Trixie aquarium heater (25 W; 15-30 L), 3- Water bath.

The horizontal plane was used because the activity and movement of zebrafish is higher in this plane that in the vertical plane (Vogl *et al.*, 1999). The depth of water in the arenas was reduced to a minimum value (10 cm – 1.5 L) to further reduce the vertical movement, but this reduction did not affect the overall level of fish activity.

With this video-tracking program, the fish were kept individually in each arena, because the program couldn't detect correctly various organisms in the same arena, when they were close to each other, due to overlapping. This problem is also an obstacle to other screening programs, because it can lead to fish incorrect identifications (Delcourt *et al.*, 2009).

3.3. Recording Areas

The arenas were placed in three recording areas (figure 2), each one with one water bath, (48x17x36 cm; length x width x height), that had a transparent base, an Trixie aquarium heater (25 Watts; 15-30 L), and a Aqua Pro internal filter M200 water pump (5 Watts; up to 45 L). The walls were covered by silver foil to reflect the light to the arenas, and about 10 liters of water which were always changed before each trial. Underneath the water bath were placed twelve LED cylindrical lamps of 60 Watts (four for each area) to uniformly illuminate the arenas. The basis of the arenas was made of glass and the base of the water baths was transparent, because in this case the light can pass through those surfaces and better highlight the fish silhouette which improves the footage quality. The LED lamps have been selected because unlike incandescent lamps, they do not heat significantly and therefore do not increase the temperature of the water bath and consequently of the arenas.

Through the aquarium resistances previously mentioned and with the help of four Digital Internal/External Thermometers (Model RT 801 Version 12) with an accuracy of ±0.5ºC, the temperatures of the aquariums and of the arenas were

set and maintained to 28±0.5ºC, the ideal for the species in question. The culture medium used in the arenas consisted of tap water aged at least 48h, in a 50 litter aquarium, with no fish and water pumps with chemical and biological cleaning.

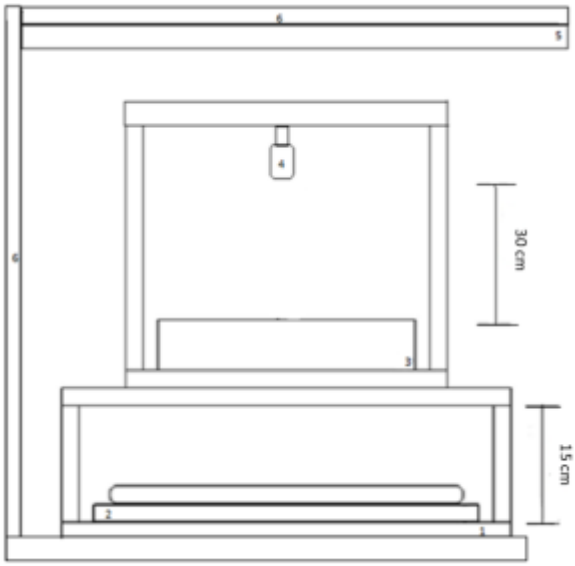

Figure 3: Scheme of one recording area. 1- Polyurethane isolation, 2- Led lamps, 3- Water bath, 4- 540L IR camera, 5- Polystyrene, 6- Expanded cork.

To minimize possible disturbance such as vibrations originating from the ground, but also sounds that could disturb the experience, specifically noises, the walls (sides and back) of the recording area were covered with expanded cork boards, and above the cameras, expanded cork boards and polystyrene were also placed in supports (these last ones facing inwards to reflect the light). On the surface of the table where the recordings took place, polyurethane isolation was also applied to minimize possible vibrations originating from the ground (figure 3). When recordings occurred, expanded cork boards were put in the front, to isolate completely the entire system and the arenas. Furthermore, in the expanded cork boards were fixed white cardboard to reflect the light and thereby obtain an enhanced contrast of the fish in the arena, which facilitates image processing.

Three 540L IR cameras (model CACO0008) flow electronics with super high color resolution were used in the recordings (one for each recording area). The data were stored in one Intel® Pentium® Dual CPU computer (2:00 E2180@2.00

GHz, 1.87 GB RAM) system with a Microsoft Windows XP Home Edition version 2002 Service Pack 3, through the DSS1000 program version 4.7.0041 of 2004.

3.4. Origin of Test organism

In this study, about 40 wild-type adult zebrafish (*Danio rerio)* with about 3 months of age and 2.5 to 3.0 cm length from the same batch (ORNI-EX, Lda, Arcozelo, Vila Nova de Gaia, Portugal) were used. The fish were acclimated for two month prior to any test in one aquarium tetra of 50 liters, because of the risk of mortality due to a possible disease, habituation to the new environment or simply due to the intrinsic differences between fish even being from the same batch. However in this case mortality was not observed. The fish were fed once a day with TetraMin Bio Active Formula, 1h after the end of each trial, and kept in a 12h of light/12h of dark photoperiod. The fish were used exclusively in these trials.

3.5. Exposure concentrations

The concentrations of the toxicants used must be ecological relevant (in the range of those found in natural environments), to obtain more realistic consequences of exposure, but at the same time the concentrations should be sub-lethal to zebrafish, because the objective is to see if the normal behavior could be impaired, without compromising the fish physical integrity.

To fulfill these conditions, the toxicant concentrations used were 9% of the 96h LC50 for the three toxicants. The selection of the toxicant concentrations was based in a previous experience, were the established concentration was 0.5 mg/L of SH. This concentration was extremely low because it represented 1% of the 24h LC_{50} (48 mg/L)) or approximately, 9% of the 96h LC_{50} (5.5 mg/L) (Magalhaes Dde *et al*., 2007; Pitanga, 2011), and did not cause permanent damage or death to the zebrafish. In the case of BPA, the concentration (0.891 mg/L) used is also ecological relevant, because although in the environment normally much lower concentrations are detected, higher concentrations have

already been detected in sewage sludge, for example 1.363 mg/kg according to Fromme et al., (2012). Even taking into account that higher concentrations than 100 µg/L are capable of causing endocrine effects (Lahnsteiner et al., 2005; Mandich et al., 2007), normally long exposures are required (days or weeks) to produce this effects, but in the present work the daily exposure time was much shorter (1h30m) and only for 9 days.

The detection capability and reliability of the system was tested because these concentrations were very low, perhaps to an incipient sub-lethal level.

Table 1: Toxic concentrations used (9% of 96h LC_{50}), and their respective 96h LC_{50}.

Toxicants	Concentration used (9% of the 96h LC_{50})	96h LC_{50}
Sodium Hypochlorite	0.500 mg/L	5.5 mg/L (Pitanga, 2011),
Bisphenol A	0.891 mg/L	9.9 mg/L (Hartmann, 2012)
Ethanol	1278 mg/L	14200 mg/L (Martins et al., 2007)

From a Panreac proanalysis SH solution with a purity of 7% (w/w) and a density of 1.15 kg/L, it was prepared the required concentration. For ethanol it was used ethanol absolute (Fisher Chemical), Analytical reagent grade with a purity of 99.5%. The density of ethanol was considered to be 0.790 kg/L = 790 mg/ml at room temperature, (roughly 20 ºC). For BPA to achieve the 9% concentration required (0.891 mg/L), 30 mg of 2,2-bis(4-hydroxyphenyl)propane for synthesis (BPA) were added to a 1L volumetric flask filled with water and mixed in an agitator for 24h before each trial at 25.0°C. The solubility of BPA is 300±5 mg/L at 25.0±0.5°C (Shareef *et al.*, 2006) and to allow a clear dissolution of BPA in the water, a factor 10 (ten times less) was use to prepare the stock solution (30 mg/L). The volumetric flask was wrapped in silver foil, to prevent photodegradation.

For all toxicants the dilution water was dechlorinated tap water filtered through activated carbon, (Staples *et al.,* 2011). To the respective arenas was added 9.6 µL of SH, or 46 ml of the stock solution prepared for BPA, or 2.51 mL of ethanol. To maintain the same depth of water in all the arenas, so that a possible difference in this parameter would not influence the behavior of the fish, which ultimately could had led to analysis errors, dechlorinated water at 28ºC was added to the arenas with 50 ml beakers. To the control and SH arenas was added 46 ml of dechlorinated water, and to the ethanol arenas was added 44.5 ml. All the toxicants were purchased from VWR International.

3.6. Experimental plan

At the beginning of each trial, the fish were transferred individually to the arenas where the acclimation was of 10 minutes, due to the transfer and the possible stress given to the fish. Then there were 1h30m of exposure time without toxicant. The last hour of exposure was recorded (recording time) and designated by moment "Before". This moment (first recording time) was the time before the addiction of the solutions, and this experimental plan was used in order for each fish to be used as control of himself. In the analysis it was only use the first half an hour of the recording time. After the first recording time, each arena was contaminated with the respective toxicant solution, and the controls with water (to maintain the same depth in every arena). Then the second exposure time was also of 1h30m. The last hour of exposure time was recorded (recording time), designated by moment "After". This moment (second recording time) was the time after the addiction of the solutions (figure 4). In the analysis, only the first half an hour of the recording times was used. Using the behaviors of the same organisms, with and without toxicant, the possible variability between individuals was reduced, which probably improve the quality of the statistical analysis.

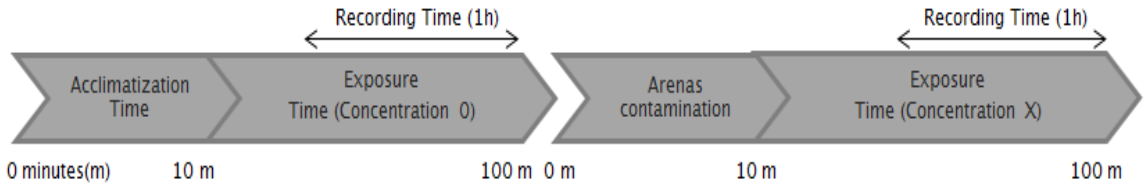

Figure 4: Experimental plan of each trial and toxicant.

In the end of the trials, the fish were kept individually in four Ciano Aqua 15 aquariums that were divided in three equal spaces. Each aquarium had three fish that were exposed to the same experimental condition, in other words, one aquarium with three fish that were the controls, the other one with three fish exposed to BPA, the other with three fish exposed to ethanol, and the last one with three fish exposed to SH. Four nets were used to transfer the fish, one for each experimental condition. The water used in the arenas was discarded after each trial, and the arenas were only filled up in the next day before a new trial.

The first half an hour of the exposure time, given before the recording times coincided with the acclimatization to the arenas of the fish, this period also allowed the toxicant dispersion in the arena. The temperature variation during the assay was 28.3ºC±0.5ºC. The variation of the temperature was small and probably this factor did not influence the behavior of the fish. To eliminate potential lesions due to handling, and additional stress only in the last trial, the fish were weighted (0.6g±0.1g).

Each arena shoot was unique, representing only one experimental unit, in a total of four in each recording area. Each trial had 3 replicates of the same condition (one replicate for recording area). The replicates were performed to allow statistical validation of the results. To improve the results and to decrease the possibility of mortality due to the daily exposures, the specimens that seemed visually bigger were selected (being larger were more visible, perhaps more resistant to the toxicant). The intention was also to select organisms with similar weight, to avoid the possible behavior influence of this factor.

Recording times may vary between studies, however others works have used recording times similar to the time used in this work (Tran and Gerlai, 2013), and also inferiors, such as 30 minutes (Nimkerdphol and Nakagawa, 2008). In relation to exposure times, 60 minutes is a very used period, especially in studies with ethanol (Gerlai *et al.*, 2000). An analysis of ethanol content of zebrafish brain revealed that ethanol has measurable levels in the brain after 20 minutes, and reached a plateau level after 40 minutes (Dlugos and Rabin, 2003). All trials were conducted between 10h and 17h as described in other works (Gerlai *et al.*, 2006).

The trials were performed during 9 days. In the first, second, third, fifth and ninth day, the trials were made as described before, and in the fourth, sixth, seventh and eighth day, the fish were also exposed in the arenas to the respective toxicants for one hour and a half, but in this cases the only difference was that no recordings were performed. This experimental design was scheduled to obtain more informative results and cover more days instead of just, five consecutive days. Daily exposures (once a day) were performed in this work because the system was designed to detect repeated contamination in time, not continuous contamination, and because they allow greater and better control over time of the toxicant concentrations than continuous exposures. In addition as the goal of the technique was to avoid prolonged exposure in the environment that could cause

irreversible effects to the populations, this design was considered more representative since it allowed to test and quickly detect the toxicants in water (ideally), without causing apparent damage to the fish. The concentrations used were very low and probably did not change significantly during the exposure time (1h30m).

3.7. Video treatment

The main objective of the video treatment was to transform the location of the fish in each recorded frame in coordinates that could be statistically analyzed. This stage was very important because the final product of each test consisted in six recording files, three of the moment Before of each recording area (Left, Center and Right) and three of the moment After, and therefore it was necessary to divide each file into four parts corresponding to the four arenas that were in the same file and whose experimental conditions were different. The format in which the files were stored (DSS) were not compatible with the statistical analysis program [(Statistica version 10 (StatSoft, 2011)] that had been used and thus they had to be converted. The first step was to convert the DSS files to AVI (video format) through the DsstoAVI program. Then through the VirtualDob program (version 1.9.11) the AVI files were converted to the format ImageSequence (series of images in Jpeg format at 25%), that corresponds to an image sequence (frames) in which it was possible to see the location of the four fish at each moment.

In the ImageJ software (version 1.40g), the entire image sequence was imported in a 50% scale (to reduce the length of the file) and converted to an 8-bit Grayscale (to be possible to use the MultiTracker tool). The Rolling Ball Background filter with light background selected was used to clean all the frames, eliminate potential shades and interferences and enhance the fish (figure 5).

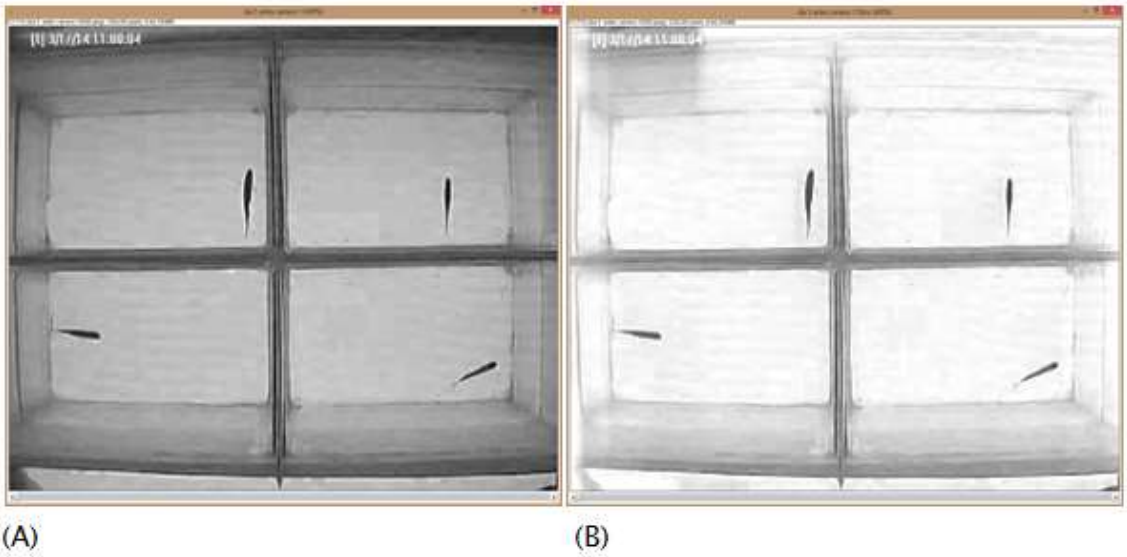

Figure 5: Image sequence, (A) before applying the Rolling Ball Background filter, (B) after applying the Rolling Ball Background filter.

In total, the next procedure was performed 240 times because 2 assays were conducted each one with five definitive experimental trials that had six image sequences that needed to be divided into four (number of arenas).

