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Sensitivity of *Triops longicaudatus* Locomotor Behaviour to Detect Short Low-Level Exposure to Pollutants

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Abstract: *Triops longicaudatus* is a crustacean typically inhabiting temporary freshwater bodies in regions with a Mediterranean climate. These crustaceans are easily maintained in the laboratory and show a set of biological features that make them good candidates for diagnosing environmental quality and health. However, information about their responses to environmental contamination is scarce. This study characterised the locomotor responses of juvenile and adult/mature *T. longicaudatus* to low concentrations of five model toxicants upon a very short 1.5 h exposure: tributyltin, mercury, lindane, sodium hypochlorite and formaldehyde. A video-tracking system was used to record the locomotor behaviour. The data were analysed with an artificial neural network to identify distinct behaviours, followed by Chi-square and Correspondence analysis to characterise the response to each toxicant. The results showed that *T. longicaudatus* is sensitive to aquatic contamination, particularly sodium hypochlorite. Six behaviour types were defined, which allowed for the characterisation and discrimination of the test toxicants. The results support the need for more investigation into this species and its behaviour types as an alternative to animal testing and the more apical and often invasive endpoints commonly recommended in standard guidelines.

Keywords: priority contaminants; metals; biomarkers; freshwater crustacean; artificial neural networks; linking exposure and effects

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1. Introduction

Tadpole shrimps or triops (*Triops* spp.) are worldwide-distributed notostracan crustaceans typically inhabiting temporary freshwater bodies [1,2]. Their morphology has remained basically unchanged since the late Cretaceous period (more than 70 million years) [3], with numerous scientific reports focusing on their living-fossil status [4,5]. At the juvenile and adult stages, they are benthic, with a very active locomotor behaviour, and omnivorous, digging through the sediment in search of detritus and small organisms for food [6,7]. The predation of mosquito larvae by triops has drawn special attention, since triops species are seen as important biological control agents of mosquito populations in ephemeral bodies of water and rice paddies [8,9] and potential vectors of a wide variety of diseases. In general, triops can display multiple reproductive strategies [10,11], including sexual reproduction (gonochoric populations), hermaphroditism and parthenogenesis (female-dominated populations). Eggs are held in egg sacs (in females and hermaphrodites) until oviposition, with the embryos developing over the following 2 or 3 days [12]. In order to survive the dry phase, a determinant stage of temporary ponds, embryo-bearing eggs are preconditioned, becoming resistant to desiccation and heat [13]. These eggs remain in a quiescent state for extended periods of time, then called resting/dormant eggs or cysts, and are capable of hatching in future flooding periods when environmental conditions become favourable [14]. The dried cysts of some species, such as *T. longicaudatus*, are commercially available; these cysts hatch about 24 h after hydration in aged tap water, making it very easy to start cultures of these organisms in the laboratory. Triops hatch as nauplii and pass through a total of five fast, mostly planktonic, larval instars, reaching the adult-like benthic juvenile stage less than 48 h after the hydration of the cysts [6,15]; maturation/oviposition in *T. longicaudatus* is reached on average at about 10 to 19 days post-hydration and with a carapace length of around 10 to 11 mm as temperature decreases from 30 to 20 °C [16], although it can occur as early as 7 days under optimal conditions [16,17]. Substantial differences in *T. longicaudatus* fecundity have been reported depending on the environmental conditions [16,18], with individuals capable of laying up to 1000 or even more eggs over their lifetime [12,17], with hatching rates reaching 80% [16,19]. The lifespan of triops is short and also dependent on environmental conditions, ranging from approximately 12 days (at a high temperature of 30 °C) to about 30 days (at moderate temperatures and good feeding conditions) in laboratory-reared *T. longicaudatus* [16,17], although some individuals may survive much longer. In summary, *T. longicaudatus* presents a set of biological features, such as (i) a full life cycle adaptation to laboratory conditions, (ii) the possibility of parthenogenetic reproduction, allowing clones to be obtained, (iii) the production of cysts that can be kept viable for long periods of time, (iv) a short life cycle/generation time, and (v) high fecundity and hatching rates, which make this species easy and cost-effective for mass rearing in the laboratory and interesting to explore as an experimental freshwater model in ecotoxicological studies. However, despite their potential, triops have seldom been used in ecotoxicity testing. Furthermore, the few available studies are limited to the context of triops (1) as biological control agents for mosquito populations, focusing on the side effects of the chemicals used as mosquito larvicides on triops populations [20,21], or (2) as pests, due to the damage they cause to seeds and plants in rice fields, with an emphasis on the effectiveness of chemicals in controlling their populations [22,23]. Thus, our study aimed at exploring the potential of *T. longicaudatus* as a model in ecotoxicological assays, in particular by investigating the suitability and sensitivity of its active swimming behaviour to assess exposure to aquatic contamination. Over the past decades, toxicological endpoints based on swimming or locomotor behaviour have been developed for various aquatic species [24–30]. Among other factors, this is due to the potential greater sensitivity of behaviour to environmental contaminants compared to other endpoints; for example, 10 to 1000 times greater than mortality [26,31]. Swimming/locomotion is of great ecological relevance, as it is at the basis of many other vital animal behaviours, such as prey finding, predator escaping or mating. Moreover, behaviour evaluations are non-invasive, allowing for assessments over time. For all of this, the swimming/locomotor behaviour is of significant importance to anticipating a loss of ecosystem quality in time to elaborate prevention or mitigation actions aiming at the maintenance of the good ecological status of affected ecosystems. The use of automated video-tracking systems [27,32–34] is particularly useful to evaluate swimming/locomotor behaviours, allowing us to gather high amounts of data amenable to analysis by sensitive statistical methods, including Artificial Neural Networks. Such analysis can provide clear identification of behaviour profiles elicited by different toxicants and has thus been used in the development of Biological Early Warning Systems (BEWS) for the diagnosis of water quality and ecosystem health [26,35]. Therefore, this work was based on a video-tracking analysis of the swimming behaviour of *T. longicaudatus* following short-term exposure (1.5 h) to priority and legacy toxicants widely detected in aquatic systems, namely, tributyltin, lindane, mercury, sodium hypochlorite and formaldehyde.

