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Locomotory reaction spectrum of *Triops longicaudatus* in response to aquatic contamination

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Abstract

Concern about aquatics habitats is always present due to their necessity both in maintaining the existent biodiversity and in a more human-interest perspective. Temporary ponds are a type of aquatic environment that is less studied than others although they play a big part in the life cycle of various organisms. Mediterranean temporary ponds, a particular type of temporary ponds, present an even higher conservation importance but ways of monitoring the status of these habitats are lacking.

Triops longicaudatus is a crustacean species that inhabit temporary ponds. They are globally spread, existing in the various regions that present a Mediterranean climate. These crustaceans could used to monitor these habitats, however information about them is severely lacking, particularly in an ecotoxicological context. This study aims to establish a rearing protocol in laboratory settings and to verify the possibility of utilizing this species in future ecotoxicological assays. This was done through various assays to determine rearing conditions and behavioral assays with exposure to low concentrations of 5 toxicants: tributyltin, mercury, lindane, sodium hypochlorite and formaldehyde. An artificial neuronal network was used to analyze the results

With the results from the assays to determine rearing conditions, a protocol was elaborated that allows the organisms to complete their life cycle. The behavior assays showed that *T. longicaudatus* are sensitive, particularly to sodium hypochlorite and mercury. These results exhibit that this species can possibly be used in assays, allowing for more studie of a protected habitat and new possibilities in a laboratory setting.

Resumo

Ambientes aquáticos sempre tiveram preocupação associada devido ao seu papel em manter a atual biodiversidade e em aspetos de interesse humano. Charcos temporários são um tipo de ambiente aquático que é menos estudado que os restantes embora tenham uma grande importância para o ciclo de vida de variados animais. Charcos temporários mediterrâneos são um tipo particular de charcos temporários e apresentam uma importância a nível de conservação superior. Contudo, formas de monitorizar a condição destes habitats são escassas.

Triops longicautus é uma espécie de crustáceo que habitam charcos temporários. Têm uma distribuição global, existindo em várias regiões que apresentam clima Mediterrâneo. Estes crustáceos poderiam ser usados para monitorizar estes habitats, mas informação eles é escassa, particularmente num contexto ecotoxicológico. Este trabalho tem como objetivo definir um protocolo para a criação e manutenção em contexto laboratorial e verificar a possibilidade de utilização desta espécie em ensaios ecotoxicológicos. Isto foi feito usando vários ensaios para determinar as condições de criação e ensaios comportamentais com exposição a baixas concentrações de 5 tóxicos: tributilestanho, mercúrio, lindano, hipoclorito de sódio e formaldeído. Uma rede neuronal artificial foi usada para analisar os resultados.

Com os resultados dos ensaios para definição de condições de cultura foi desenvolvido um protocolo que permite que os organismos completem o seu ciclo de vida. Os ensaios comportamentais mostraram que os *T. longicaudatus* são sensíveis, particularmente ao hipoclorito de sódio e mercúrio. Estes resultados mostram que esta espécie pode ser usada em ensaios, permitindo mais estudos sobre um habitat protegido e novas possibilidades num contexto laboratorial

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List of abbreviations

AChE- acetylcholinesterase

ATP- Adenosine triphosphate

BEWS- Biological Early Warning Systems

GABA- gamma-Aminobutyric acid

GABAAR- gamma-Aminobutyric acid receptor

GMA_3- 3 algae granulate

LC50- Lethal concentration 50. Compound concentration that is lethal to 50% of the exposed animals.

LOEC- Lowest Observed Effect Concentration

MTP- Mediterranean Temporary Pond

NOEC- No Observed Effect Concentration

OP- Organophosphorus

POP- Persistent Organic Pollutant

TBT- Tributyltin

1. Introduction

1.1 Temporary ponds

Temporary ponds are commonly described as water bodies that have a predictable annual dry phase of 3 to 8 months, usually occurring during summer or autumn (Ward, 1992), and appear in shallow depressions along the soils' upper layer (Collinson et al., 1995). These freshwater habitats are widespread and can be found in different regions, such as in northern Europe (Angélibert et al., 2004), West Africa (Lahr et al., 2000) and Australia (Williams, 1997). Although these environments are considered harsh due to the fluctuations of dry and wet periods, this situation presents favourable opportunities for some species. For instance, a regular dry out phase of a temporary pond can lead to the remineralization of nutrients, promoting plants productivity (Collinson et al., 1995). Moreover, they are used as feeding and reproduction areas by several species of amphibians, reptiles, mammals and invertebrates (Griffiths, 1997).