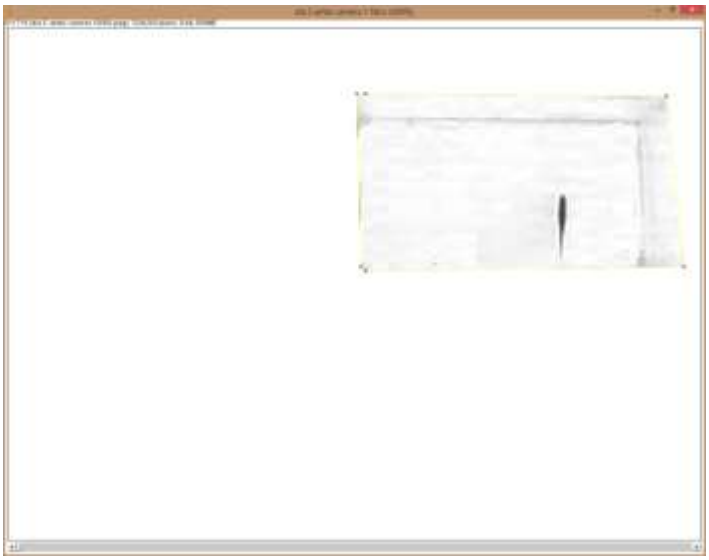

Figure 6: Selection of one arena with the tool polygon selections.

With the polygon selections tool, the space corresponding to one arena was selected in the image sequence, then the rest of the image was eliminated with the command clear outside (figure 6). This process was made 4 times for

each image sequence producing four different image sequences (one for each arena/fish). It was necessary to individualize the image sequences of the fish because the MultiTracker tool cannot track the trajectory of several fish at the same time.

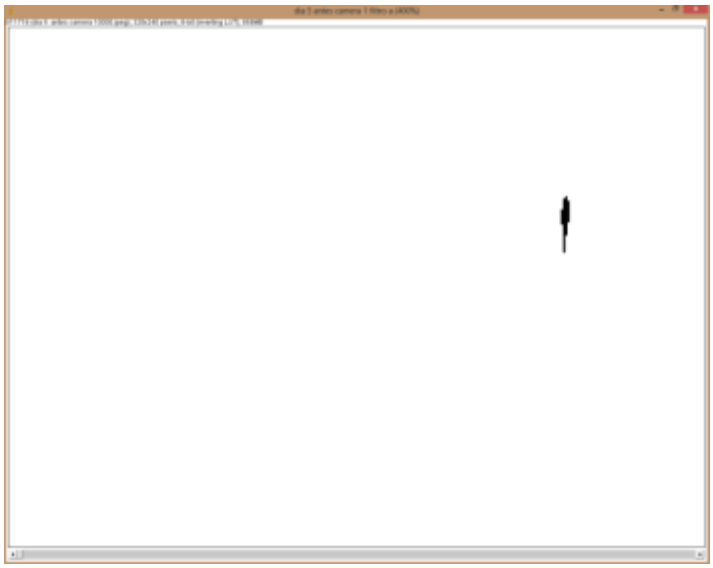

Figure 7: Application of the Threshold command in one fish before applying the MultiTracker tool.

From this point, and using the same program it was possible through the Threshold command to turn each fish in to a black spot while everything else in the pictures turned white (figure 7). Several thresholds were applied according to the fish size in order to improve the quality of detection and data collection. A minimum threshold size was used so that smaller objects (e.g. feces, shades) were not taken into account. In a treated frame, only the detected pixels of each targeted organism appear. Then by applying the MultiTracker tool the program was able to calculate the coordinates of the organism within the arena for each image. The final product for each fish, was a table in excel format with two columns of coordinates (X and Y) and another with the number of each frame to which the program exported the data about the location of the fish in each image sequence.

The coordinates calculated by the program were relative to the geometric center of the "black spots" defining an organism. For each fish in each trial was created an excel document with the coordinates corresponding to the moments Before and After. These documents had also detailed information about the

repetition (assay 1 or 2), date, trial day (1, 2, 3, 5 or 9), the begging of each time (hour), Moment Before (-) or After (+), camera (Left, Center, Right), arena (A, B, C, D), fish (1 to 12), replica (a, b, c) toxicant (Control, SH, Ethanol, BPA) and the number of each frame.

Using these coordinates were also determined in the program excel, nine movement descriptors of behavior of each zebrafish. These movement descriptors were selected, because they were the same that were used in a previous work done by the same research group. These movement descriptors were the following: linear velocity (mm/s), angular velocity (degrees/s), average value of X coordinates (mm), average value of the Y coordinate (mm), angle (degrees) that was the angle between the segments formed by the coordinates of the fish in three successive frames, linear acceleration (mm/s^2), angular acceleration ($degrees/s^2$), meander absolute (degrees/mm), that is the degree of curvature per unit displacement and the product of the standard deviation of X/Y coordinates (mm) that measures the distribution (dispersion) of the fish in space. The coordinates of the x and y axes had also been reversed and centered in order to have the same spatial correspondence, as if the arenas were all overlapped.

3.8. Statistical Analysis

3.8.1. Cluster Analysis

It was not possible to use the entire recording time (one hour) of each file for analysis due to limitations of the hardware and of the Statistica software himself, whose capability of data processing is approximately 150.000 lines (frames). It was determined that the most representative time interval that was less subject to external disturbances was the first half an hour of the recording time, and because of that this time interval was selected for analyze.

In the statistical analysis only the first 6000 frames corresponding to the first half an hour of the recorded Moments were used. This means that the time analyzed was the second half an hour after the contamination of the arena. The movements descriptors used were not instantaneous values. To use the

maximum data possible, all the frames were used in successive averages of 64 frames. For example frame 1 to 64, represents one average; frame 2 to 65 represents another average, and so on. These resulting averages were numbered from 1 to 20 in successive series, but only the averages 1 and 11 were selected, meaning that all the frames of the half hour were used in the average values. Due to the limited capacity of data storage of the Statistica software it was necessary to perform the previous step. To avoid minor disturbances that might occur at the beginning of recording, the first 200 frames were eliminated (approximately 1 minute). In the rare occasions where the fish jumped, the last 20 frames before the jump were also eliminated.

The Statistica program was used to analyze the data about the behavior of the zebrafish. Initially the data were analyzed through a cluster analysis. This analysis was executed by an ANNs, more precisely the SOM model because they are relatively simple to fit, and were successfully used in previous studies. The objective of the cluster analysis was to define different behavior categories of the fish submitted to the different experimental conditions with the information about the movement descriptors, but without knowing anything about the experiment conditions, without any identification. The cluster analysis allowed to classify the behavior of each fish in each moment and assigned it a behavior category.

The information in the Microsoft Excel files about all the fish in each day recorded including all the movement descriptors calculated, were placed in the program properly identified, in a total of 138283 frames. So the input variables were the movement descriptors of behavior. The ANNs requires three separate time series to perform the cluster analysis, which were defined as Training, Testing and Validation. In the series of Training the model adjusts the values and decreases the error, the Testing series helps the model to adapt to the results and the Validation series as the name implies validates the model.

The random sampling method for the training series was of 40%, for the testing series was 30% and for the validation series was also 30%. The parameters of the calibration were for the training cycle: 1000, for the learning rate: start = 0.01 and end = 0.0001, neighborhoods: start = 2 and end = 0, network randomization: normal, and 4x3 topological dimensions (12 behavior categories) because with more than 12 dimensions, a few of them started to appear with nearly zero frequency. These parameters were set by trial and error through the

analysis of the error found in the Training and Testing series. All the other parameters were maintained as initially, in other words as default.

3.8.2. Anova and post-Hoc Test

First the ANOVA (analysis of variance) was performed to see if there were statistically significant differences between the groups of behavior categories defined by cluster analysis in relation to the averages of all movement descriptors. Then the post-Hoc test (Scheffe's), and the homogeneous subset, were performed to analyze the average values of the movement descriptors of each category and compare it. These tests were executed in the SPSS (Software version 21) program because the Statistica program doesn't perform the homogeneous subset.

3.8.3. Correspondence Analysis

To, obtain a quantifiable measure of the behavior effect caused by each toxicant, several correspondence analysis were performed using different partitions of the data in terms of day, assay and toxicant. Of all correspondence analysis tested, the partitions of data with all the toxicants, by day and assay had the best results in terms of significance level and conclusions consistency.

In the correspondence analysis the 12 behavior categories defined by the cluster analyses were used as row variable. As column variable the conditions W, BPA, Et and SH were used. These conditions classify the experimental units. W had all experimental units in the Moment Before (without toxicant) and also the controls at the Moment After (after adding the toxicants) in others words this category had all experimental conditions without toxicant. The BPA, Et and SH conditions represent the experimental units with toxicant at the Moment After (after adding the respective toxicants, BPA, ethanol and SH). The case selection conditions were day=i (i= 1, 2, 3, 5 e 9) and assay=k (k=1 e 2). The supplementary column points were the frequencies profile of the different

behavior categories in each experimental unit organized by replica (a, b and c), toxicants (Control, BPA, Et and SH), Moment (Before and After), day (1, 2, 3, 5 and 9) and assay (1 and 2).

Analyzing the projection of the categories of the column variable it's was possible to see that all types of experimental conditions (W, BPA, Et and SH) were clearly discriminated spatially in the three dimensions defined by the different correspondence analysis, as represented in figure 8. In the multidimensional space defined by each correspondence analysis (by day and assay), three vectors (Toxicant vectors) that represent the effects caused by the toxicant on the fish behavior were determined. These vectors (vectors BPA, Et and SH) were determined by the difference between point W and the respective toxicant point. The points W, BPA, Et and SH represent the group of experimental units that belong to each of these types of experimental units. These points were the midpoints of each group of experimental conditions defined by the Column variable in each Correspondence Analysis.

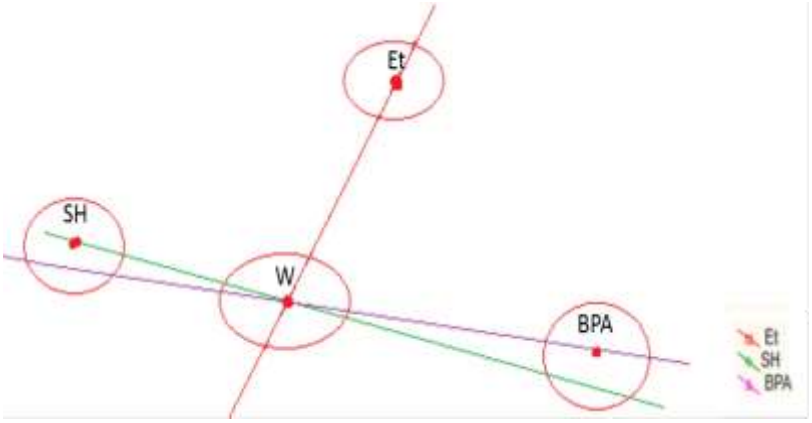

Figure 8: Correspondence analysis, plot of row column coordinates (experimental conditions W, SH, Et, BPA): dimension 2x3, day 1, assay 1.

The Euclidean distance of each point (experimental unit) to the respective Toxicant vector (BPA, Et and SH) was calculated through the scalar projection of the vector formed by each point with the corresponding Toxicant vector. The objective of these Distances BPA, SH and Ethanol was to have a measure of the position of each point within the gradient formed by the respective Toxicant vector. These Distances measure the degree of modification of each experimental unit in the direction of the three Toxicant vectors. These Distances were standardized to allow comparisons between the different days and assays. In the calculation of the Distances to avoid subjective selections of the dimensions for

the representation of the effect caused by the toxicant and to simplify the comparison of the different measures, all the three dimensions created by the correspondence analysis were used and the standard procedures were always the same.

3.8.4. Saturated Orthogonal Multiple Linear Regression Analysis

The degree of alteration (Distances BPA, Ethanol and SH) observed in each experimental unit over time (days) was analyzed by different models of saturated orthogonal multiple linear regression analysis (Box *et al.*,1978). These analysis were made by toxicant (BPA, Et, SH), and by assay. The assays were individually analyzed (assay 1 and assay 2) but also in ensemble. When the factors associated with the assay were not significant the two assays were tested in an ensemble analysis, without discriminating them because the differences were not significant. Thus, significant effects observed in one of the assays are highlighted if they are also significant in the ensemble analysis. However a significant effect in the ensemble analysis that isn't significant in the individual analyzed was more relevant than if it were only significant in the individual analyzed.

3.8.4.1. Presence/Absence Model

This regression model was used to test if the changes in the behavior profiles of the fish measured by the Distances Ethanol, BPA and SH were significantly correlated statistically with the presence or the absence of the respective toxicant in the water.

The independent variables were with and without toxicant in the water (ToxW), Day and Assay. The dependent variable was the Distance to the respective Toxicant vector and they were analyzed separately. The independent variables, were combined in a full factorial design, and were coded in orthogonal polynomial coefficients. The independent variable ToxW has the value +1 in the Moment After in the toxicant experimental units, and the value -1 in the remaining experimental units and times (Moment Before in the toxicant

experimental units and the two moments in the control experimental units). The independent variable Day, with 5 levels, one for each day analyzed, were decomposed in linear, quadratic and cubic terms, with a regressor for each one (table 2). The independent variable Assay takes the value -1 for assay 1 and +1 for assay 2. The subsequent 16 regressors were Intercept (b0), Assay, AssayD1, AssayD2, AssayD3, AssayToxW, AssayToxWD1, AssayToxWD2, AssayToxWD3, D1, D2, D3, ToxW, ToxWD1, ToxWD2, ToxWD3.

Table 2: Encoding of the independent variable Day.

Day	1	2	3	5	9
D1 (linear)	-2	-1	0	1	2
D2 (quadratic)	2	-1	-2	-1	2
D3 (cubic)	-1	2	0	-2	1

3.8.4.2. Moment/Control Model

This regression model was used to analyze the progress of the behavior response, in the moment Before and After for the control experimental units. The independent variables were the Moment, Day and Assay, the dependent variable was the distance to the respective Toxicant vector (Et, SH or BPA), in the control experimental units. These distances were analyzed separately in this model. The independent variables, were combined in a full factorial design, and were coded in orthogonal polynomial coefficients. The variable Moment has the value -1 in the experimental units before adding the toxicant and the value +1 after adding the toxicant. The two other independent variables Day and Assay were encoded in the same way as in the previous model (Presence/Absence model). The subsequent 16 regressors were Intercept (b0), Assay, AssayD1, AssayD2, AssayD3, Assay Moment, Assay MomentD1, Assay MomentD2, Assay MomentWD3, D1, D2, D3, Moment, MomentD1, MomentD2, MomentD3.

3.8.4.3. Moment/Toxicant Model

This regression model was used to analyze the progress of the behavior response, before and after adding each toxicant, in the experimental units with toxicant. The independent variables were the Moment, Day and Assay. The dependent variable was the distance to the respective Toxicant vector (Et, SH or BPA). Each of the toxicants was analyzed separately in this model. The distances to the Toxicant vector Et were used for the ethanol experimental units, the distances to the Toxicant vector BPA were used for the BPA experimental units, and the distances to the Toxicant vector SH were used for the SH experimental units. The independent variables, were combined in a full factorial design, and were coded in orthogonal polynomial coefficients. The three independent variables (Moment, Day and Assay) were encoded in the same way as in the previous model (Moment/Control). The resulting 16 regressors were equal to the regressors of the previous model (Moment/Control).

4. Results

4.1. Custer analysis

The cluster analysis was used in order to define behavior categories using the behavior of the fish exposed to the test conditions. In table 3 the error in the validation series (final error) of the cluster analysis is presented. In this analysis, the error was only of 0.064550, which represent only 6%.

Table 3: Error of each time series of the ANN.

Summary of active networks

Index	Net. name	Training error	Test error	Validation error	Training algorithm
1	SOM	0,063409	0,065490	0,064550	Kohonen 1000

The weight of each movement descriptor for each category is presented in table 4. However, to facilitate the analysis and the interpretation of these results it was necessary to use another type of statistical analysis.

Table 4: Weight of each movement descriptor in the 12 behavior categories.

	Weights spreadsheet Network: SOM											
Inputs	(1, 1)	(1, 2)	(1, 3)	(1, 4)	(2, 1)	(2, 2)	(2, 3)	(2, 4)	(3, 1)	(3, 2)	(3, 3)	(3, 4)
x	0,469460	0,666010	0,539894	0,195429	0,562658	0,561095	0,534147	0,172508	0,208168	0,434182	0,092717	0,453063
y	0,098187	0,805421	0,698500	0,282405	0,561844	0,562168	0,532942	0,288518	0,539331	0,665257	0,027285	0,460879
linear velocity	0,281267	0,358619	0,319665	0,280286	0,561900	0,563935	0,530978	0,238209	0,437431	0,635211	0,078665	0,676980
acceleration	0,518993	0,444219	0,675960	0,005767	0,561346	0,533199	0,536514	0,271894	0,256849	0,003001	0,244314	0,016565
angle°	0,565855	0,357991	0,786611	0,193541	0,560258	0,541036	0,497569	0,317780	0,437576	0,062912	0,013676	0,420385
angular velocity	0,485816	0,338952	0,870925	0,153768	0,563161	0,539872	0,507156	0,523804	0,340905	0,006682	0,013072	0,212205
angular acceleration	0,690898	0,530303	0,226620	0,021971	0,561535	0,526166	0,154548	0,545206	0,102200	0,006432	0,636553	0,061037
meander	0,650675	0,265406	0,176606	0,253030	0,561527	0,518319	0,438583	0,533915	0,440129	0,012027	0,239778	0,284600
stander deviation xy	0,842940	0,579657	0,300117	0,305368	0,561049	0,533653	0,297994	0,432318	0,622114	0,005288	0,688125	0,306079

The same data used in the cluster analysis, was introduced in the SPSS (SPSS 21 Software) program to execute other types of tests, such as the Post Hoc test.

