Aquatic eco-neurotoxicology

High-throughput behavioral biotests

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INTRODUCTION

Aquatic environments face an ever-increasing burden of the daily, human, introduction of contaminants. Many of these contaminants have shown to be neurotoxic or neuro-modulating, meaning that they are harmful for both humans and animals through contaminated drinking water and accumulation in aquatic foods. However, this form of pollution should be, and currently is, gaining awareness considering the impact it has on animal behavior.

This white paper, written in collaboration with Prof. Donald Wlodkowic, an expert in eco-neurotoxicology, touches upon this subject, and shows how Noldus' DanioVision and EthoVision® XT can advance your research.

Analyzing animal behavior is an increasingly popular end-point since it is seen as the holistic readout of how an individual functions as a whole.

CURRENT STATUS

Currently, it is estimated that less than one percent of chemicals in production have been evaluated for their potential neurotoxicity, indicating the considerable backlog in the risk assessment of existing and new chemicals. Therefore, one of the most pressing challenges is the development of high-throughput laboratory screening for potential neurotoxic chemicals.

New methods and laboratory techniques to rapidly identify the impact of pollutants are badly needed. Analyzing animal behavior is an increasingly popular end-point since it is seen as the holistic readout of how an individual functions as a whole. Multi-faceted changes at a neurological, neuroendocrine, muscular, and metabolic levels underly the behavioral phenotypes, which eventually influence the survival of the individuals as well as the populations. Below we outline behavioral tests currently available and advocate that video-based analysis of behavior is key in the development of low-cost, high throughput eco-neurotoxicity tests.

BEHAVIORAL ASSAYS IN ECO-NEUROTOXICOLOGY

Ecotoxicological and predictive neurotoxicological behavioral biotests using small aquatic organisms are gaining an increasing interest. Vertebrate models such as larval and juvenile zebrafish (*Danio rerio*), but also diverse lower invertebrates such as frog tadpoles are very popular research models.

Functional tests include straightforward locomotor activity, but also more complex sensory-motor behavioral testing and even cognitive (learning and memory) functions. With their relatively complex nervous systems, small aquatic model organisms are well suited to explore the mechanisms underlying neuro-behavioural alterations upon exposure to pollutants.

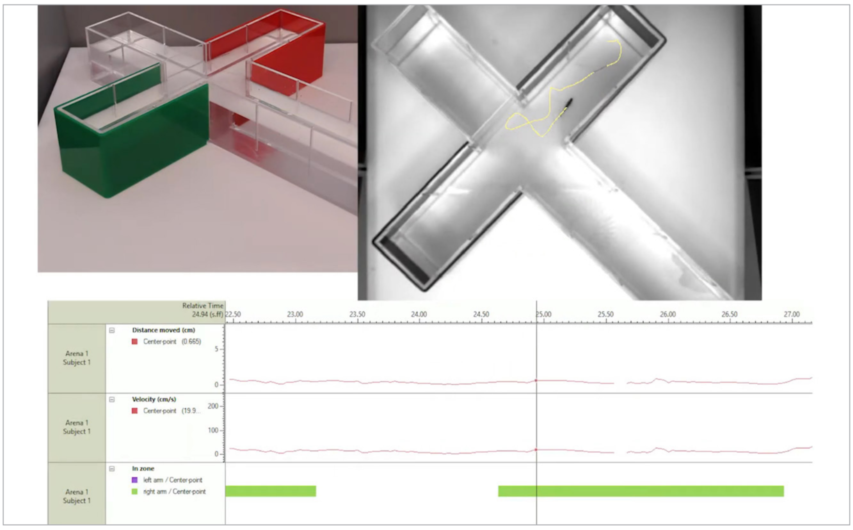
The progress in the development of ultra-high-throughput analysis of aquatic animal behavior has, however, been relatively slow compared to for example cell-based high-throughput screening. Recent progress in multi-arena tracking, multi-animal tracking and batch processing of video files provide promising avenues to address the existing analytical bottlenecks and can act as a spearhead for the development of low-cost and high-throughput neuro-behavioral toxicity tests.

A BRIEF HISTORY

The zebrafish (*Danio Rerio*), a small and robust behavioral research model, has been used as a model organism since the 1960s. Although it's actual scientific popularity has been picked up since the 1990s/2000s.