Particular attention has been given to Mediterranean Temporary Ponds (MTPs), a priority habitat under the Habitats Directive of European Union's Natura 2000 Network (Evans, 2006). The MTPs are present in 5 different regions of the world with Mediterranean climate: Western USA (Kneitel and Lessin, 2010), Europe (Zacharias et al., 2007), North and South Africa (Rhazi et al., 2006; Seaman et al., 1995) and Australia (Williams, 1997) and exhibit some of the same characteristics as other temporary ponds but with additional conservation importance. However, these areas suffer an ever-increasing degradation rate even though a wide variety of rare and endangered species inhabit them (Aponte et al., 2010; Beja and Alcazar, 2003; Brendonck and Williams, 2000). Additionally, they can be considered early warning systems of long-term changes in larger bodies of water (rivers and lakes), as a result of their reduced size and simple community structure (Hulsmans et al., 2008; De Meester et al., 2005). This community structure can be related to the origin of the temporary pond, ranging from ponds created by melting snows in colder northern zones to inundated coastal pools (Nilsson and Söderström, 1988; Serrano and Serrano, 1996). Nowadays, despite an improving public perception of wetlands over recent years, MTPs are disappearing or losing their quality due to anthropogenic activities, such as intensive agriculture (*i.e.*, increased use of fertilizers and biocides) or land abandonment, overgrazing, groundwater extraction, urbanization, touristic/recreational uses, forest fires, introduction of exotic species and climate changes

(Aponte et al., 2010; Beja and Alcazar, 2003; Brendonck and Williams, 2000; Zacharias et al., 2007). In fact, these pressures are magnified in MTPs since they are typically characterized by shallow waters and frequently small surface areas (Boix et al., 2016). Thereafter, there is a clear need to develop ecotoxicological approaches useful to assess the quality status of these ecosystems.

1.2 Triops longicaudatus

Triops spp., also referred as tadpole shrimps (TPS), are widely distributed crustaceans that inhabit temporary freshwater ponds (Horn and Cowley, 2016; Sassaman et al., 1997). They belong to the phylum Arthropoda, sub-phyllum Crustacea, class Branchiopoda, sub-class Phyllopoda, order Notostraca, and family Triopsidae. Their morphology has remained largely the same since the late Cretaceous period (more than 70 million years) (Suno-uchi et al., 1997), with numerous scientific reports focusing on its living fossil status (Mantovani et al., 2008; Seong et al., 2016). In fact, many of the TPS features come from their benthic nature. Dorsally they have a continuous domed carapace, presenting a line of demarcation, with an equally thick head integument (Fryer, 1988). In the head there are sessile compound eyes that, along with the entire head, are integrated into the carapace. Posteriorly, it has a long abdomen with various appendages (thoracopods) with movement and respiratory functions (Fryer, 1988; Martin and Boyce, 2004). The abdomen ends in a filamentous caudal furca.

These benthic branchiopods feed by burrowing into the sediments in search for detritus and different small organisms (Scholnick and Snyder, 1996). Generally, they eat bacteria, microalgae, protozoa, rotifers, branchiopods, oligochaete, insect larvae, frog eggs, and also small tadpoles, being either facultative detritus feeders, scavengers or predators (Cvetkovic-Milicic and Petrov, 1999). Nevertheless, their feeding behaviours depend on their habitat conditions. For instance, in high population density scenarios, food becomes scarce and, as a consequence, mortality increases due to cannibalistic behaviours, together with development inhibition and reduced fecundity (Scholnick and Snyder, 1996). These freshwater invertebrates are also a key organism of the diet of many species of amphibians and birds (Fry et al., 1994; Fryer, 1988), while they still provide for more food to filter-feeder as a result of the constant digging in the pond substrate, which stirs up the sediment. *Triops* spp. have also shown potential as a biological control agent of mosquito populations and weeds, in paddy fields (Fry et al., 1994; Tietze and Mulla, 1991). Mosquito predation is of particular interest, as it helps in controlling insect vectors of a wide variety of diseases (Reiter, 2001; Vinauger et al., 2014). Hence, tadpole shrimps play a crucial role in the functioning and health of temporary freshwater ponds (Fry et al., 1994).