4.2. Anova and post-Hoc Test

In table 5 are presented the results of the ANOVA conducted to compare the 9 movement descriptors (dependent variables) between the behavior categories. In the table is indicated that the significance values were lower than 5% (<0.05). These values indicate that the results between the categories were statistically significant in all variables analyzed, and therefore the null hypothesis (There aren't differences between the average values of the behavior categories) was rejected. Significant differences between the average values of the behavior categories were detected.

Table 5: Analysis of variance performed between behavior categories.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
X	Between Groups	66779340.617	11	6070849.147	28502.973	0.000
	Within Groups	29450344.329	138271	212.990		
	Total	96229684.946	138282			
Y	Between Groups	21299877.055	11	1936352.460	28739.619	0.000
	Within Groups	9316107.769	138271	67.376		
	Total	30615984.824	138282			
Linear velocity	Between Groups	37636484.643	11	3421498.604	14442.871	0.000
	Within Groups	32756232.888	138271	236.899		
	Total	70392717.530	138282			
Acceleration	Between Groups	624.208	11	56.746	4.145	.000
	Within Groups	1892775.213	138271	13.689		
	Total	1893399.421	138282			
Angle	Between Groups	353409.565	11	32128.142	225.398	0.000
	Within Groups	19709101.829	138271	142.540		
	Total	20062511.394	138282			
Angular Velocity	Between Groups	1181015626.015	11	107365056.910	38941.710	0.000
	Within Groups	381222958.178	138271	2757.071		
	Total	1562238584.193	138282			
Angular acceleration	Between Groups	29630861843.518	11	2693714713.047	39394.699	0.000
	Within Groups	9454638283.130	138271	68377.594		
	Total	39085500126.648	138282			
Meander	Between Groups	95017168.950	11	8637924.450	20625.191	0.000
	Within Groups	57908528.008	138271	418.805		
	Total	152925696.958	138282			
Stander deviation X/Y	Between Groups	7749203.875	11	704473.080	52054.357	0.000
	Within Groups	1871278.477	138271	13.533		
	Total	9620482.353	138282			

Post Hoc tests are a posteriori analysis that in this case, has the purpose to find patterns (relationships) between groups or subgroups of data. Homogeneous subset is the same test with a different data organization that helps to interpret the results in a more intuitive way. The homogeneous subset is done by different

statistical tests such as the Scheffe's or Tukey's test, and they help the analysis by pairing of means to see if there is a difference. In this case although the two tests were performed, the Scheffe's test was used because it was a more conservative multiple comparisons technique.

Table 6: Summary of the homogeneous group analysis for the movement descriptors, average value of coordinate x and y, and linear velocity. The numbering indicates the subsets of each behavior category.

		95% Confidence Interval for Average			95% Confidence Interval for Average			95% Confidence Interval for Average	
Categories	Average value of X (mm)	Lower Bound	Upper Bound	Average value of Y (mm)	Lower Bound	Upper Bound	Linear Velocity (mm/s)	Lower Bound	Upper Bound
1.1	72.288[1]	72.163	72.414	39.974[1]	39.904	40.043	47.837[1]	47.723	47.951
1.2	22.615[2]	22.212	23.018	33.837[2]	33.492	34.183	35.856[2]	35.065	36.647
1.3	46.582[3]	46.303	46.861	32.165[3]	31.988	32.342	63.516[3]	63.227	63.805
2.1	78.538[4]	78.375	78.701	46.373[4]	46.273	46.474	41.491[4]	41.341	41.641
2.2	84.736[5]	84.395	85.077	26.826[5]	26.623	27.030	59.906[5]	59.484	60.328
2.3	74.676[6]	74.452	74.900	49.900[6]	49.794	50.006	59.697[5]	59.488	59.907
3.1	101.748[7]	101.232	102.264	46.966[7]	46.578	47.355	1.228[6]	1.165	1.292
3.2	96.730[8]	96.451	97.010	58.748[8]	58.601	58.895	41.132[4]	40.841	41.425
3.3	122.279[9]	121.956	122.602	30.778[9]	30.551	31.005	32.198[7]	31.643	32.753
4.1	98.522[10]	98.049	98.994	57.247[10]	57.025	57.469	4.725[8]	4.580	4.869
4.2	117.208[11]	116.948	117.469	65.362[11]	65.215	65.510	53.271[9]	52.912	53.630
4.3	57.161[12]	56.754	57.568	71.543[12]	71.440	71.645	64.081[3]	63.587	64.575

The results obtained in the Scheffe's test are compiled in tables 6, 7 and 8. The average value of the x coordinate and the average value y were the movement descriptors that had maximum ability to discriminate the behavior categories, because all the values for this two movement descriptors were significantly different. The coordinates of the center of the arena were 88 mm and 50 mm (x,y), which means that values much inferior or superior to these indicates that the fish were close to the walls of the arena. The behavior category 1.2 for the movement descriptor average value of x coordinate presented the lowest value 22.615 mm which indicates that the fish were frequently in the left side of the arena, close to the glass of the arena and the category 3.3 presented the highest value 122.279 mm which indicates that the fish were frequently in the right side of the arena, close to the glass. The behavior category 2.2 for the movement descriptor average value of Y coordinate presented the lowest value 26.826 mm which indicates that the fish were frequently in the underside of the

arena close to the glass. The category 4.3 presented the highest value 71.543 mm which indicates that the fish were frequently on the top side of the arena, close to the glass. The analysis of the movement descriptor linear velocity revealed that the behavior category 4.3 had the highest value (64.081 mm/s), followed by the behavior category 1.3 (63.516 mm/s). The behavior categories 3.1 and 4.1 presented the two lowest linear velocities, 1.228 mm/s and 4.725 mm/s respectively. The linear velocity had 9 subsets in total which reveals a good discriminatory capacity of this movement descriptor.

Table 7: Summary of the homogeneous group analysis for the movement descriptors, linear acceleration, angle and angular velocity. The numbering indicates the subsets of each behavior category.

		95% Confidence Interval for Average			95% Confidence Interval for Average			95% Confidence Interval for Average	
Categories	Linear Acceleration (mm/s^2)	Lower Bound	Upper Bound	Angle (degrees)	Lower Bound	Upper Bound	Angular Velocity (degrees/s)	Lower Bound	Upper Bound
1.1	0.019[1]	-0.017	0.055	0.811[1 2 3]	0.706	0.916	105.010[1]	104.743	105.277
1.2	-0.042[1]	-0.159	0.075	1.735[4]	1.348	2.121	291.875[2]	289.210	294.540
1.3	0.042[1]	-0.050	0.134	1.491[3 4]	1.272	1.710	203.713[3]	202.681	204.744
2.1	-0.056[1]	-0.097	-0.015	-0.281[5]	-0.416	-0.147	117.173[4]	116.787	117.558
2.2	-0.112[1]	-0.210	-0.014	-1.485[6]	-1.723	-1.248	197.229[5]	196.107	198.351
2.3	0.106[1]	0.044	0.168	1.033[2 3 4]	0.881	1.186	162.942[6]	162.341	163.544
3.1	-0.027[1]	-0.067	0.013	0.900[1 2 3]	0.678	1.122	185.474[7]	183.219	187.730
3.2	-0.062[1]	-0.137	0.013	0.623[1 2]	0.430	0.817	217.375[8]	216.409	218.340
3.3	-0.030[1]	-0.115	0.055	0.192[1 5]	-0.157	0.540	357.258[9]	355.463	359.053
4.1	-0.080[1]	-0.150	-0.010	1.210[2 3 4]	0.924	1.496	372.292[10]	370.686	373.898
4.2	0.070[1]	-0.007	0.146	-4.010[7]	-4.375	-3.646	363.324[11]	362.125	364.523
4.3	0.095[1]	0.000049	0.190	-2.691[8]	-3.041	-2.342	293.769[2]	292.302	295.236

The behavior categories relatively to the linear acceleration in the case of the Scheffe's test were in the same group for all categories, which indicates that this movement descriptor was uninformative. For the movement descriptor angle the behavior categories 1.2 and 1.3 presented the highest values, 1.735 degrees and 1.491 degrees respectively which indicate that the fish in this category turned predominantly to the right. The behavior categories 4.2 and 4.3 for the movement descriptor angle presented the two the two lowest values, -4.010 degrees and -2.691 degrees respectively which indicate that the fish in this category turned predominantly to the left. This descriptor movement revealed a good discriminatory capacity because it had 8 subsets in total. For the angular

velocity the categories 4.1 and 4.2 presented the two highest values, 372.292 degrees/s and 363.324 degrees/s respectively which indicate that the fish turned at high speed. The categories 1.1 and 2.1 presented the two lowest values for the angular velocity, 105.010 degrees/s and 117.173 degrees/s respectively which indicate that the fish turned at low speed.

Table 8: Summary of the homogeneous group analysis for the movement descriptors, angular acceleration, meander and standard deviation of X/Y coordinates. The numbering indicates the subsets of each behavior category.

		95% Confidence Interval for Average			95% Confidence Interval for Average			95% Confidence Interval for Average	
Categories	Angular acceleration (degrees/s^2)	Lower Bound	Upper Bound	Meander (degrees/mm)	Lower Bound	Upper Bound	Stander deviation of X/Y(mm)	Lower Bound	Upper Bound
1.1	585.417[1]	583.715	587.120	1.460[1]	1.433	1.488	23.787[1]	23.752	23.822
1.2	1468.710[2]	1449.456	1487.963	30.228[2]	28.911	31.544	8.923[2]	8.737	9.108
1.3	1236.015[3 4]	1230.365	1241.665	3.187[3]	3.110	3.264	25.616[3]	25.537	25.695
2.1	723.110[5]	720.495	725.724	2.964[3]	2.884	3.043	16.958[4]	16.920	16.997
2.2	1234.596[3 4]	1229.219	1239.972	5.742[4]	5.499	5.985	17.156[4]	17.058	17.254
2.3	962.019[6]	958.510	965.528	2.510[1 3]	2.437	2.583	25.191[5]	25.139	25.243
3.1	283.705[7]	277.909	289.501	45.668[5]	44.669	46.666	0.627[6]	0.584	0.670
3.2	1244.809[3]	1240.097	1249.521	13.345[6]	12.986	13.704	15.720[7]	15.637	15.802
3.3	1741.866[8]	1734.202	1749.530	38.547[7]	37.391	39.703	7.998[8]	7.886	8.110
4.1	1222.722[4]	1213.206	1232.238	118.952[8]	117.742	120.162	2.306[9]	2.238	2.374
4.2	1873.655[9]	1869.198	1878.112	6.595[4]	6.414	6.775	10.559[10]	10.486	10.633
4.3	1784.101[10]	1778.087	1790.116	6.266[4]	6.027	6.505	11.366[11]	11.279	11.452

For the angular acceleration the categories 4.2 and 4.3 presented the two highest values, 1873.655 degrees/s^2 and 1784.101 degrees/s^2 respectively. The categories 1.1 and 3.1 presented the two lowest values for the angular acceleration, 585.417 degrees/s^2 and 283.705 degrees/s^2 respectively. This movement descriptor revealed a great discriminatory capacity because it had 10 subsets in total. For the meander the behavior category 4.1 and had highest value, 118.952 degrees/mm followed by the category 3.1 with 45.668 degrees/mm. These values indicate that the fish has a pattern of movements composed by many changes of directions. The categories 1.1 and 2.3 presented the two lowest values for the meander, 1.460 degrees/mm and 2.510 degrees/mm respectively which indicate that the fish has a pattern of movements composed by few changes in direction with more straight trajectories. The meander had 8 subsets in total, which reveals a good discriminatory capacity of

this movement descriptor. For the standard deviation of X/Y the categories 1.3 and 2.3 presented the two highest values, 25.616 mm and 25.191 mm respectively which indicates that the fish circulated throughout the arena. The categories 3.1 and 4.1 presented the two lowest values for the standard deviation of X/Y, 0.627 mm and 2.306 mm respectively which indicates that the fish were frequently in a very small space of the arena. This movement descriptor and the angular velocity had the second most discriminative capacity with 11 subsets in each one.

4.3. Correspondence Analysis

Using different partitions of the data in terms of day, assay and toxicant several correspondence analyses were performed. The objective of the correspondence analysis was to have quantifiable measure of the effect caused by the toxicant.

The Chi-squared test (appendix) of all the analyzes carried by day and assay (10 in total, because of the 5 recorded days and 2 assays) ranged between 1587.4 and 8446.6, with 30-33 has a degree of freedom (df) (on day 5 of the first assay the df was 30 because one of the behavior categories had zero occurrences) and a significance level (p) of less than 0.0000 for all analyzes. This means that the difference of the frequency profile of the behavior categories (Row variable in the correspondence analysis) between the different types of experimental units analyzed (W, BPA, Et and SH; Column variable in the correspondence analysis) was statistically significant.

4.4. Presence/Absence Model

In this regression, the objective was to analyze if the fish behavior (measured by the Distances Ethanol, BPA and SH) was linked statistically with the presence or the absence of each toxicant. The results regarding ethanol, BPA and SH are presented in tables 9, 10 and 11, respectively and in figures 9, 10 and 11.

In this regression the linear, quadratic and cubic term of the variable day was analyzed, however these factors were gradually eliminated until the regression was significant. This procedure made the model simpler, which increased the degrees of freedom and the quality of the model.

All regressions were statistically significant. The factor Assay was never statistically significant, and in this case the two assays were treated as if they were one. The advantage of doing this was that the model became simpler and accurate because fewer factors were used, and the degrees of freedom for error increased. All the analysis with the factor Assay (Ethanol, $F(15,104)=2.8192$ $p<0.00103$; BPA, $F(15,104)=3.7291$ $p<0.00003$; SH, $F(15,104)=3.8243$ $p<0.00002$) had worse results than the two assays together (Ethanol, $F(7,112)=6.1989$ $p<0.00000$; BPA, $F(7,112)=6.2933$ $p<0.00000$; SH, $F(5,114)=11.853$ $p<0.00000$).

Table 9: Regression analysis Presence/Absence of the dependent variable Distance Ethanol, considering the two assays as one. Statistically significant regressors ($p \leq 0.05$), appear in red.