2. Materials and Methods

2.1. Triop Rearing

Triops (*T. longicaudatus*) were hatched from commercial cysts (Triops King, Germany), and reared for up to seven days (juveniles) and fourteen days (adults, mature animals) according to the general procedures described in [16]. Briefly, the animals were cultivated in 30 L aquariums containing fine sand and dechlorinated water, at a temperature of 25 ± 1 °C, and with continuous aeration; the food consisted of a granulate for aquarium fish composed of a mixture of three algae (3-Algae Granulat, Tropical), supplied daily.

2.2. Chemicals

Sodium hypochlorite (NaOCl-5%, CAS 7681-52-9) was acquired from PanReac AppliChem. Tributyltin (TBTO, CAS 56-35-9), formaldehyde (HCHO, CAS 50-00-0), mercury chloride ($HgCl_2$, CAS 7487-94-7) and lindane (γ-BHC, CAS 58-89-9) were obtained from Sigma-Aldrich (St. Louis, MI, USA).

2.3. The Video-Tracking System and Exposure Experiments

The swimming behaviour was evaluated using a custom-made video-tracking system for capture and recording, followed by analysis with an adapted algorithm. Four recording video vigilance cameras (Flow Electronic 540L IR camara, with CCD 1/3″ Sony sensor, resolution 795 × 596 PAL, model CACO008, connected to a Camtronics DVR 38 AHD Plus capture device) were placed in an isolated temperature- and light-controlled recording chamber. Each camera filmed 12 circular arenas, in a total of 48 arenas per recording. Each set of 12 arenas contained two randomly distributed replicates of the control and the five toxicants. At seven and fourteen days old, the animals were exposed to the test toxicants. For this, the animals were randomly transferred into the recording arenas (one per arena) and allowed a non-recording exposure period of 1 h. The recording started from this point onward and lasted for 30 min, for a total assay period of one and a half hours. The recording procedure was repeated six times throughout the study with animals aged seven and fourteen days. The exposures were carried out in circular arenas suited for video recording, filled with 100 mL of either control medium (culture medium) or a toxic solution of sodium hypochlorite (0.5 mg/L), tributyltin (0.243 μg/L), formaldehyde (3.69 mg/L), mercury chloride (4.5 μg/L) or lindane (10 μg/L). For comparative purposes, the toxicant concentrations were the same as those previously tested in investigations of the locomotor behaviour in the zebrafish, in a similar system [35]. The test solutions were prepared by dilution in the culture medium of the respective stock solutions in ultrapure water.