HOW TO ANALYZE BEHAVIOR OF SMALL MODEL ORGANISMS

To assess the ecological relevance of the impact pollutants have on animal behavior, researchers perform large-scale animal tracking. Digital video recording coupled with software-based animal tracking like EthoVision XT makes it possible to run complex assays using multi-well plates, Petri dishes, or simple tanks.



With EthoVision XT: movement, velocity and time spent in zones (among others) can be easily visualized and scored real-time!

EXPERT TIP

Most video-based animal tracking algorithms are optimized for contrast-based detection, requiring a clear separation between the background and tracked specimens. Analysis of simple behavioral responses using this method such as general activity and swimming speed does not require any additional layers of hardware. However, analysis of more complex behaviors such as e.g. sensory-motor functions also requires a more complex approach. For example, inducing behaviors that require light stimuli, requires hardware modules that deliver the appropriate stimuli that is integrated with the video stream and analyzed real-time.

BEHAVIORAL BIOTESTS: DATA ANALYSIS

Below we outline a few examples of neuro-behavioral assays that are particularly well suited for high-throughput prioritization of toxicants using the earlier mentioned small aquatic model species such as larval and juvenile zebrafish (Danio rerio) and some invertebrate species.

The overall changes in motility (hyperactivity, hypoactivity) and specific swimming patterns such as thigmotaxis ("wall hugging") are common experimental endpoints.

SPONTANEOUS LOCOMOTOR ACTIVITY

Measuring general activity, commonly referred to as swimming speed alteration tests, are popular because they are reasonably simple and inexpensive to perform in most laboratory settings. These tests are usually performed in multi-well plates and simply measure the impact of toxicants on the spontaneous locomotor activity of the organisms.

The overall changes in motility (hyperactivity, hypoactivity) and specific swimming patterns such as thigmotaxis ("wall hugging") are common experimental endpoints. Their simplicity is what makes these tests rapid, inexpensive and very amendable to high-throughput testing. On the downside, they provide little information on the impact of toxicants on higher neurological functions.

SENSORY-MOTOR BIOTESTS

All aquatic animals are able to respond to external stimuli and alter their behaviors accordingly. The analysis of more complex behaviors can be very advantageous due to their sensitivity as well as high physiological and ecological relevance.

STARTLE RESPONSES

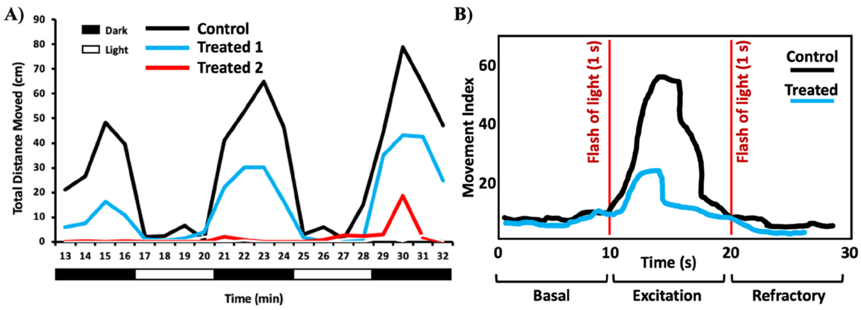
The startle response is an example of sensory-motor testing. It is the animal's defensive reaction to a sudden and unexpected stimulus, such as a sound, vibration, light change or a moving shadow simulating a predator. You usually see a pronounced increase in non-directional locomotion, often followed by more complex behaviors such as avoidance or shelter seeking.

ZEBRAFISH LARVAL PHOTOMOTOR RESPONSE (LPR)

The larval photomotor response (LPR) assay is a specific test performed on freely swimming zebrafish larvae at the age of 5 to 7 days post fertilization (dpf). At that developmental stage, fish show stereotypic non-directional light seeking behavior: rapid increases in locomotion in response to turning off the light.

For example, Holloway *et al.* [2] studied the neurotoxicity of the polycyclic aromatic hydrocarbon Benzo(a)pyrene, which caused locomotor hypoactivity at dpf 6 in zebrafish larvae, as measured with the LPR test. Using this test they measured changes in swimming patterns and overall distance moved. Indeed Bownik and Wlodkowic [1] describe the LPR test as a tool to observe a reproducible startle response to repeated light-dark changes. Habituation and sensitization to these light/dark changes can in turn then also be used to study the impact of toxicants on non-associative learning.