	F(7,112)=6,1989 p<0,00000 Std.Error of estimate: 1,02					
N= 120	b*	Std.Err. (of b*)	b	Std.Err. (of b)	t(112)	p-value
Intercept			0.4572	0.1085	4.2142	0.0001
ToxW	0.5200	0.0802	0.7032	0.1085	6.4822	0.0000
ToxWD1	-0.0424	0.0926	-0.0351	0.0767	-0.4573	0.6484
ToxWD2	-0.0395	0.0926	-0.0277	0.0648	-0.4268	0.6703
D1	0.0351	0.0926	0.0291	0.0767	0.3789	0.7055
D2	-0.0343	0.0926	-0.0240	0.0648	-0.3702	0.7119
ToxWD3	0.0440	0.0926	0.0365	0.0767	0.4752	0.6356
D3	0.0606	0.0926	0.0502	0.0767	0.6546	0.5141

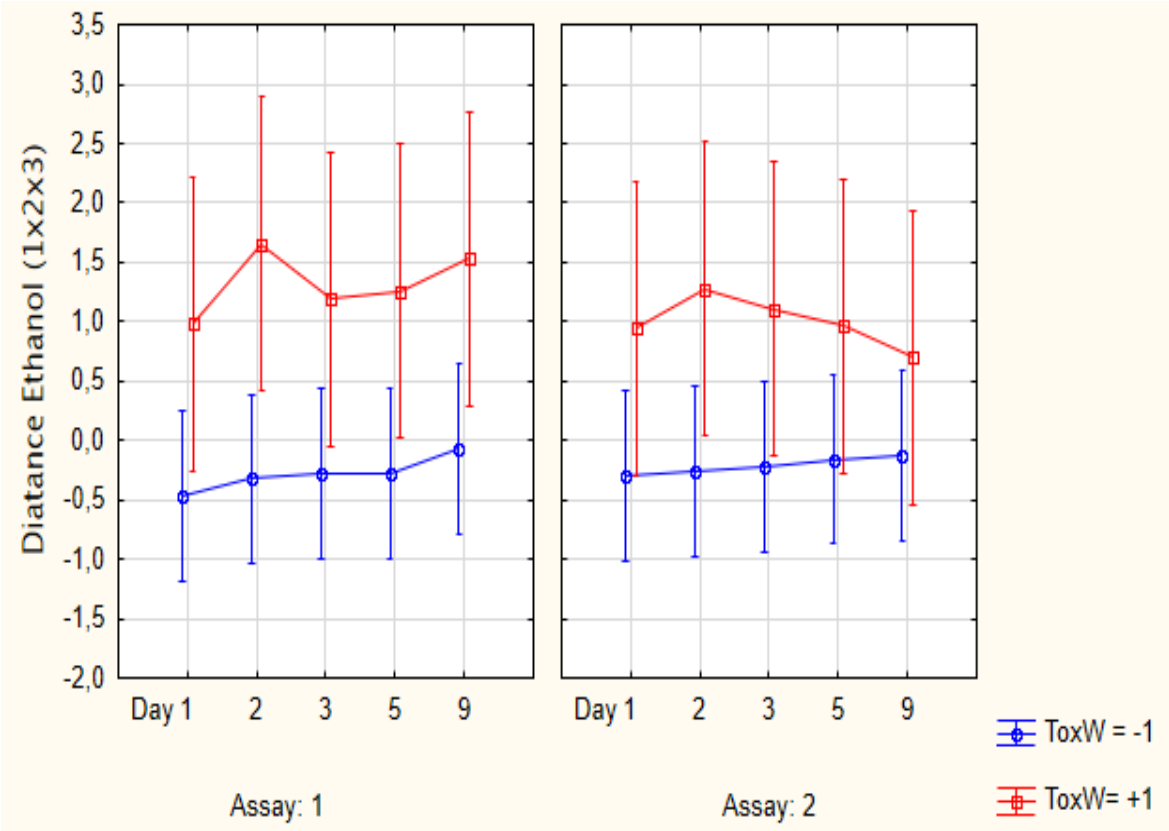

Figure 9: Representation of the dependent variable Distance Ethanol, in function of the Assay, Day and ToxW (Presence/Absence model). Vertical bars denote 0.95 confidence intervals. ToxW=+1, represents the experimental units with toxicant. ToxW=-1 represents the experimental units without toxicant.

The regressor ToxW was statistically significant in all analyzes, with significance levels always below 0.0000 (tables 9, 10 and 11). This means that the fish behavior with and without the toxicants was statistically different. The regression coefficient (b) of this regressor was positive in all the cases, which indicates that the corresponding dependent variables (Distance Ethanol, BPA and SH) had, on average, higher values when the toxicant was present in the experimental units, than when it was absent.

Table 10: Regression analysis Presence/Absence of the dependent variable Distance BPA, considering the two assays as one. Statistically significant regressors (p≤0.05), appear in red.

N=120	F(7,112)=6,2933 p<,00000 Std.Error of estimate: 1,0350					
	b*	Std.Err. (of b*)	b	Std.Err. (of b)	t(112)	p-value
Intercept			0.4552	0.1091	4.1724	0.0001
ToxW	0.4861	0.0801	0.6624	0.1091	6.0720	0.0000
ToxWD1	-0.2218	0.0924	-0.1851	0.0771	-2.3997	0.0181
ToxWD2	-0.0390	0.0924	-0.0275	0.0652	-0.4223	0.6736
D1	-0.0351	0.0924	-0.0293	0.0771	-0.3797	0.7049
D2	-0.0096	0.0924	-0.0068	0.0652	-0.1043	0.9171
ToxWD3	-0.0355	0.0924	-0.0296	0.0771	-0.3838	0.7018
D3	-0.0525	0.0924	-0.0438	0.0771	-0.5678	0.5713

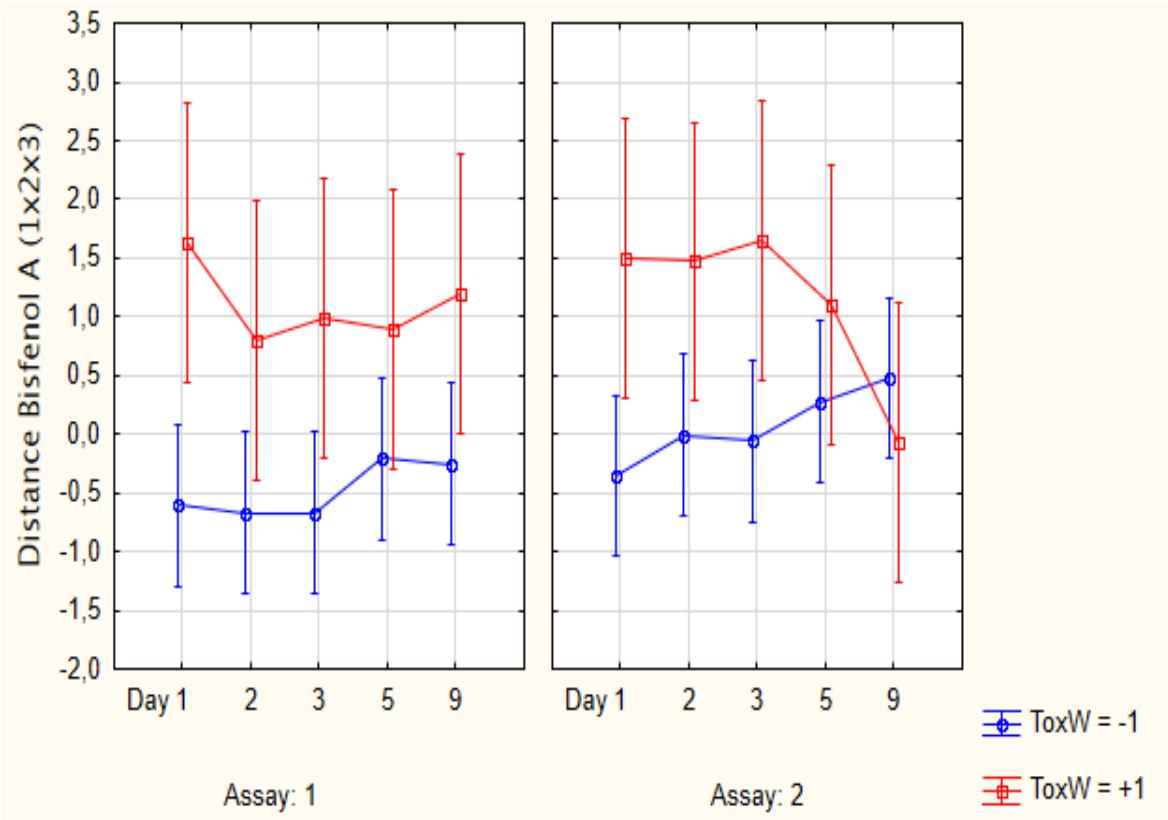

Figure 10: Representation of the dependent variable Distance BPA, in function of the Assay, Day and ToxW (Presence/Absence model). Vertical bars denote 0.95 confidence intervals. ToxW=+1, represents the experimental units with toxicant. ToxW=-1 represents the experimental units without toxicant.

The regressor ToxWD1 in the regressions for the dependent variables Distances BPA (b=-0.1851) and SH (b=-0.1499) was also statistically significant. As their regression coefficients were negative, this indicated that the ToxW effect

described above decreased linearly over the days in both cases. In this two cases, the values of the Distances, decreased over the days in the Moment After (ToxW=+1), and increased over the days in the Moment Before and in the controls (ToxW=-1). This tendency is visible in figures 10 and 11.

Table 11: Regression analysis Presence/Absence of the dependent variable Distance SH, considering the two assays as one. Statistically significant regressors (p≤0.05), appear in red.

	F(5,114)=11,853 p<,00000 Std.Error of estimate: 0,97509					
N=120	b*	Std.Err. (of b*)	b	Std.Err. (of b)	t(114)	p-value
Intercept			0.5297	0.1028	5.1539	0.0000
ToxW	0.5581	0.0760	0.7551	0.1028	7.3462	0.0000
ToxWD1	-0.1749	0.0877	-0.1449	0.0727	-1.9932	0.0486
ToxWD2	0.0378	0.0877	0.0265	0.0614	0.4313	0.6670
D1	-0.0754	0.0877	-0.0624	0.0727	-0.8591	0.3921
D2	0.0990	0.0877	0.0693	0.0614	1.1288	0.2613

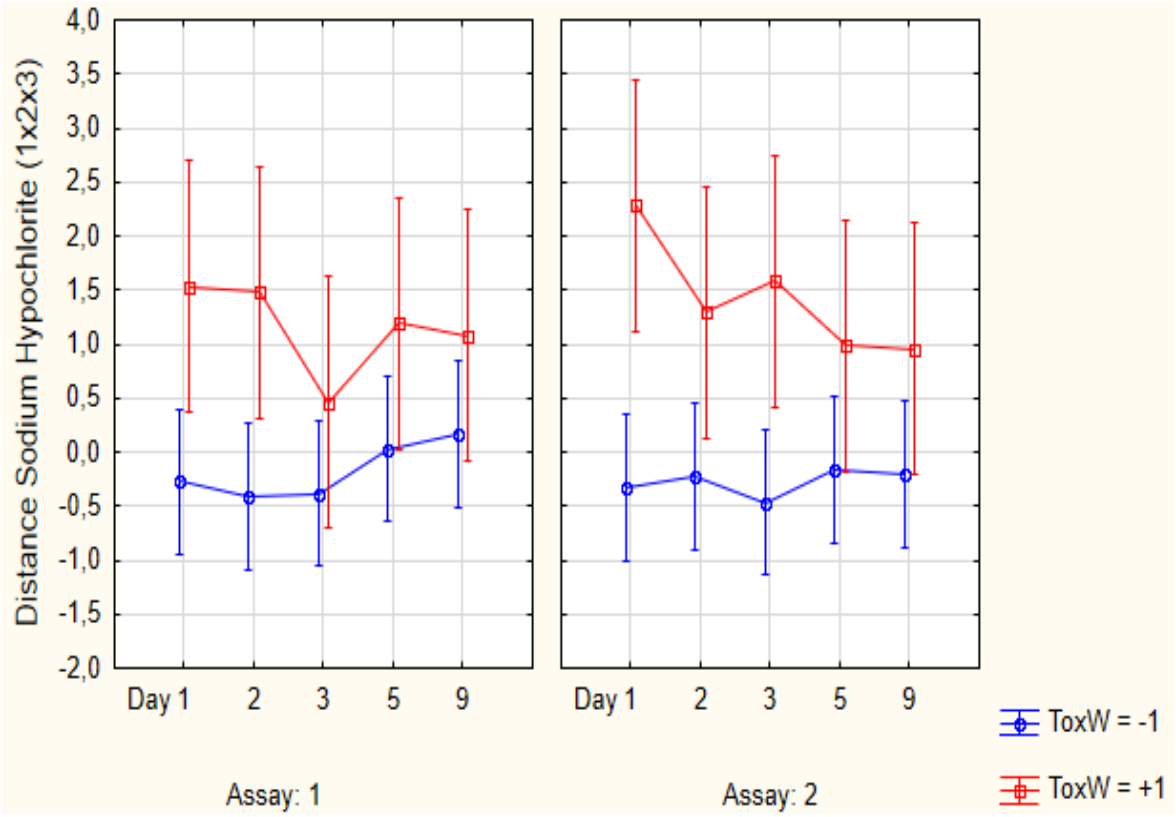

Figure 11: Representation of the dependent variable Distance SH, in function of the Assay, Day and ToxW (Presence/Absence model). Vertical bars denote 0.95 confidence intervals. ToxW=+1, represents the experimental units with toxicant. ToxW=-1 represents the experimental units without toxicant.

The standardized regression coefficients values, a standardized regression coefficient (b*) that is a standard measure that enables the comparison between different toxicants, indicated that in relative terms the effect measured by ToxW (adding the toxicant) was strongest with SH (b*=0.558) than with ethanol (b*= 0.520) and BPA (b*=0.486). The decrease of this effect over days (ToxWD1) was more pronounced with BPA (b*= -0.222) than with SH (b*= -0.175).

Nevertheless, it would be interesting to understand some aspects of the fish behavior from the repeated exposure to the toxicants, including the progress of the response, before and after adding each toxicant. For this reason this analysis was performed and its results are presented below.

4.5. Moment/Control Model

This regression model had the objective of understanding the progress of the behavior response, in the moment Before and After for the control experimental units. In this regression the linear, quadratic and cubic term of the variable was also analyzed, however as in the previous analysis, these factors were gradually eliminated until the regression was significant. As in the previous model, when the factor Assay was not statistically significant, the two assays were treated as if they were one. In this model, the dependent variables (Distances BPA, SH and Ethanol), registered only in the control groups, were analyzed based on moment Before and After (Moment), the assay (Assay) and also based in the repetition (Day). The regression analysis for dependent Variable SH in the controls is shown in table 12. The distances SH for the controls are represented in figure 12.

Table 12: Regression analysis (Moment/Control) of the dependent variable Distance SH in the control group considering the two assays as one. Statistically significant regressors ($p \leq 0.05$), appear in red.

	F(3,56)=,21727 **p<0,88402** Std.Error of estimate: 0,89416					
N=60	b*	Std.Err. (of b*)	b	Std.Err. (of b)	t(56)	p-value
Intercept			-0.4294	0.1154	-3.7199	0.0005
Moment	-0.0193	0.1329	-0.0168	0.1154	-0.1454	0.8849
D1	-0.0155	0.1329	-0.0095	0.0816	-0.1167	0.9075
MomentD1	-0.1044	0.1329	-0.0641	0.0816	-0.7855	0.4355

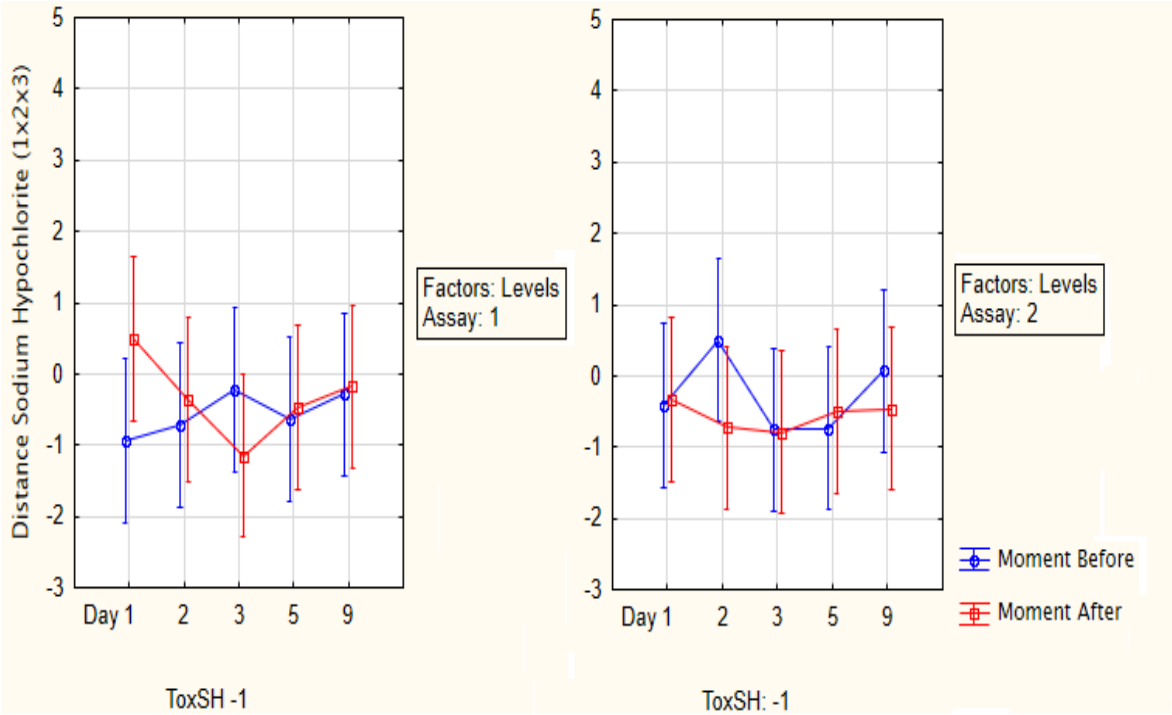

Figure 12: Representation of the dependent variable Distance SH in the control group, in function of the Assay, Moment and Day (Moment/Control model). Vertical bars denote 0.95 confidence intervals.