The algorithm multiwellTracker [36] was adapted by the team for the analysis of the behaviour videos recorded with the above video-tracking system. The adaptation included the automatic detection of the circular arenas, tracking and analysis of the trajectories, as well as validation and extraction of nine behaviour parameters: distance covered (derived from the Y and X coordinates, i.e., the position of the organism in the Y and X axes); mean velocity (mm/s); mean angular velocity (degrees/s); the degree formed by movement vectors; instantaneous velocity (mm/s); the square root of the standard deviations of X and Y (dispersion measure); and the mean meander (degrees/mm) and instantaneous acceleration (mm/s^2). The adapted algorithm ran through three phases, each responsible for different but equally important steps in making the video easier to analyse. It worked by differential brightness between the organism of interest and the background i.e., it followed a dark object in a bright background. The first phase (pre-processing) corrected various aspects of the provided video. Here, possible fishbowl effects were removed, diminishing the distortion observed in the extremities. Furthermore, the contrast between the object and background was increased, making it an almost black-and-white image. The second phase was the detection of the trajectory, where the algorithm detected the number of circular arenas, delimited them and then detected the trajectories created by the moving organisms. This tracking was done through the variation in colour intensity in each frame. This approach requires a stable zoom and camera position while recording, and a suitable lighting orientation to ensure the best possible contrast between the background and the organism. The last phase was post-processing. Here, the detected trajectories were analysed and the behaviour parameters were derived from them. The obtained data were exported as Excel files. Additionally, a verification file was created, which allowed us to check the detection of the arenas and trajectories for the validation of the data obtained (Figure 1).

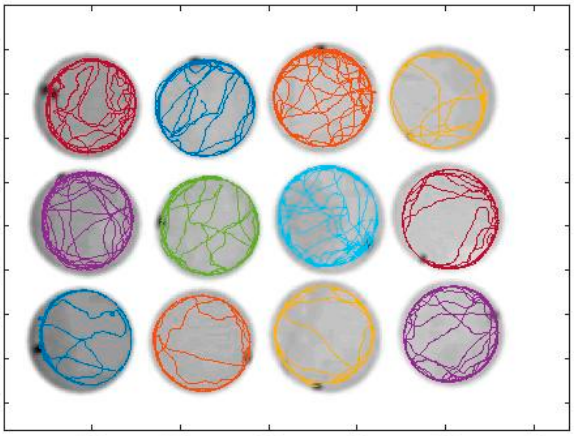

Figure 1. Final output example of one of the videos created for validation of the data obtained from the animal tracking. Such videos were used to verify the automated tracking and perform a first inspection of the overall behaviour of the recorded animals.

2.4. Data Analysis

A Cluster Analysis, using an artificial neuronal network (ANN) algorithm as indicated in [26], was performed to describe the six main behavioural patterns recorded. One-way Analysis of Variance with the Tukey HSD was used to characterise the behavioural types defined. A Chi-square analysis with behaviour types and toxicants as independent factors was then carried out for each exposure age (seven and fourteen days old) to investigate possible differences among test substances in the frequency of the behaviour types. A residual analysis was then carried out to investigate specific differences among treatments; the statistical significance of each residual was determined by comparing the respective Chi-square contribution (i.e., partial Chi-square value) against the critical distribution value determined with the Bonferroni correction for the total number of cells. All statistical analyses were performed using Statistica 14.0.0.15v (TIBCO Statistica, StatSoft GmbH, Germany). Correspondence analysis further depicted the pattern of behavioural responses to short-term exposures.