Typical endpoints in EthoVision XT - Total distance moved and Total activity measured in different time frames (baseline, stimulus, refractory phase, etc.). DanioScope can also be used for analysis of frequency of photo-induced embryo body flexions in embryo photo-motor response (PMR) bioassay.



This image taken from from Bownik and Wlodkowic [1] shows the results obtained from performing the LPR test in zebrafish

PHOTOTAXIS

Phototaxis is a directional response to light. It can be positive when the test animal moves toward the light, and negative when the animal moves away from it. Qian *et al.* [3] found a significant inhibition of the phototactic response in zebrafish larvae following exposure to Boscalid, a neurotoxic fungicide.

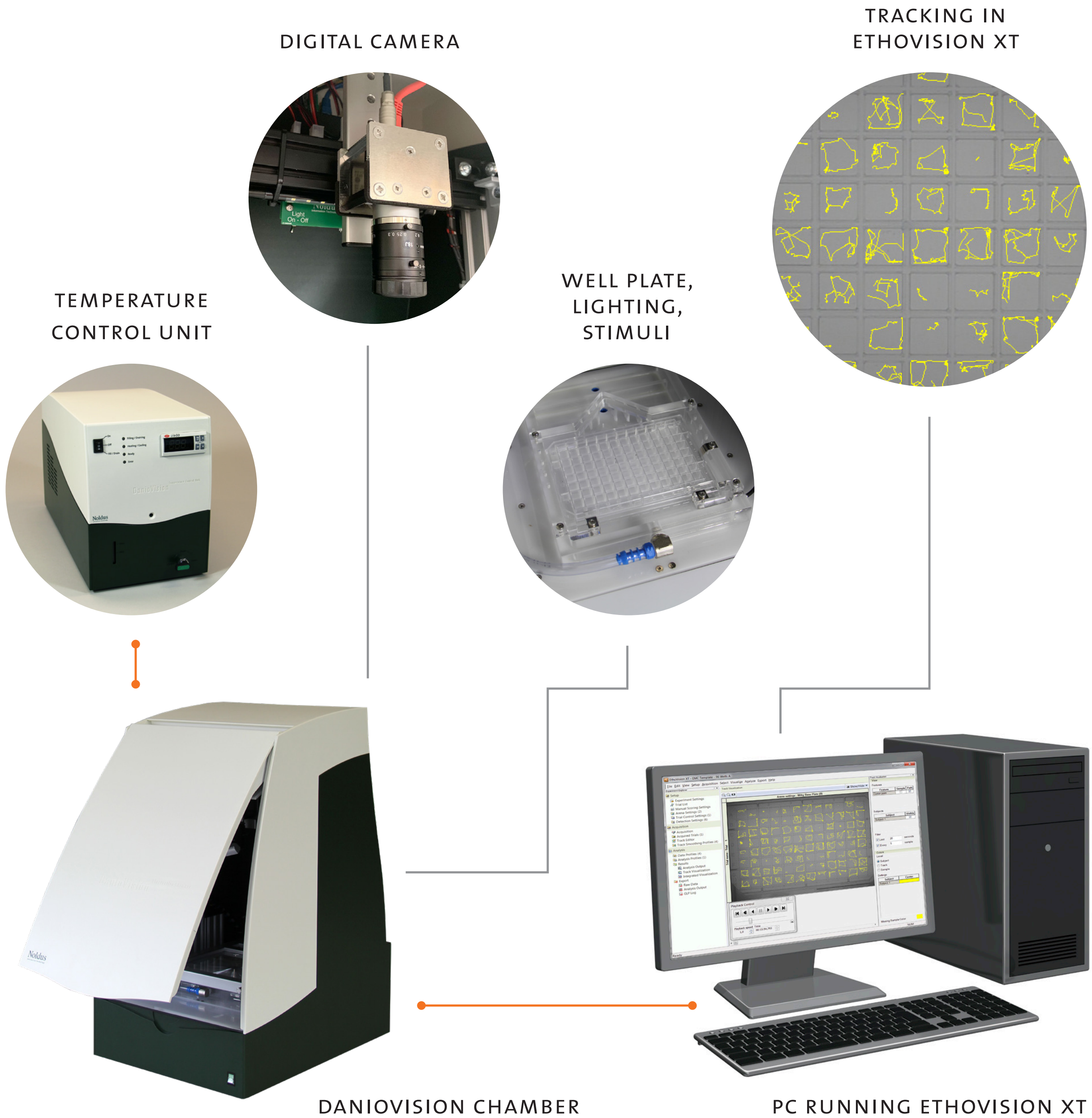
There have been studies demonstrating value in toxicity tests utilizing cladocerans (water fleas) [4] and amphipods (freshwater shrimp) [5]. In addition, freshwater planarians (flatworms) have been reported to exhibit strong phototactic behaviors, but their value in classification of aquatic neurotoxic and neuro-modulating pollutants requires further investigations [6].