The dependent variable Distance SH in the control groups (table 11) didn't show any statistically significant trend in relation to the analyzed factors and the interaction between them. The significance levels were always above 0.05 in both assays, separately (data not represented) and together, for all regressors analyzed. This result indicate that there weren't differences between the Moment Before, and the Moment After in the controls ($p < 0.88402$) and also that the factors Assay and Day did not influenced the behavior as it can be seen in figure 12. The interactions between all the factors were also not statistically significant.

The regression analysis for dependent variable BPA in the controls is sown in table 13. The distances BPA for the controls are represented in figure 13.

Table 13: Regression analysis (Moment/Control) of the dependent variable Distance BPA in the control group, considering the factor Assay. Statistically significant regressors ($p \leq 0.05$), appear in red.

N=60	F(7,52)=2,3354 p<0,03765 Std.Error of estimate: 0,92967					
	b*	Std.Err. (of b*)	b	Std.Err. (of b)	t(52)	p-value
Intercept			-0.3518	0.1200	-2.9309	0.0050
Assay	0.3932	0.1210	0.3901	0.1200	3.2505	0.0020
AssayMoment	-0.0746	0.1210	-0.0740	0.1200	-0.6164	0.5403
AssayD1	0.1239	0.1210	0.0870	0.0849	1.0246	0.3103
AssayMomentD1	0.1533	0.1210	0.1075	0.0849	1.2670	0.2108
Moment	-0.0564	0.1210	-0.0560	0.1200	-0.4663	0.6430
D1	0.1865	0.1210	0.1308	0.0849	1.5417	0.1292
AssayD1	0.0473	0.1210	0.0332	0.0849	0.3912	0.6972

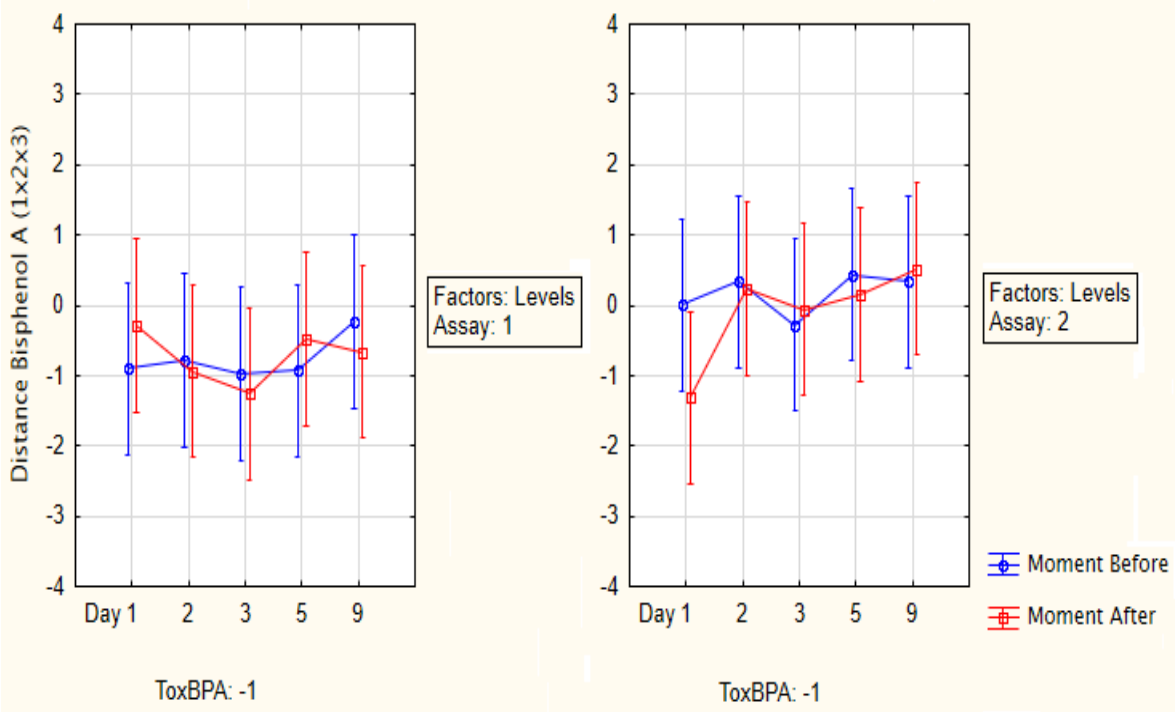

Figure 13: Representation of the dependent variable Distance BPA in the control group, in function of the Assay, Moment, and Day (Moment/Control model). Vertical bars denote 0.95 confidence intervals.

In the case of BPA, because the regressor Assay was statistically significant, the factor Assay had to be considerate. The control groups presented values of Distance BPA (table 13), on average, higher in assay 2 than in assay 1 (b_{Assay}=0.3901; sig=0.0002) but this measure was not significantly influenced by any other factor analyzed, and the interaction between them. As in the previous

case, differences between the Moment Before and the Moment After in the controls were not detected, and also that the factor Day did not influenced the behavior as it can be seen in figure 13. The interactions between all the factors were also not statistically significant.

The regression analysis for dependent variable Ethanol in the controls is demonstrated in table 14. The distances Ethanol for the controls are represented in figure 14.

Table 14: Regression analysis (Moment/Control) of the dependent variable Distance Ethanol in the control group, considering the factor Assay. Statistically significant regressors ($p \leq 0.05$), appear in red.

	F(4,55)=3,1104 p<0,02228 Std.Error of estimate: 0,76979					
N=60	b*	Std.Err. (of b*)	b	Std.Err. (of b)	t(55)	p-value
Intercept			-0.5658	0.0994	-5.6937	0.0000
AssayMomentD1	0.2626	0.1218	0.1516	0.0703	2.1569	0.0354
Moment	-0.2728	0.1218	-0.2226	0.0994	-2.2400	0.0292
D1	0.0616	0.1218	0.0356	0.0703	0.5062	0.6148
MomentD1	0.1931	0.1218	0.1115	0.0703	1.5861	0.1184

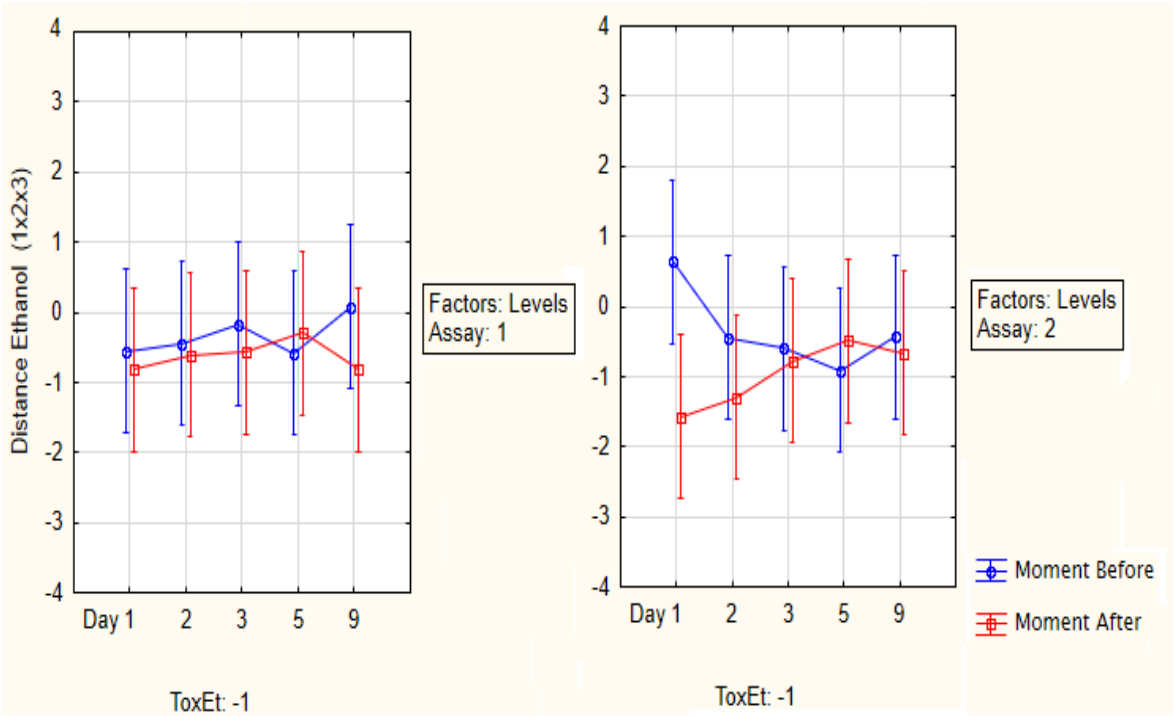

Figure 14: Representation of the dependent variable Distance Ethanol in the control group, in function of the Assay, Moment and Day (Moment/Control model). Vertical bars denote 0.95 confidence intervals.

In the measure Distance Ethanol, the control fish did not present values with an evolution as neutral as in the previous toxicants. The Before and After effect (Moment) was statistically significant and negative (b_{Moment} = -0.2226, sig = 0.0292), which means that the measure Distance Ethanol decreased between the moment Before and After in this group of fish (table 14). But this difference tends to decrease over the days ($b_{AssayMomentD1}$=0.1516, sig=0.0354). This tendency is particularly visible in assay 2 being practically zero on the last day (figure 14).

4.6. Moment/Toxicant Model

This regression model had the objective of understanding the progress of the behavior response, before and after adding each toxicant, in the toxicant experimental units. In this regression the linear, quadratic and cubic term of the variable was also analyzed, however as in previous analyzes, these factors were gradually eliminated until the regression was significant. As in previous models, when the factor Assay was not statistically significant, the two assays were treated as if they were one. In this model, the dependent variables (Distance BPA, SH and Ethanol), registered only in the toxicant groups, were analyzed based on moment Before and After (Moment), the assay (Assay) and also based in the repetition (Day).

With all the toxicants, the respective measures (Distance) of the exposed fish were not influenced by the factor assay throughout their range (table 15, 16 and 17). No differences were detected between the two assays, and the factor assay didn't affect the other factors.

In all toxicant groups the factor Moment influenced in a statistically significant way (and in this case positive way), the respective average Distance (Ethanol, b=0.3834, sig=0.0061; BPA, b=0.5179, sig=0.0004; SH, b=0.5510, sig=0.0001), which means that the Distances of the Moment After were higher than the Distances of the Moment Before (tables 15, 16 and 17).

Table 15: Regression analysis (Moment/Toxicant) of the dependent variable Distance Ethanol in the ethanol group, considering the two assays as one. Statistically significant regressors (p≤0.05), appear in red.

N=60	F(3,56)=2,9783 p<0,03909 Std.Error of estimate: 1,0422					
	b*	Std.Err. (of b*)	b	Std.Err. (of b)	t(56)	p-value
Intercept			0.7770	0.1345	5.7750	0.0000
Moment	0.3536	0.1241	0.3834	0.1345	2.8496	0.0061
D1	0.0752	0.1241	0.0576	0.0951	0.6058	0.5471
MomentD1	-0.0830	0.1241	-0.0636	0.0951	-0.6690	0.5062

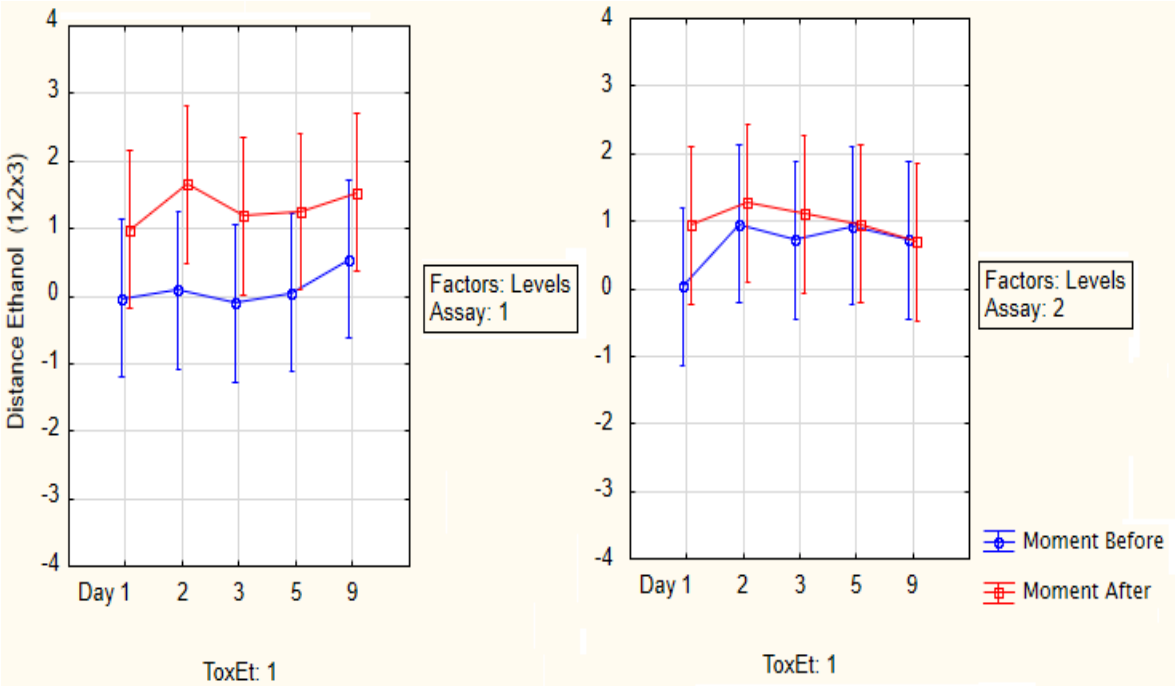

Figure 15: Representation of the dependent variable Distance Ethanol in the ethanol group, in function of the Assay, Moment and Day (Moment/Toxicant model). Vertical bars denote 0.95 confidence intervals.

For ethanol the differences between the Moment Before and After adding ethanol, were similar in all the days ($b_{MomentD1}$=-0.0636, sig=0.5062), especially in assay 1 as is shown in figure 15.

Table 16: Regression analysis (Moment/Toxicant) of the dependent variable Distance BPA in the BPA group, considering the two assays as one. Statistically significant regressors ($p \leq 0.05$), appear in red.

	F(7,52)=2,9264 p<0,01167 Std.Error of estimate: 1,0567					
N=60	b*	Std.Err. (of b*)	b	Std.Err. (of b)	t(52)	p-value
Intercept			0.5997	0.1364	4.3963	0.0001
Moment	0.4459	0.1175	0.5179	0.1364	3.7966	0.0004
D1	-0.0052	0.1175	-0.0043	0.0965	-0.0447	0.9645
D2	-0.0322	0.1175	-0.0223	0.0815	-0.2738	0.7853
MomentD1	-0.2558	0.1175	-0.2101	0.0965	-2.1782	0.0340
MomentD2	-0.0173	0.1175	-0.0120	0.0815	-0.1474	0.8834
D3	-0.1251	0.1175	-0.1028	0.0965	-1.0654	0.2916
MomentD3	0.0357	0.1175	0.0294	0.0965	0.3043	0.7621

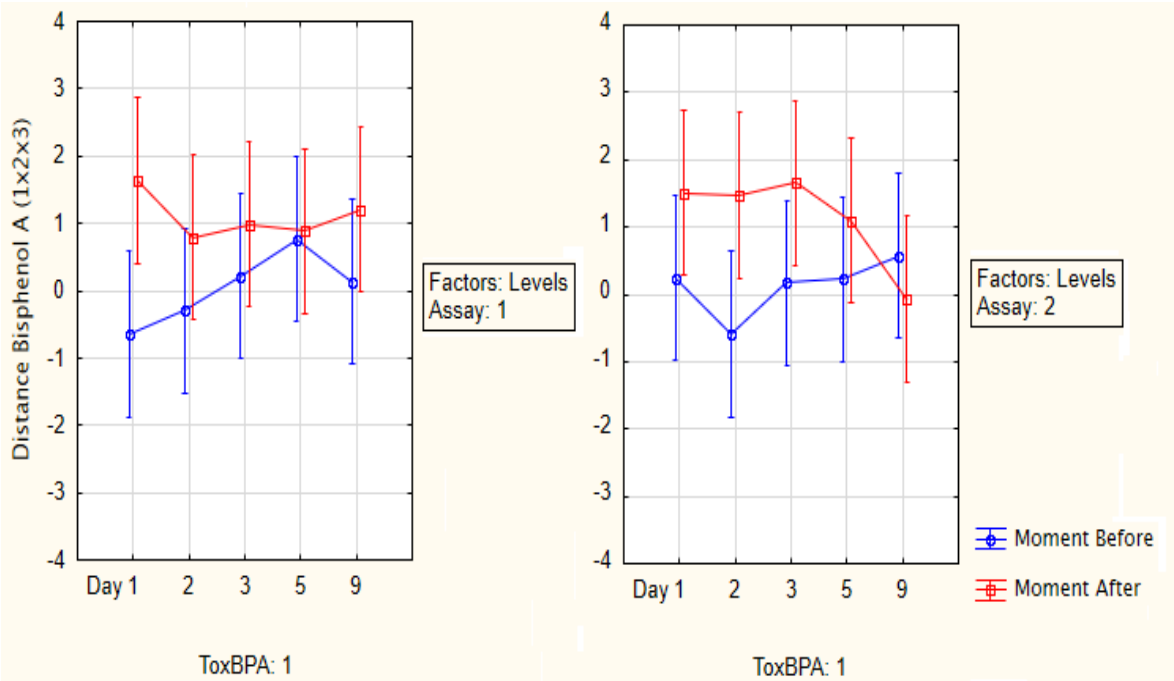

Figure 16: Representation of the dependent variable Distance BPA in the BPA group, in function of the Assay, Moment and Day (Moment/Toxicant model). Vertical bars denote 0.95 confidence intervals.