3. Results and Discussion

Six swimming behaviour types (A to F) were defined by the Cluster Analysis based on the ANN, each exhibiting different average values of relevant movement variables (Figure 2, top); the remaining variables were found to be redundant or to have a meagre contribution to the behaviour types defined. All movement data were used in the analysis, irrespective of the experimental condition or age of the animals. The behaviour types represented a gradient of variation to which the measured variables showed different contributions (Figure 2, bottom). The mean velocity and distance to the centre of the arena were the variables showing the highest contribution to the behavioural types (Figure 2, bottom). Each behavioural type thus showed its typical locomotion, though they shared some similarities (as indicated by a Cluster Analysis) in terms of velocity; slow swimming in behaviour types A, D and C, versus fast swimming in behaviour types B, E and F. For instance, when exhibiting behaviour type B, animals tended to swim fast, rotate quickly and wander, mostly in the centre of the arena. When exhibiting behaviour type C, animals tended to swim slowly and mostly in the periphery of the arena. In behaviour type F, the animals tended to change much their swimming velocity as indicated by the instantaneous acceleration and swim in the periphery of the arena.

Behaviour type	A	D	C	B	E	F
Instantaneous acceleration	−3.16 b	−4.41 a	−2.38 c	3.06 e	2.04 d	17.6 f
Instantaneous velocity	3.54 a	6.89 c	5.41 b	27.4 e	20.5 d	37.8 f
Mean velocity	7.33 a	10.2 b	7.67 a	23.7 e	15.7 c	19.1 d
Mean angular velocity	33.7 a	68.6 d	92.3 e	99.4 f	48.8 b	57.4 c
Mean meander	49.8 a	61.4 d	75.2 e	87.0 f	53.2 b	57.0 c
Distance to the centre	26.8 d	20.0 a	29.6 f	21.9 b	25.4 c	29.1 e

Behaviour type	A	D	C	B	E	F
Instantaneous acceleration	0.45	0.41	0.45	0.54	0.50	0.61
Instantaneous velocity	0.03	0.14	0.08	0.38	0.31	0.50
Mean velocity	0.99	0.99	0.99	0.99	0.99	0.99
Mean angular velocity	0.19	0.43	0.50	0.55	0.30	0.35
Mean meander	0.39	0.40	0.41	0.46	0.39	0.40
Distance to the centre	0.73	0.61	0.73	0.61	0.69	0.69

Figure 2. Characteristics of the behaviour types defined by the Cluster Analysis based on the Artificial Neural Network. (**Top**) Mean values in each behaviour type of the most relevant movement parameters. Significant differences among types were identified for the parameters investigated; homogenous subsets identified through the Scheffé test are indicated by small letters (different letters indicate significant differences at $p < 0.05$). (**Bottom**) Contribution (weight) of each movement parameter to the different behaviour types. The darker the green shade, the stronger the contribution. The results are grouped according to similarities in behavioural types across toxicants as found through cluster analysis.

The Chi-square analysis identified significant differences among the experimental conditions in the exposure of both juveniles ($Chi^2 = 3753$, $df = 25$, $p < 0.0001$) and mature animals ($Chi^2 = 4453$, $df = 25$, $p < 0.0001$). Figure 3 shows the residuals of the analysis and their statistical significance as determined by comparing each partial Chi-square value with the critical distribution value corresponding to a 0.05 significance level corrected by the Bonferroni method. From these results, behavioural profiles can be drawn for each experimental condition and used to distinguish the toxicants from the control, as indicated also by a Correspondence Analysis (Figure 4). Juvenile controls (7-d-old triops) tended to exhibit mostly the behaviour type B (swimming fast, with quick rotations, in the centre of the arena) (Figures 3 and 4). Mature controls (14-d-old triops) tended to show a lower frequency of behaviour C and increased frequencies of behaviour types E and F (intermediate mean velocity and swimming away from the centre of the arena). As for the toxicants, triops were sensitive to all toxicants and two main responses were found;