Typical endpoints in EthoVision XT - Heading angles, Total distance moved, Speed of movement toward specific zones.

LIGHT-DARK PREFERENCE ASSAYS

The light-dark preference test is a simplified version of the phototaxis assay and has been a well-validated test in zebrafish larvae [7] and adults [8]. Upon acclimation the animals are exposed to a binary light stimulus: one half of the chamber is illuminated while another half is kept in darkness.

Typical endpoints in EthoVision XT - Total distance moved in the dark vs light zones, the number of individuals, the visits and the time spent in the two zones, the thigmotaxis in the two zones and more.



Daniovision is a high-througput behavioral system for multi-well plates and petridishes. This system is customizable with several add-ons and powered by our signature EhtoVision XT software.

COGNITIVE ECO-NEUROTOXICOLOGY

The impact of neurotoxic and neuro-modulating pollutants on learned behaviors (broadly defined as cognitive effects) is currently one of the most understudied aspects in eco-neurotoxicology. Learned behaviors represent the highest-level cognitive function of the nervous system and are highly relevant for both individual's and population survival, however they are also inherently difficult to study experimentally in aquatic animals because the effect of substances may be subtle and thus gone undetected.

NON-ASSOCIATIVE LEARNING

Non-associative learning is the simplest form of cognition [9]. It encompasses learned behaviors that are not conditioned by any specific stimulus, such as habituation. When the animal 'gets used to something', it decreases its response to this repeatedly presented stimulus. An example of this is habituation to light, which is applicable in higher-throughput and automated assays and has been demonstrated in aquatic risk assessment in fish and a handful of invertebrate species [10].

Typical endpoints in EthoVision XT - Total distance moved before vs. after the end of the stimulus.