For BPA and SH the effect of the factor Moment tends to decrease with days (BPA, $b_{MomentD1} = -0.2101$, sig=0.0340; SH, $b_{MomentD1} = -0.2368$, sig=0.0112) (tables 16 and 17) reaching practically zero in the last days. This tendency is particularly visible in assay 2 for BPA (figure 16) and in assay 1 for SH (figure 17).

Table 17: Regression analysis (Moment/Toxicant) of the dependent variable Distance SH in the SH group, considering the two assays as one. Statistically significant regressors ($p \leq 0.05$), appear in red.

N=60	F(7,52)=4,1648 p<0,00105 Std.Error of estimate: 0,98571					
	b*	Std.Err. (of b*)	b	Std.Err. (of b)	t(52)	p-value
Intercept			0.7338	0.1273	5.7666	0.0000
Moment	0.4806	0.1110	0.5510	0.1273	4.3298	0.0001
D1	0.0364	0.1110	0.0295	0.0900	0.3280	0.7443
MomentD1	-0.2921	0.1110	-0.2368	0.0900	-2.6318	0.0112
D2	0.0239	0.1110	0.0164	0.0760	0.2151	0.8305
MomentD2	0.1160	0.1110	0.0795	0.0760	1.0450	0.3008
D3	-0.1337	0.1110	-0.1084	0.0900	-1.2047	0.2338
MomentD3	0.0982	0.1110	0.0796	0.0900	0.8847	0.3804

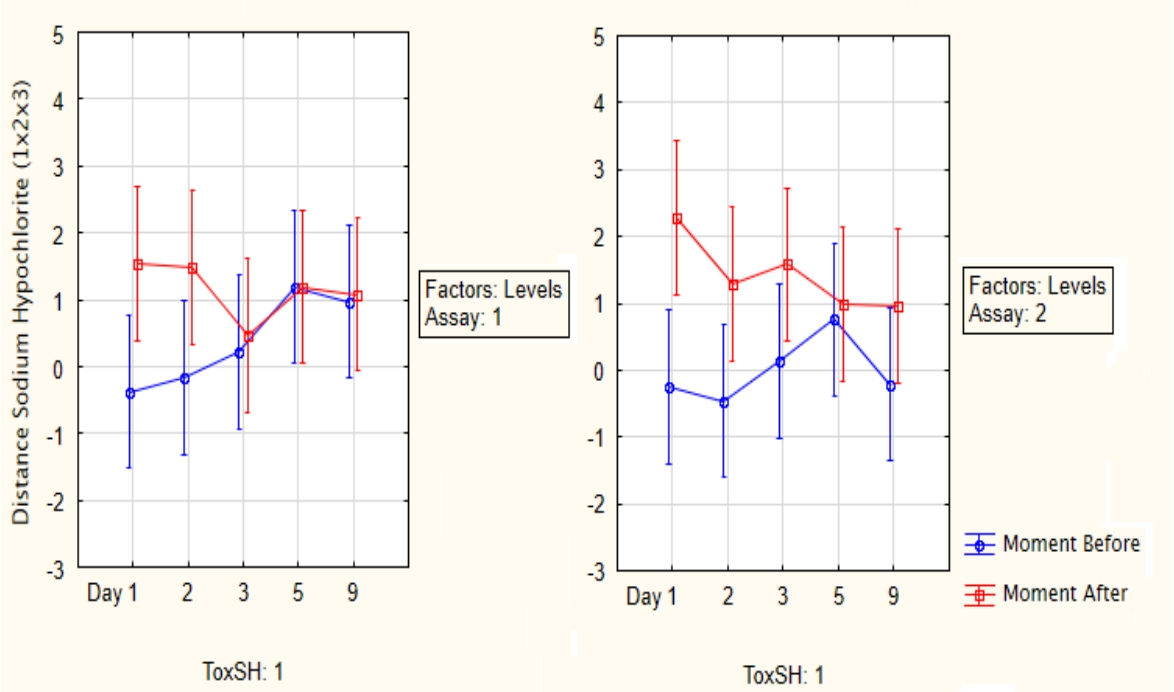

Figure 17: Representation of the dependent variable Distance SH in the SH group, in function of the Assay, Moment and Day (Moment/Toxicant model). Vertical bars denote 0.95 confidence intervals.

5. Discussion

5.1. Characterization of the Behavior Categories

Using the cluster analysis it was possible to define 12 behavior categories of the fish submitted to the different experimental conditions. The 6% error of the validation series of the cluster analysis (table 3) was minimal and represented the quality of the cluster analysis, the precision quality of the system. The ANOVA and the post-Hoc test allowed realizing that these behavior categories had statistically significant differences, and that all the categories were different from each other.

Due to the massive amount of data and especially the number of days used in the analysis, it was difficult to establish associations between the behavior categories with each toxicant throughout the days, but the post-hoc test permitted the characterization of the behavior categories based on the movement descriptors. The categories 3.1 and 4.1 presented the two lowest linear velocities (1.228 mm/s and 4.580 mm/s respectively), the two lowest standard deviation x/y (0.627 mm and 2.238 mm respectively), the two highest meander ((45.668 rad/mm and 118.95 respectively) and in the case of the category 4.1 the highest angular velocity (372.292 rad/s). Curiously because the Angle values were positive the fish in these two categories also turned predominantly to the right. These results indicated that the fish in those categories presented slow movement and probably more stops, with low dispersion in space and many changes of direction that in the case of the category 4.1 were done at high angular velocity. When in states of heighted anxiety the zebrafish specie tends to display erratic movement (many changes of direction in a relatively small space), and more stops (freezing, when stationary) (Egan *et al.*, 2009). Base on the movement descriptors the categories 3.1 and 4.1, fit in this type of behaviors, and several substances as acute caffeine and alarm pheromone had be proven to triggered these behaviors in zebrafish (Egan *et al.*, 2009). In a previous work (for publication) acutely exposed zebrafish to SH (1h30m), also presented this type of behavior (freezing, increased changes of direction, decreased velocity).

The categories 1.3, 2.2 and 2.3 had high linear velocities (63.227 mm/s, 59.906 mm/s and 59.697 mm/s respectively), low meander values (3.187

rad/mm, 5.742 rad/mm and 2.510 rad/mm respectively) and high standard deviations of x/y (25.616 mm, 17.156 mm and 25.191 mm respectively) and these parameters indicate fast movements and less stops with few changes of direction and high dispersion in space. These types of behaviors are usually related with normal exploratory behavior and activity.

The categories described in this section were the most distinguishable and for that reason were highlighted, as stated above it was impossible to establish associations between the behavior categories with each toxicant throughout the days, but this was not one of the objectives of the work.

5.2. Analysis of the Regressions

The results obtained by the Presence/Absence model (table 9, 10 and 11) were within the expectations, all the differences between the Distances BPA, Ethanol and SH were statistically significantly and detected the presence of the respective toxicant in water (ethanol, b_{ToxW}=0.7032; BPA, b_{ToxW}=0.6624; SH, b_{ToxW}=0.7551; with sig<0.0000 in all analyzes).

In relation to ethanol the differences were similar over the days (table 9). This conclusion was confirmed by the model Moment/Toxicant (table 15), where only the experimental units in which ethanol was added were analyzed, and the Distances between the Moments Before and After were similar over the days. In this work the development of behavior tolerance to ethanol was not observed, which was beneficial for the detection capability of the system that remained unchanged over the days. However, Gerlai *et al*. (2006) found that zebrafish exposed to ethanol for two weeks to 14% of the 96h LC_{50}, had developed behavior tolerance, and only an acute exposure to 56% of the 96h LC_{50} after the chronic exposure attenuated the development of tolerance. Several other works also have reported the development of tolerance in zebrafish chronically exposed, but the concentrations that caused this response were always superior to the 9% 96h LC_{50} used in the present work because they were normally between 14% of the 96h LC_{50} and 28% (Dlugos and Rabin, 2003; Gerlai *et al*., 2009; Dlugos *et al*., 2011; Tran and Gerlai, 2013; Tran *et al*., 2014). Contrary to the reported in

several works and the initial expectations, the zebrafish did not develop tolerance to ethanol in this work, (because the fish reacted always to the presence of ethanol), and the same had happened with wild-type zebrafish in a study performed by Dlugos and Rabin (2003) that have exposed zebrafish for two weeks to 28% of the 96h LC_{50} of ethanol and did not detect the development of tolerance. In this previous work, another strain of zebrafish (long-fin striped zebrafish) exposed chronically for two weeks, in contrary to the other strain, developed tolerance to ethanol (the average distance between each fish and its nearest neighbor were similar to the pre-exposure values). So it's possible that like in the previous study that in this work the wild-type zebrafish strain used was less resistant to the treatment (although they analyze the shoal behavior instead of the locomotor), and like advanced by Dlugos and Rabin (2003) possibly due to differences in both the response of the central nervous system, as well as the ability of the CNS to adapt to ethanol. Maybe in this work the exposure time wasn't enough to elicit this response because a 24h continual exposure wasn't employed, more a chronic daily exposure (once a day). Probably in the present work the concentration or the exposure time wasn't enough to cause the brain neurotransmitters pattern adaptation (Gerlai *et al.,* 2009; Tran *et al*., 2014), which highlights the sensibility of the system to quickly detect exposure situations. It's also possible that the zebrafish in this work, experienced an exposure that was sufficient to cause behaviors effects (hyperactivity), typical observed in acute exposure to low concentrations such as 14% of the 96h LC_{50} of ethanol (anxiolytic, decreased erratic movements and increased exploration) (Gerlai *et al*., 2000), in each day, which explains the similar behavior response along the days, although normally this don't happens with chronic exposures.

In the case of SH and BPA and contrary to what happened with ethanol, through the distances analyzed in the Presence/Absence model (tables 9, 10 and 11) the system was always able to detect the presence of the respective toxicant, although these differences diminished over the days which lead to a decrease of the detection capability over the days (BPA, b_{ToxWD1}=-0.1851; SH, b_{ToxWD1}=-0.1449; with sig<0.0486 in all analyzes) (tables 9 and 10). This decreased, is caused by the decrease of the differences of the distances between the Moments Before and After demonstrated by the model Moment/Toxicant (BPA, $b_{MomentD1}$=-0.2101, sig=0.0340; SH, $b_{MomentD1}$=-0.2368, sig=0.0112) (tables 15 and 16). The

fish in the last days may had a similar behavior in the two moments, as shown in figure 16 and 17.

The deterioration over the days, for BPA and SH was most likely due to the fish behavior and the toxicokinetics of the toxicants. The repeated exposures to the toxicant throughout the days may have induced alterations in physiological mechanisms (acclimation) which led to ceasing of the response of the fish to the toxicant, or the toxicants may have caused damage to the fish that accumulated throughout the days. It is possible that a behavior recuperation may have happened with the SH and BPA in this work due to acclimation. Cases of fish able to recover in terms of behavior after continuously exposed to a toxicant at concentrations in terms of LC_{50} higher than those used in this work have been reported. Juvenile rainbow trout (*Oncorhynchus mykiss*) following sub-lethal exposure to a concentration of 45% 96h LC_{50} (5 time superior to the concentration used in the present work) of the insecticide deltamethrin, revealed a reduction on critic swimming speed (measure of aerobic swimming utilizing mainly red muscle) after days 1 and 4 of exposure, but this response was fully recovered (acclimation) after the day 7 of exposure (Goulding *et al.*, 2013). It is also possible that the toxicants (SH and BPA) may have caused damages to the fish, that accumulated over time. SH is capable of causing gill damage (hypertrophy, lamellar fusion) and a decreased response of detoxification mechanisms (catalase and GST), maybe due to increased production of ROS, in fish (López-Galindo *et al.*, 2010b), and BPA is capable of causing vitellogenin induction, gonad structural changes in male, reduced sperm quality, delayed ovulation in females, also in different species of fish (Lahnsteiner *et al.*, 2005; Mandich *et al.*, 2007) and the damage caused by this toxicants can ultimately be represented in the fish behavior. This possibility explains, the fact that the fish did not present significant statistically behavior differences between the Moment Before and After in the last two days, which means that the behavior of the fish before the exposure may have approximated the behavior of the fish after the exposure.

The capacity of this system to detect behavior changes caused by the toxicants was quite satisfactory in comparison with the results of others works. For example in one study carried out by Magalhaes *et al.* (2007), the lowest concentration of SH that caused behavior responses (hyperactivity) was about ten times higher than the concentration used in this work, and the zebrafish were

also exposed about 3 times longer. In relation to BPA, in one development study (where more sensitive states are used), a concentration of 2280 µg/L, that is more than 2 times higher than the concentration used in this work, did not caused behavior effects in larval zebrafish (Saili *et al*., 2012). In the present work relatively to ethanol, the system was able to detect behavior changes in zebrafish, even though the exposure time and the concentrations used were much shorter, that the chronic concentrations (14%-28% 96h LC_{50}) and the exposure time, normally two weeks or even more, commonly used in other works (Dlugos and Rabin, 2003; Gerlai *et al*., 2006; Gerlai *et al*., 2009; Tran and Gerlai, 2013; Tran *et al*., 2014). In another study the concentration used in an acute exposure (2h) to ethanol was only 7% of the 96h LC_{50}, a concentration very similar to the concentration detected in the present work (9% of the LC_{50}), but no significant behavior alteration were detected (Dlugos *et al*., 2011). In other works with others toxicants the concentrations used may be lower, but on the other hand the exposures times are also much longer, allowing the accumulation of the effects caused by the toxicants. For example adult male zebrafish (*Danio rerio)* exposed to 5 ng/L 17α-Ethinyl estradiol (an active component of oral contraceptive pills) demonstrated anxiogenic-like behaviors in the test tank Novel (Reyhanian *et al*., 2011), but the exposure time used in this study (14 days) was 224 times higher than the time used in the present work (1h30m) which ultimately highlights the fast detection capability of the system used in the present work. These comparisons highlight the sensitivity of the system used in this work, indicating that the detecting capability of behavioral changes is very high and fast since lower concentrations and shorter exposure times were used. The goal of the system was to promptly detect the presence of toxicants and In the case of ethanol the system was resistant to the repeated exposures and was able to detect changes in behavior. These results clearly demonstrate the effectiveness and applicability of the system, that in one case remained unchanged over the days (ethanol) and in other two cases deteriorated with the repeated exposures (bisphenol A and sodium hypochlorite).

The results obtained in the Moment/Control model were excellent, because they indicate that the behavior of the controls in relation to the Distances BPA and SH were not influenced, by the factors Moment and Day. The behavior was similar in all days and between the two moments. The factor Assay although it was statically different for the distances BPA, this factor also did not influence any of

the others factors. In relation to the Distances Ethanol the results in this model indicate that the behavior of the controls were initially influenced by the factor Moment, but this influence decreases over time, going in the opposite direction of the gradient (Toxicant vector).