toxicants with increased frequencies of behaviour types C and D (lindane and bleach) and toxicants with increased frequencies of behaviour types E and F (mercury, formaldehyde and tributyltin). Sodium hypochlorite elicited the strongest changes, suggesting triops were particularly sensitive to it, as found previously for other species [26,35]. Juvenile and mature triops responded in a fairly similar way to NaOCl. At 7 d of age, the exposure tended to markedly increase the frequency of behaviours A, D and C, and decrease the frequency of behaviour types B, E and F, compared to expected values and the remaining treatments. At 14 d of age, the exposure tended to decrease the frequency of behaviour types A, B, E and F, and increase the frequency of behaviour types C and D. Globally, triops exposed to bleach were the slowest and less erratic (as indicated by the instantaneous acceleration) swimmers, as observed in zebrafish [26,35]. This tendency for slow wandering swimming may be related to the cellular depletion of ATP (adenosine triphosphate) elicited by the exposure [37], which may limit the energy available for locomotion. Exposure to lindane also tended to slow down the animals, at both 7 and 14 days of age, as reflected by the significant increase in the frequency of behaviour types D and C (Figures 3 and 4) and decrease in behaviours B, E and F. Lindane is known to be neurotoxic through its interaction with glycine receptors, which are major inhibitory receptors in the spinal cord and the brain stem [38]. In contrast, TBTO tended to elicit fast swimming responses. The exposure caused marked differences from the control profile, notably decreasing the frequency of behaviour types A, D and C and increasing the frequency of behaviour types B and E in 7 d-old triops and B and F in 14 d-old animals; the latter are the behaviours showing a higher mean velocity. Tributyltin is a ligand of retinoid X (RXR) and the ecdysteroid (EcR) receptors, which, in arthropods, act as homologs of RXR. Furthermore, it is a well-recognised endocrine disrupter that causes neurotoxicity and physiological stress in exposed animals [39]. While little is known about the mechanistic effects of tributyltin in *T. longicaudatus*, the recent protein-ligand modelling of EcR revealed the great evolutionary conservation of the protein across species when comparing the interaction amino acids (ascribed to RXR in *Homo sapiens*), with little conformational variation in the interaction pocket [40]. Exposure to formaldehyde tended to increase the frequency of behaviours B and E in 7 d-old triops, and A, E and F in 14 d-old triops, although at more moderate levels, and in opposition to the changes observed in the bleach group. Globally, the animals tended to show very irregular swimming in the periphery, with interspersed periods of lower and higher mean velocity, particularly at 14 d of age (Figures 3 and 4). Formaldehyde was previously found to alter the behaviour and respiration (time-dependent decrease in oxygen consumption) of zebrafish, with the detection of abnormal swimming movements, reduced opercular beats and increased mucus secretion that may be lethal [41]. These alterations were linked to increased metabolic costs due to damage in the gills, impairing movement ability. The response to mercury chloride was very similar to that observed in the formaldehyde-exposed group, except for behaviour type F, which also tended to be increased in frequency in juvenile triops. Mercury is a known neurotoxicant affecting the brain, but also possibly the central and peripheral nervous system [42].

Overall, the results showed that *T. longicaudatus* is sensitive to very low concentrations of hazardous contaminants in a very short exposure period (1.5 h). Using an ANN analysis, the species was useful in discriminating the toxicants from the control group. Both the juvenile and the mature animals showed high sensitivity and the ability to discriminate among the toxicants. The best-discriminated toxicants were bleach, lindane and tributyltin at 14 d of age. The use of mature animals in testing may be an advantage for the possibility of combining behavioural with reproductive endpoints. The results presented herein are comparable to those previously reported for zebrafish, using an analogous video-tracking system, the same toxicants and test concentrations, though the duration of the exposure was slightly shorter (45 min) [35]. In zebrafish, alterations in the swimming parameters (e.g., instantaneous and angular velocity, meander) were also found, producing a spectrum of sensitivities discriminating the contaminants.