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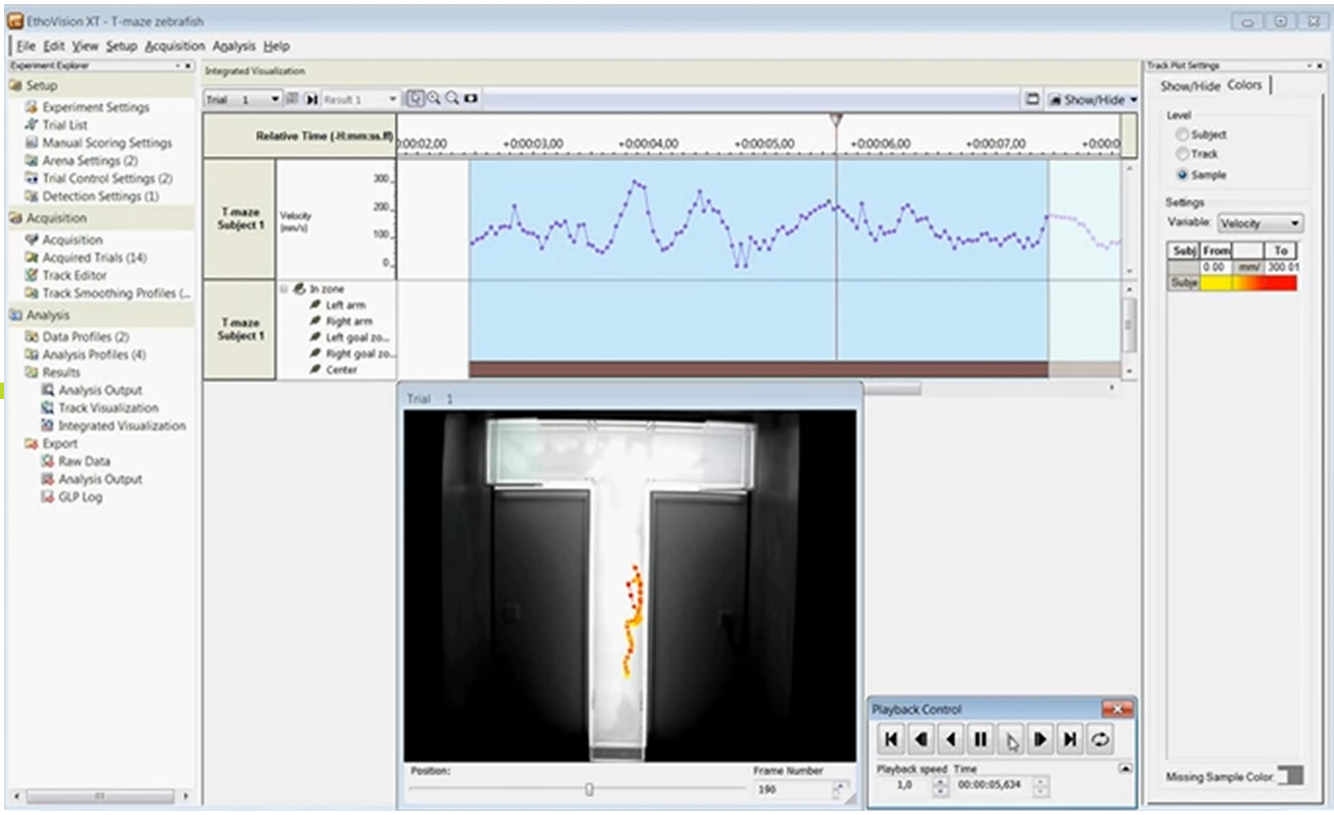
ASSOCIATIVE LEARNING

Associative learning is more complex: cognitive responses where learning and memory formation occurs [11]. The animal associates related and unrelated (neutral) stimuli, which we call classical conditioning. This can also occur when the association is made between a behavior and its direct consequence, such is the case during reward or punishment scenarios in operant conditioning tasks. This type of testing is performed most often using adult fish in multi chambered setups such as T-, Y and radial mazes. These tests include stimuli such as enrichment, shelter, light, vibration startle, electric startle, swimming restrictions and predator simulation.

Barreiros *et al.* [12] studied this the complex brain processes responsible for associative learning. They used visual, sound and movement stimuli associated with food, in addition to assessing the response of the zebrafish



A T-maze is a test that is often used to study learning in zebrafish.

in decision making during conditioning activities. They found that when subjected to two stimuli for decision-making in the food reward, zebrafish responded better to red light stimuli as opposed to vibration.

Typical endpoints in EthoVision XT - Latency to first zone entry, Number of entry errors.

SOCIAL BEHAVIOR

Zebrafish larvae do not display social behavior, but adults do. Dreosti *et al.* [13] specifically investigated at what stage of development zebrafish begin to interact with and prefer other fish. This develops between the 1 to 3 week mark, which coincides with the development of vision and coordination of movement. Fu *et al.* [14] consequently investigated whether silver, which is known to cause physiological damages to aquatic animals, affects social preference and social recognition. They indeed found altered social behavior as a consequence of silver accumulation, which could cause reduced neural activity in critical brain nuclei responsible for social behavior.

Typical endpoints in EthoVision XT - Mean distance between subject, Time spent next to conspecific vs. alone, Time spent moving toward conspecifics.

CONCLUDING REMARKS

Small model organisms, such as zebrafish, are playing an essential role in the behavioral phenotyping in the neurotoxicological field. This is due to the limitations of conventional in vivo rodent models, but also due to the standard the zebrafish is becoming in the domain of high-throughput behavioral testing. Although full integration and automation of large-batch testing of zebrafish is still underdeveloped, semi-automated batch animal tracking has already solid bases, and proves to be flexible and accurate [6, 15].

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