A behavior analysis on daily basis, along the chronic exposure was not described in the literature for any of the toxicants (SH, BPA and ethanol), although for ethanol a behavior analysis after each week have already been described (Dlugos and Rabin, 2003; Dlugos *et al.*, 2011). This type of analysis (on daily basis) was innovative and proved to be very efficient in the detection of the behavioral response along time. It is also important to mention that the zebrafish behavior was successfully used through the Kohonen ANNs to define and identify classes of fish behavior, and that through the ANNs, the correspondence analysis and the linear and orthogonal regressions was possible to evaluate the conditions to which the fish were exposed. In a real situation and to prevent the loss of detection capability in the last days, as those observed relatively to BPA and SH, an exchange of the fish whenever they detect some important disturbance, can be performed.

6. Conclusion

Aquatic pollution is one of the main threats to the ecosystems, and often contaminations occur intentionally or due to negligence, being important in this sense to find better ways to rapidly detect these situations. Behavior changes have proven to be a fast and sensible indicator, and therefore several BEWSs use various behavioral parameters. In the present work, the system used was able to detect different behavior responses of zebrafish exposed daily for 9 days to 9% of the 96h LC_{50}, of SH, ethanol and BPA. Using the ANNs, the correspondence analysis and the linear and orthogonal regressions it was possible, to use the zebrafish behavior changes caused by the toxicants as a way to detect them, analyze the behavior response and relate it with the presence or absence of toxicants, and analyze the detection capability over the days.

The Presence/Absence regression, revealed that all measures used (Distances BPA, Ethanol and SH), have detected in a statistically significant way the presence of the respective toxicant in the water, indicating that each toxicant caused behavior responses in the zebrafish. Its particular important to highlight that for ethanol this capacity remained unchanged throughout the daily exposures, but for SH and BPA the detection capability deteriorated over the days. The behavior was similar between all days for ethanol, because the differences between the Distances in the moments Before and After were maintained over time. This indicates that the system was resistant to the repeated exposure and a decreased behavior response over the days was not observed. In this work the zebrafish did not develop tolerance to ethanol. Maybe, the differences between strains, the low concentration used or the repeated short exposure time may have prevented the development of tolerance. In the case of SH, and BPA it was detected a decrease of the difference between the moments Before and After, measured in terms of behavior of the fish associated with the presence of toxicants in the water, which explains the detection deterioration over the days. To avoid this loss of detection capability, an exchange of fish when they detect some important disturbance in water can be performed, to maintain the detection efficiency. The Moment/Control model analysis showed that the behavior of the controls in the moment After was less or equally related to the presence of the toxicants in the water (in relation to the Toxicant vectors) than in the moment

Before. This demonstrates that the behavior changes of the fish observed after the addition of the toxicants, which occurred towards the respective Toxicant vectors, are explained solely by the presence of the toxicants and not by the practical procedure.

In summary, through this system it was possible to detect all toxicants using the behavior effects caused in zebrafish. This is more remarkable, when taking into account that the concentrations used for each toxicant were very small (9% of 96h LC_{50}), and the daily exposure time was only of 1h30m. This detection capability remained unchanged after 9 days of repeated exposure to ethanol, but, for the two other toxicants tested (SH and BPA), the detection capability decreased significantly over the days, being almost zero in last days.

Behavioral responses should be included in the evaluation of water quality, because its rapid response and high sensitivity have the potential to allow an early detection of contaminants and avoid more serious consequences. The zebrafish behavior demonstrated to be exceptionally useful, especially when used in a video-tracking system analysis. This system can be a very important tool in the monitoring of water quality, and it can function like a BEWS for the protection of the aquatic ecosystems, because, it has a relatively low cost, and it's very fast and sensible to low concentrations of toxicants. In the future it would be interesting to have a more integrative approach, to try understand the effects in behavior by analyzing neurotransmitters levels, such as, dopamine, serotonin and GABA in the case of ethanol, other endpoints such as AChE, testosterone and estradiol levels in the case of BPA, and catalase, GST, ROS in the case of SH. To assess the detection capability, it should be important to test other factors, such as, different exposures times, or/and other types of toxicants, bacterias and compounds (cyanotoxins).

7. References

Bae, M.J., Park, Y.S., 2014. Biological early warning system based on the responses of aquatic organisms to disturbances: A review. Science of the Total Environment 466-467, 635-649.

Befyaeva, N.F., Kashirtseva, V.N., Medvedeva, N.V., Ipatova, O.M., Archakov, A.I., Khudoklinova, Y.Y., 2010. Zebrafish as a model system for biomedical studies. Biomeditsinskaya Khimiya 56 (1), 120-131.

Belfroid, A., Van Velzen, M., Van der Horst, B., Vethaak, D., 2002. Occurrence of bisphenol A in surface water and uptake in fish: Evaluation of field measurements. Chemosphere 49 (1), 97-103.

Blankenship, A.L., Coady, K., 2005. Bisphenol A. In Encyclopedia of Toxicology, (Wexler, P., Anderson, B., Peyster, A.d., Gad, S., Hakkinen P.J., Kamrin, M., Locey, B., Mehendale, H., Pope, C., Shugart, L. edition), pp. 314-317. New York: Elsevier Inc,.

Blaser, R., Gerlai, R., 2006. Behavioral phenotyping in zebrafish: comparison of three behavioral quantification methods. Behavior Research Methods 38 (3), 456-469.

Box, G.E.P., Hunter, W.G., Hunter, J.S., 1978. Statistics for experimenters: an introduction to design, data analysis, and model building. (2th edition). New York: John Wiley & Sons, Inc.

Brewer, S.K., Little, E.E., DeLonay, A.J., Beauvais, S.L., Jones, S.B., Ellersieck, M.R., 2001. Behavioral dysfunctions correlate to altered physiology in rainbow trout (*Oncorynchus mykiss*) exposed to cholinesterase-inhibiting chemicals. Archives of Environmental Contamination and Toxicology 40 (1), 70-76.

Canistro, D., Melega, S., Ranieri, D., Sapone, A., Paolini, M., Gustavino, B., Monfrinotti, M., Rizzoni, M., 2012. Modulation of cytochrome P450 and induction of DNA damage in *Cyprinus carpio* exposed in situ to surface water treated with chlorine or alternative disinfectants in different seasons. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis 729 (1/2), 81-89.

Cardoso, R.A., Brozowski, S.J., Chavez-Noriega, L.E., Harpold, M., Valenzuela, C.F., Harris, R.A., 1999. Effects of ethanol on recombinant human neuronal nicotinic acetylcholine receptors expressed in Xenopus oocytes. Journal of Pharmacology and Experimental Therapeutics 289 (2), 774-780.

Chapman, P.M., 2002. Integrating toxicology and ecology: putting the "eco" into ecotoxicology. Marine Pollution Bulletin 44 (1), 7-15.

Cousins, I.T., Mackay, D., Staples, C.A., Klečka, G.M., 2002. A multimedia assessment of the environmental fate of bisphenol A. Human and Ecological Risk Assessment 8 (5), 1107-1135.

Davies, D.L., Asatryan, L., Kuo, S.T., Woodward, J.J., King, B.F., Alkana, R.L., Xiao, C., Ye, J.H., Sun, H., Zhang, L., Hu, X.-Q., Hayrapetyan, V., Lovinger, D. M., Machu, T. K., 2006. Effects of ethanol on adenosine 5'-triphosphate-gated purinergic and 5-hydroxytryptamine receptors. Alcoholism: Clinical And Experimental Research 30 (2), 349-358.

de Esch, C., Slieker, R., Wolterbeek, A., Woutersen, R., De Groot, D., van der Linde, H., Willemsen, R., 2012. Locomotor activity assay in zebrafish larvae: Influence of age, strain and ethanol. Neurotoxicology and Teratology 34 (4), 425-433.

Delcourt, J., Becco, C., Vandewalle, N., Poncin, P., 2009. A video multitracking system for quantification of individual behavior in a large fish shoal: Advantages and limits. Behavior Research Methods 41 (1), 228-235.

Delcourt, J., Denoël, M., Ylieff, M., Poncin, P., 2013. Video multitracking of fish behaviour: a synthesis and future perspectives. Fish and Fisheries 14 (2), 186-204.

Diehl, P., Gerke, T., Jeuken, A., Lowis, J., Steen, R., van Steenwijk, J., Stoks, P., Willemsen, H.-G., 2006. Early Warning Strategies and Practices Along the River Rhine. In: The Rhine (Knepper, T.P. edition), pp. 99-124. Berlin: Springer.

Dildy-Mayfield, J.E., Harris, R.A., 1995. Ethanol inhibits kainate responses of glutamate receptors expressed in Xenopus oocytes: Role of calcium and protein kinase C. Journal of Neuroscience 15 (4), 3162-3171.

Dlugos, C.A., Brown, S.J., Rabin, R.A., 2011. Gender differences in ethanol-induced behavioral sensitivity in zebrafish. Alcohol 45 (1), 11-18.

Dlugos, C.A., Rabin, R.A., 2003. Ethanol effects on three strains of zebrafish: Model system for genetic investigations. Pharmacology Biochemistry and Behavior 74 (2), 471-480.

Dorn, P.B., Chou, C.-S., Gentempo, J.J., 1987. Degradation of bisphenol A in natural waters. Chemosphere 16 (7), 1501-1507.

Egan, R.J., Bergner, C.L., Hart, P.C., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A.V., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S. I., Bartels, B. K., Tien, A. K., Tien, D. H., Mohnot, S., Beeson, E., 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behavioural Brain Research 205 (1), 38-44.

Elia, A.C., Anastasi, V., Dörr, A.J.M., 2006. Hepatic antioxidant enzymes and total glutathione of *Cyprinus carpio* exposed to three disinfectants, chlorine dioxide, sodium hypochlorite and peracetic acid, for superficial water potabilization. Chemosphere 64 (10), 1633-1641.

Emmanuel, E., Perrodin, Y., Blanchard, J.M., Vermande, P., Keck, G., 2004. Toxicological effects of disinfections using sodium hypochlorite on aquatic organisms and its contribution to AOX formation in hospital wastewater. Environment International 30 (7), 891-900.

Environmental Protection Agency of United States (USEPA). 2012. Attachment H Total Residual Chlorine Discharge Limit Calculation NPDES Permit No. MA0103284. Boston, Massachusetts.

European Commission. 2011. Commission Directive 2011/8/EU of 28 January 2011 amending Directive 2002/72/EC as regards the restriction of use of Bisphenol A in plastic infant feeding bottles. Official Journal of the European Union.

Faingold, C.L., N'Gouemo, P., Riaz, A., 1998. Ethanol and neurotransmitter interactions--from molecular to integrative effects. Progress in Neurobiology 55 (5), 509-535.

Farabollini, F., Porrini, S., Della Seta, D., Bianchi, F., Dessì-Fulgheri, F., 2002. Effects of Perinatal Exposure to Bisphenol A on Sociosexual Behavior of Female and Male Rats. Environmental Health Perspectives 110 (3): 409–414.

Flint, S., Markle, T., Thompson, S., Wallace, E., 2012. Bisphenol A exposure, effects, and policy: A wildlife perspective. Journal of Environmental Management 104, 19-34.

Freitas, J.G., Barker, J.F., 2013. Denatured ethanol release into gasoline residuals, Part 1: Source behaviour. Journal of Contaminant Hydrology 148, 67-78.

Fromme, H., Küchler, T., Otto, T., Pilz, K., Müller, J., Wenzel, A., 2002. Occurrence of phthalates and bisphenol A and F in the environment. Water Research 36 (6), 1429-1438.

Gerhardt, A., 1995. Monitoring behavioural responses to metals in *Gammarus pulex* (L.) (Crustacea) with impedance conversion. Environmental Science and Pollution Research 3 (2), 15-23.

Gerhardt, A., 2007. Aquatic behavioral ecotoxicology - Prospects and limitations. Human and Ecological Risk Assessment 13 (3), 481-491.

Gerlai, R., Chatterjee, D., Pereira, T., Sawashima, T., Krishnannair, R., 2009. Acute and chronic alcohol dose: population differences in behavior and neurochemistry of zebrafish. Genes, Brain and Behavior 8 (6), 586-599.

Gerlai, R., Lahav, M., Guo, S., Rosenthal, A., 2000. Drinks like a fish: zebra fish (*Danio rerio)* as a behavior genetic model to study alcohol effects. Pharmacology Biochemistry and Behavior 67 (4), 773-782.

Gerlai, R., Lee, V., Blaser, R., 2006. Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (*Danio rerio*). Pharmacology Biochemistry and Behavior 85 (4), 752-761.

Gonzalez, C., Greenwood, R., Quevauviller, PP., 2009. Rapid Chemical and Biological Techniques for Water Monitoring. Chichester: John Wiley & Sons, Inc.

Goulding, A.T., Shelley, L.K., Kennedy, C.J., Ross, P.S., 2013. Reduction in swimming performance in juvenile rainbow trout (*Oncorhynchus mykiss*) following sublethal exposure to pyrethroid insecticides. Comparative Biochemistry and Physiology, Part C 157 (3), 280-286.

Hartmann, C. Bisphenol A: Environmental Fate and Behaviour, Human Health Effects – Implications for EU-chemical Policy. Vien: University of Vien. 2012. Master's thesis.

Holmstrup, M., Sørensen, J.G., Overgaard, J., Bayley, M., Bindesbøl, A.-M., Slotsbo, S., Fisker, K.V., Maraldo, K., Waagner, D., Labouriau, R., Asmund, G., 2011. Body metal concentrations and glycogen reserves in earthworms (*Dendrobaena octaedra)* from contaminated and uncontaminated forest soil. Environmental Pollution 159 (1), 190-197.

Houtman, C.J., 2010. Emerging contaminants in surface waters and their relevance for the production of drinking water in Europe. Journal of Integrative Environmental Sciences 7 (4), 271-295.

Jenner, H.A., Taylor, C.J.L., van Donk, M., Khalanski, M., 1997. Chlorination by-products in chlorinated cooling water of some European coastal power stations. Marine Environmental Research 43 (4), 279-293.

Klerks, P.L., 1999. Acclimation to contaminants by the grass shrimp Palaemonetes pugio: Individual contaminants vs. mixtures. Ecotoxicology 8 (4), 277-286.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A., 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139 (10), 4252-4263.

Kwak, I.S., Chon, T.S., Chung, N.I., Kang, H.M., Kim, Y.S., Kim, J.S., Koh, S.C., Lee, S.K., 2002. Pattern recognition of the movement tracks of medaka *(Oryzias latipes)* in response to sub-lethal treatments of an insecticide by using artificial neural networks. Environmental Pollution 120 (3), 671-681.

Lahnsteiner, F., Berger, B., Kletzl, M., Weismann, T., 2005. Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, Salmo trutta f. fario. Aquatic Toxicology 75 (3), 213-224.

Lee, H.J., Chattopadhyay, S., Gong, E.-Y., Ahn, R.S., Lee, K., 2003. Antiandrogenic Effects of Bisphenol A and Nonylphenol on the Function of Androgen Receptor. Toxicological Sciences 75 (1), 40-46.

Lindholst, C., Bjerregaard, P., Wynne, P.M., Marriott, P., Pedersen, S.N., 2003. Metabolism of bisphenol A in zebrafish *(Danio rerio)* and rainbow trout *(Oncorhynchus mykiss*) in relation to estrogenic response. Comparative Biochemistry and Physiology, Part C 135 (2), 169-177.

Little, E., Archeski, R., Flerov, B., and Kozlovskaya, V., 1990. Behavioral indicators of sublethal toxicity in rainbow trout. Archives of Environmental Contamination and Toxicology 19 (3), 380-385.

Little, E., Finger, S.E., 1990. Swimming behavior as an indicator of sublethal toxicity in fish. Environmental Toxicology and Chemistry 9 (1), 13-19.

Liu, J., Wang, R., Huang, B., Lin, C., Wang, Y., Pan, X., 2011a. Distribution and bioaccumulation of steroidal and phenolic endocrine disrupting chemicals in wild fish species from Dianchi Lake, China. Environmental Pollution 159 (10), 2815-2822.

Liu, Y., Chon, T.S., Lee, S.H., 2011b. Analysis of behavioral changes of zebrafish (*Danio rerio*) in response to formaldehyde using Self-organizing map and a hidden Markov model. Ecological Modelling 222 (14), 2191-2201.

López-Galindo, C., Garrido, M.C., Casanueva, J.F., Nebot, E. 2010a. Degradation models and ecotoxicity in marine waters of two antifouling compounds: Sodium hypochlorite and an alkylamine surfactant. Science of the Total Environment 408 (8), 1779-85.