Behaviour type	A	D	C	B	E	F
Control		−9.7		14		
$HgCl_2$	−4.6	−7.8		3.7	8.4	7.0
HCHO	−4.4	−12	−4.6	9.3	9.7	
TBTO	−4.2	−7.3	−3.3	3.7	9.3	
γ-BHC	−4.4	9.3	4.0	−5.3		
NaOCl	20	27	9.2	−26	−25	−8.1

Behaviour type	A	D	C	B	E	F
Control		−4.3	−12	5.1	9.6	9.6
$HgCl_2$			−13		8.7	12
HCHO	8.8	−6.7	−11	−6.3	7.8	8.0
TBTO	−6.9	−4.7	−7.0	18		5.6
γ-BHC			17	−8.4	−4.5	−12
NaOCl	−3.7	20	26	−5.8	−25	−22

Figure 3. Results of the residual analysis conducted to identify significant differences among treatments (control; formaldehyde, HCHO; tributyltin, TBTO; mercury, $HgCl_2$; lindane, γ-BHC; sodium hypochlorite, NaOCl) in juvenile (**top**) and mature (**bottom**) triops. Only the significant residuals are shown; the statistical significance of each residual was determined by comparing the respective partial Chi-square value against the critical distribution value estimated with the Bonferroni correction for a significance level of 0.05.

The present results point out the interest in triops as a suitable model for developing biological early warning systems (BEWS) to diagnose environmental contamination. Historically, BEWS were thought to fill the gap and complement the traditionally available water quality testing [43]. Behavioural types of mostly fish, the first model employed, but also crustaceans and mussels, were used to develop real-time monitoring. Toxicant-induced behavioural manifestations or types often preceded endpoints at the physiological, developmental or reproductive level, which are indicative of detrimental effects on the exposed populations [43]. Though BEWS based on behavioural analysis are usually non-specific, the approach taken here provides a strategy to develop exposure profiles to different toxicants and a suitable alternative species under the ethical legislation of animal experimentation and welfare. A crucial actual challenge in environmental toxicology is to move from the current paradigm of animal testing, based to a high extent on mortality and vertebrate species. A biological effects assessment is unavoidable for diagnosing environmental quality and mixture effects in systems with complex mixtures of contaminants, i.e., comprising many unknown chemicals, chemicals for which no analytical methods are yet available or that are below the limits of detection and unrecognised transformation products of many parental toxicants. There is, thus, a need to develop more sensitive alternatives, based on less invasive endpoints and reducing the number of animals employed, useful for anticipating potentially detrimental impacts before the systems attain the tipping point or threshold beyond which unstoppable detrimental effects take place and any intended protection or recovery action will not be effective. Triops appear to be a model of high interest for such an approach. They are easy to maintain in the lab and are accessible for follow-ups, owing to their colourful shell. They show a suitable size for video-tracking but are still small enough to allow for the development of easily operating and affordable testing systems. Most importantly, triops exhibit very active swimming, with naturally different behavioural types and types specifically elicited by distinct toxicants, which is useful for profiling. Lastly, they are also amenable to complementary molecular investigations that can bring knowledge on the modes of action of chemicals on exposed animals [40,44,45]. In particular, compared with the well-established freshwater model *Daphnia magna*, triops are about ten times bigger, which facilitates carrying out analyses in individuals, such as the determination of biochemical and other molecular biomarkers. The video-tracking of swimming behaviours becomes easier because of the size of the animals and their intense movement. The lab maintenance is comparatively simpler, as they can be cultured in dechlorinated water and fed with commercially available food, in contrast to daphnids, which need a specific culture medium with nutritional supplements and nourish well only on freshly cultured microalgae. Adding to this, triops' cysts are commonly dehydrated and can be stored for long periods, up to the moment they are required for the assays, in the same way artemia is used. Lastly, they cover an important need in freshwater ecotoxicology as they are primarily benthic, spending most of their time digging and sifting through bottom substrate of ponds and pools in search of food. Further testing with this species should focus on evaluating other toxicants (with similar and dissimilar modes of action), singularly and in mixtures. This should be combined with resilience and diagnostic stress tests, including sensitivity and accuracy determinations for diagnosis quality (false positives and false negatives in diagnosis tests).