López-Galindo, C., Nebot, E., Rubio, D., Vargas-Chacoff, L., Mancera, J.M., Casanueva, J.F., Solé, M., 2010b. Biomarker responses i*n Solea senegalensis* exposed to sodium hypochlorite used as *antifouling*. Chemosphere 78 (7), 885-893.

López-Galindo, C., Nebot, E., Rubio, D., Vargas-Chacoff, L., Mancera, J.M., Casanueva, J.F., Solé, M., 2010c. Sublethal responses of the common mussel (*Mytilus galloprovincialis*) exposed to sodium hypochlorite and Mexel®432 used as antifoulants. Ecotoxicology and Environmental Safety 73 (5), 825-834.

Magalhaes Dde, P., Armando da Cunha, R., Albuquerque dos Santos, J.A., Buss, D.F., Baptista, D.F., 2007. Behavioral response of zebrafish *Danio rerio* Hamilton 1822 to sublethal stress by sodium hypochlorite: ecotoxicological assay using an image analysis biomonitoring system. Ecotoxicology 16 (5), 417-422.

Mandich, A., Bottero, S., Cevasco, A., Massari, A., Pedemonte, F., Benfenati, E., Maggioni, S., Erratico, C., Viganò, L., 2007. In vivo exposure of carp to graded concentrations of bisphenol A. General and Comparative Endocrinology 153 (1/3), 15-24.

Manduzio, H., Monsinjon, T., Galap, C., Leboulenger, F., Rocher, B., 2004. Seasonal variations in antioxidant defences in blue mussels *Mytilus edulis* collected from a polluted area: Major contributions in gills of an inducible isoform of Cu/Zn-superoxide dismutase and of glutathione S-transferase. Aquatic Toxicology 70 (1), 83-93.

Martins, J., Teles, L.O., Vasconcelos, V., 2007. Assays with *Daphnia magna* and *Danio rerio* as alert systems in aquatic toxicology. Environment International 33 (3), 414-425.

Matsuda, S., Matsuzawa, D., Ishii, D., Tomizawa, H., Sutoh, C., Nakazawa, K., Sajiki, J., Shimizu, E., Amano, K., 2012. Effects of perinatal exposure to low dose of bisphenol A on anxiety like behavior and dopamine metabolites in brain. Progress in Neuro-Psychopharmacology and Biological Psychiatry 39 (2), 273-279.

Maximino, C., da Silva, A.W.B., Herculano, A.M., Gouveia, A., 2011. Pharmacological analysis of zebrafish (*Danio rerio*) scototaxis. Progress in Neuro-Psychopharmacology and Biological Psychiatry 35 (2), 624-631.

Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: A meta-analysis. Chemosphere 93 (10), 2217-2223.

Meyer, J.N., Di Giulio, R.T., 2003. Heritable adaptation and fitness costs in killfish (*Fundulus heteroclitus)* inhabiting a polutted estuary. Ecological Appplications 13 (2), 490-503.

Mihic, S.J., Ye, Q., Wick, M.J., Koltchine, V.V., Krasowski, M.D., Finn, S.E., Mascia, M.P., Valenzuela, C.F., Hanson, K.K., Greenblatt, E.P., Harris, R. A.. Harrison, N. L., 1997. Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. Nature 389 (6649), 385-389.

Moriyama, K., Tagami, T., Akamizu, T., Usui, T., Saijo, M., Kanamoto, N., Hataya, Y., Shimatsu, A., Kuzuya, H., Nakao, K., 2002. Thyroid hormone action is disrupted by bisphenol A as an antagonist. Journal of Clinical Endocrinology and Metabolism 87 (11), 5185-5190.

Nacci, D., Coiro, L., Champlin, D., Jayaraman, S., McKinney, R., Gleason, T.R., Munns Jr, W.R., Specker, J.L., Cooper, K.R., 1999. Adaptations of wild populations of the estuarine fish *Fundulus heteroclitus* to persistent environmental contaminants. Marine Biology 134 (1), 9-17.

Nimkerdphol, K., Nakagawa, M., 2008. Effect of Sodium Hypochlorite on Zebrafish Swimming Behavior Estimated by Fractal Dimension Analysis. Journal of Bioscience and Bioengineering 105 (5), 486-492.

Ownby, D.R., Newman, M.C., Mulvey, M., Vogelbein, W.K., Unger, M.A., Arzayus, L.F., 2002. Fish *(Fundulus heteroclitus)* populations with different exposure histories differ in tolerance of creosote-contaminated sediments. Environmental Toxicology and Chemistry 21 (9), 1897-1902.

Pan, Y., Kaiguo, M., Gerlai, R., Razak, Z., Westwood, J.T., 2011. Chronic alcohol exposure induced gene expression changes in the zebrafish brain. Behavioural Brain Research 216 (1) , 66-76.

Park, Y.S., Chung, N.I., Choi, K.H., Chon, T.S., Cha, E.Y., Lee, S.K. (2005). Computational characterization of behavioral response of medaka *(Oryzias latipes*) treated with diazinon. Aquatic Toxicology 71 (3), 215-228.

Perez-Coll, C.S., Herkovits, J., Daniel, P., D'Eramo, J.L., Fridman, O., 1999. Metallothionein induction and cadmium uptake in *Bufo arenarum* embryos following an acclimation protocol. Environmental Pollution 106 (3), 443.

Pitanga, F.L. The effect of sodium hypochlorite in different aquatic organisms. Aveiro: University of Aveiro, 2011. Master's thesis.

Reyhanian, N., Volkova, K., Hallgren, S., Bollner, T., Olsson, P.E., Olsen, H., Hallstrom, I.P., 2011. 17alpha-Ethinyl estradiol affects anxiety and shoaling behavior in adult male zebra fish (*Danio rerio).* Aquatic Toxicology 105 (1/2), 41-48.

Rico, E.P., Rosemberg, D.B., Dias, R.D., Bogo, M.R., Bonan, C.D., 2007. Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. Toxicology Letters 174 (1/3), 25-30.

Saili, K.S., Corvi, M.M., Das, S.R., Przybyla, J., Anderson, K.A., Tanguay, R.L., Weber, D.N., Patel, A.U., 2012. Neurodevelopmental low-dose bisphenol A exposure leads to early life-stage hyperactivity and learning deficits in adult zebrafish. Toxicology 291 (1/3), 83-92.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquatic Toxicology 68 (4), 369-392.

Scott, G.R., Sloman, K.A., Wood, C.M., Rouleau, C., 2003. Cadmium disrupts behavioural and physiological responses to alarm substance in juvenile rainbow trout *(Oncorhynchus mykiss)*. Journal of Experimental Biology 206 (11), 1779-1790.

Shareef, A., Angove, M.J., Wells, J.D., Johnson, B.B., 2006. Aqueous solubilities of estrone, 17β-estradiol, 17α- ethynylestradiol, and bisphenol A. Journal of Chemical and Engineering Data 51 (3), 879-881.

Staples, C.A., Tilghman Hall, A., Friederich, U., Klecka, G.M., Caspers, N., 2011. Early life-stage and multigeneration toxicity study with bisphenol A and fathead minnows (*Pimephales promelas)*. Ecotoxicology and Environmental Safety 74 (6), 1548-1557.

Storey, M.V., van der Gaag, B., Burns, B.P., 2011. Advances in on-line drinking water quality monitoring and early warning systems. Water Research 45 (2), 741-747.

Strohm, B.H., Sweet, L.I., 2005. Ethanol. In: Encyclopedia of Toxicology (Wexler, P., Anderson, B., Peyster, A.d., Gad, S., Hakkinen P.J., Kamrin, M., Locey, B., Mehendale, H., Pope, C., Shugart, L. edition), pp. 263-266. New York: Elsevier Inc.

Swift, R., 2003. Direct measurement of alcohol and its metabolites. Addiction 98, 73-80.

Teles, L.O., Vasconcelos, V., Pereira, E., Saker, M., 2006. Time series forecasting of cyanobacteria blooms in the Crestuma Reservoir (Douro River, Portugal) using artificial neural networks. Environmental Management 38 (2), 227-237.

Tierney, K.B., 2011. Behavioural assessments of neurotoxic effects and neurodegeneration in zebrafish. Biochimica et Biophysica Acta 1812 (3), 381-389.

Tran, S., Chatterjee, D., Gerlai, R., 2014. An integrative analysis of ethanol tolerance and withdrawal in zebrafish *(Danio rerio)*. Behavioural Brain Research. In press.

Tran, S., Gerlai, R., 2013. Time-course of behavioural changes induced by ethanol in zebrafish (*Danio rerio*). Behavioural Brain Research 252, 204-213.

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of biomarkers in biomonitoring: A 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comparative Biochemistry and Physiology, Part C 146 (3), 281-300.

Vogl, C., Spieser, O.H., Grillitsch, B., Scholz, W., Wytek, R., 1999. Qualification of spontaneous undirected locomotor behavior of fish for sublethal toxicity testing. Part I. Variability of measurement parameters under general test conditions. Environmental Toxicology and Chemistry 18 (12), 2736.

Wang, X., Dong, Q., Chen, Y., Jiang, H., Xiao, Q., Wang, Y., Li, W., Bai, C., Huang, C., Yang, D., 2013. Bisphenol A affects axonal growth, musculature and motor behavior in developing zebrafish. Aquatic Toxicology 142–143, 104-113.

Wirgin, I., Waldman, J.R., 2004. Resistance to contaminants in North American fish populations. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis 552 (1/2), 73-100.

Wright, D.A., Welbourn, P., 2002. Environmental Toxicology. (1st edition). Cambridge: Cambridge University Press.

Wright, J.M., Peoples, R.W., Weight, F.F., 1996. Single-channel and whole-cell analysis of ethanol inhibition of NMDA-activated currents in cultured mouse cortical and hippocampal neurons. Brain Research 738 (2), 249-256.

Yamamoto, T., Yasuhara, A., Shiraishi, H., Nakasugi, O., 2001. Bisphenol A in hazardous waste landfill leachates. Chemosphere 42 (4), 415-418.

Ying, G.-G., Kookana, R.S., 2005. Sorption and degradation of estrogen-like-endocrine disrupting chemicals in soil. Environmental Toxicology and Chemistry 24 (10), 2640-2645.

Zhang, G., Eddy Patuwo, B., Y. Hu, M., 1998. Forecasting with artificial neural networks:: The state of the art. International Journal of Forecasting 14 (1), 35-62.

8. Appendix

Number of Dims.	Singular Values	Eigen-Values	Perc. of Inertia	Cumulatv Percent	Chi Squares
Eigenvalues and Inertia for all Dimensions Input Table (Rows x Columns): 12 x 4 Total Inertia=,26198 Chi²=3547,7 df=33 p=0,0000 Include condition: Day=1 and Assay=1					
1	0,40125	0,16100	61,4589	61,458	2180,37
2	0,28419	0,08076	30,8306	92,289	1093,77
3	0,14212	0,02020	7,7104	100,000	273,54

Table 1: Chi-squared test of the correspondence analysis for assay 1, day 1.

Number of Dims.	Singular Values	Eigen-Values	Perc. of Inertia	Cumulatv Percent	Chi Squares
Eigenvalues and Inertia for all Dimensions Input Table (Rows x Columns): 12 x 4 Total Inertia=,60679 Chi²=8446,6 df=33 p=0,0000 Include condition: Day=1 and Assay=2					
1	0,66253	0,43895	72,3390	72,3391	6110,17
2	0,33152	0,10991	18,1134	90,452	1529,96
3	0,24069	0,05793	9,5475	100,000	806,441

Table 2: Chi-squared test of the correspondence analysis for assay 2, day 1.

Number of Dims.	Singular Values	Eigen-Values	Perc. of Inertia	Cumulatv Percent	Chi Squares
Eigenvalues and Inertia for all Dimensions Input Table (Rows x Columns): 12 x 4 Total Inertia=,18914 Chi²=2632,9 df=33 p=0,0000 Include condition: Day=2 and Assay=1					
1	0,30551	0,09333	49,3485	49,348	1299,27
2	0,25887	0,06701	35,4318	84,780	932,87
3	0,16966	0,02878	15,2196	100,000	400,711

Table 3: Chi-squared test of the correspondence analysis for assay 1, day 2.

Number of Dims.	Eigenvalues and Inertia for all Dimensions Input Table (Rows x Columns): 12 x 4 Total Inertia=,35963 Chi²=4810,8 df=33 p=0,0000 Include condition: Day=2 and Assay=2				
	Singular Values	Eigen-Values	Perc. of Inertia	Cumulatv Percent	Chi Squares
1	0,41662(	0,17357	48,2637(	48,263(	2321,87
2	0,35394(	0,12527	34,8348(	83,098(	1675,83(
3	0,24654	0,06078	16,9013(	100,000(	813,09

Table 4: Chi-squared test of the correspondence analysis for assay 2, day 2.

Number of Dims.	Eigenvalues and Inertia for all Dimensions Input Table (Rows x Columns): 12 x 4 Total Inertia=,11404 Chi²=1587,4 df=33 p=0,0000 Include condition: Day=3 and Assay=1				
	Singular Values	Eigen-Values	Perc. of Inertia	Cumulatv Percent	Chi Squares
1	0,23112(	0,05341(	46,8431(	46,8431	743,588(
2	0,22465	0,05046(	44,2567	91,099(	702,533(
3	0,10074(	0,01014(	8,9001(	100,000(	141,281(

Table 5: Chi-squared test of the correspondence analysis for assay 1, day 3.

Number of Dims.	Eigenvalues and Inertia for all Dimensions Input Table (Rows x Columns): 12 x 4 Total Inertia=,35145 Chi²=4892,2 df=33 p=0,0000 Include condition: Day=3 and Assay=2				
	Singular Values	Eigen-Values	Perc. of Inertia	Cumulatv Percent	Chi Squares
1	0,39648	0,15720	44,7292	44,729	2188,24
2	0,35918	0,12901	36,7084(	81,437	1795,85(
3	0,25541(	0,06523	18,5622(	100,000(	908,10(

Table 6: Chi-squared test of the correspondence analysis for assay 2, day 3.

Number of Dims.	Eigenvalues and Inertia for all Dimensions Input Table (Rows x Columns): 11 x 4 Total Inertia=,24976 Chi²=3476,7 df=30 p=0,0000 Include condition: Day=5 and Assay=1				
	Singular Values	Eigen-Values	Perc. of Inertia	Cumulatv Percent	Chi Squares
1	0,36218(	0,13117(	52,5194(	52,519(	1825,95(
2	0,26014	0,06767(	27,0955	79,615(	942,03
3	0,22564	0,05091	20,3850	100,000(	708,72(

Table 7: Chi-squared test of the correspondence analysis for assay 1, day 5.

Number of Dims.	Eigenvalues and Inertia for all Dimensions Input Table (Rows x Columns): 12 x 4 Total Inertia=,19064 Chi²=2653,7 df=33 p=0,0000 Include condition: Day=5 and Assay=2 Singular Values	Eigen-Values	Perc. of Inertia	Cumulatv Percent	Chi Squares
1	0,32339(	0,104581	54,8585!	54,858!	1455,77
2	0,248277	0,061641	32,3341!	87,1927	858,047
3	0,15625!	0,02441!	12,8072(	100,000(	339,864

Table 8: Chi-squared test of the correspondence analysis for assay 2, day 5.

Number of Dims.	Eigenvalues and Inertia for all Dimensions Input Table (Rows x Columns): 12 x 4 Total Inertia=,29618 Chi²=4124,0 df=33 p=0,0000 Include condition: Day=9 and Assay=1 Singular Values	Eigen-Values	Perc. of Inertia	Cumulatv Percent	Chi Squares
1	0,41913!	0,175677	59,3142!	59,314!	2446,12
2	0,25303(	0,06402!	21,6170!	80,931!	891,48!
3	0,23765(	0,05647!	19,0687(	100,000(	786,39!

Table 9: Chi-squared test of the correspondence analysis for assay 1, day 9.

Number of Dims.	Eigenvalues and Inertia for all Dimensions Input Table (Rows x Columns): 12 x 4 Total Inertia=,29036 Chi²=4041,8 df=33 p=0,0000 Include condition: Day=9 and Assay=2 Singular Values	Eigen-Values	Perc. of Inertia	Cumulatv Percent	Chi Squares
1	0,36829(	0,135639	46,7142!	46,714(	1888,09(
2	0,33194!	0,110190	37,9496(	84,663!	1533,84!
3	0,21102(	0,04453(	15,3361!	100,000(	619,85!

Table 10: Chi-squared test of the correspondence analysis for assay 2, day 9.