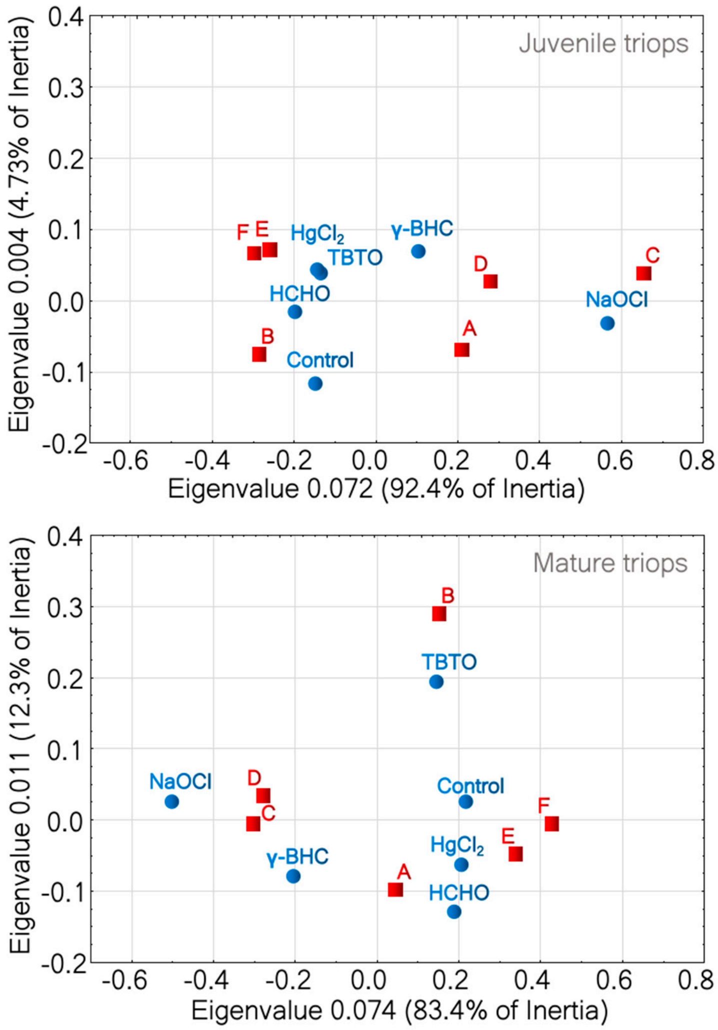

Figure 4. Behavioural types (A to F) exhibited by triops exposed for a short-term period (1.5 h) to formaldehyde (HCHO), tributyltin (TBTO), mercury ($HgCl_2$), lindane (γ-BHC) or sodium hypochlorite (NaOCl), as indicated by a Correspondence Analysis.

4. Conclusions

As revealed by the statistical pipeline refined herein (artificial neural network analysis followed by Correspondence analysis), the present work is a first approach to the investigation of triops as a potentially sensitive model to detect and assess low levels of detrimental aquatic pollutants in very short exposures, as an alternative to animal testing. Behavioural patterns observed in control and toxicant groups were identified. Based on previous studies employing a similar approach, the results were in the sensitive range of adult zebrafish. Exposure of mature animals appeared to provide a slightly better discrimination of all toxicants. Future work should focus on investigating the sensitivity of this model and behavioural tools of other contaminants, singularly and in mixture, and validate its potential for development as a biological early warning system. Combining the results with the evaluation of molecular and biochemical biomarkers in this species, as well as health indicators, will further bring insight into the modes of action and molecular initiating and secondary events linked to the toxicant response. This will help to streamline the conditions for application to environmental diagnoses and monitoring, and their implications for ecosystem health. The model species and behavioural analysis can be easily applied to these goals and others (e.g., evaluating remediation actions and ecosystem recovery), under the common frameworks and guidance available, including before/after designs and site-specific risk assessments.

Author Contributions: Conceptualization, methodology, validation, supervision, project administration and funding acquisition L.G., A.P.C. and L.O.-T.; software, L.G., N.S. and L.O.-T.; experimentation and formal analysis, P.R., C.T., A.P., J.A. and L.O.-T.; writing—original draft preparation, L.G., A.P.C., P.R. and L.O.-T.; writing—review and editing, All authors. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data can be shared upon request.

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