According to Korn et al., (2010), morphology-based identification of *Triops* spp. is complex appearing as reasonable only at the genus level, whereas reliable identification of individual specimens requires the application of genetic markers. Currently, there are 11 identified species of the *Triops* genus: *T. australiensis*, *T. baeticus*, *T. cancriformis*, *T. emeritensis*, *T. gadensis*, *T. granarius*, *T. longicaudatus*, *T. mauritanicus*, *T. newberryi*, *T. simplex* and *T. vicentinus* (Korn and Hundsdoerfer, 2006; Korn et al., 2006, 2010; Sassaman et al., 1997; Tyler et al., 1996). Among them, *T. longicaudatus* is the only species which cysts are commercialized.

Triops longicaudatus can be found worldwide, in regions including the American continent, Pacific Islands and Saudi Arabia (Aloufi and Obuid-Allah, 2014; Kwon et al., 2010). The reproduction of this species is achieved by oviposition, with the embryos developing in the following 2 or 3 days (Takahashi, 1977). In order to survive the dry phase, a determinant stage of temporary ponds, eggs harbouring embryos are preconditioned, by making them resistant to heat and desiccation (Carlisle, 1968). Thus, these eggs remain in quiescent state and are capable of hatching in future flooding periods (Su and Mulla, 2002a). If a dry stage does not take place, an "egg bank”, which is a multigenerational assemblage of eggs, is created (Su and Mulla, 2002b). Eggs are burrowed into the pond sediments in order to survive from potential predators, including TPS themselves. This behaviour, typical of *Triops* spp., leads to an increase in water turbidity and, thus, can be indicative of TPS presence in ponds. (Fry-O’Brien and Mulla, 1996).

To summarize, *Triops longicaudatus* possess interesting biological attributes, such as a: (i) widespread distribution; (ii) short life cycle; (iii) high fecundity; and (iv) a dormant egg (cyst) phase (Fry-O’Brien and Mulla, 1996; Su and Mulla, 2001; Takahashi, 1977). Although it has seldom been used in toxicity testing (Fry-O’Brien and Mulla, 1996; Grigarick et al., 1961; Kumar and Hwang, 2006; Mullié et al., 1999; Su and Mulla, 2005; Su et al., 2014; Tsukimura et al., 2006; Walton et al., 1990), the above characteristics make of them a good candidate for use as test species in ecotoxicological studies and interesting laboratorial model, given the relative ease to get a large number of animals, in a short period of time.

1.3 Ecotoxicology and its importance to environmental disturbances

In 1969, the term “Ecotoxicology” was created in order to describe a new subdivision of Toxicology that aimed to study the fate and effects of chemical pollution on the ecosystems (Truhaut, 1977), and has become one the most relevant multidisciplinary fields of study. As such, Ecotoxicology not only seeks to understand the fate and adverse effects of chemical contaminants on organisms, but also to relate them with different levels of organization within the ecosystem. Hence, it’s two ultimate objectives are to prevent environmental disturbances caused by toxicants and to resolve them (Amorim et al., 2017a; Forbes, 1993). However, Ecotoxicology still has to face the gap between agriculture and industrial development, discovery of new compounds and the assessment of their potential risk to the environment (Bushnell et al., 2010). Over the years, several approaches have been developed in order to detect and quantify the effects of contaminants on various levels of biological organization. When applied in ecotoxicology, these include toxicity assays and biomarker usage (Klaassen and Amdur, 2013). The prevention of environmental problems requires early and significant knowledge of the effects that the exposure can cause on individuals, populations communities and ecosystems. To reach this objective, a set of different and complementary early warning environmental biomarkers are used, providing information about exposure, effects and modes of action of chemical contaminants (Rodrigues and Pardal, 2014). These are key diagnostic tools that may suggest if the organism was exposed to contamination, the extent of the response and the possibility of recovery (Cajaraville et al., 2000; Costa and Guilhermino, 2015). A sensitive biomarker that can help close that gap may be the usage of animal behaviour analysis in ecotoxicological assays.

1.4 Behaviour as a toxicity endpoint

In classical ecotoxicological assays, the most used endpoint is lethality/survival (Hellou, 2011) providing the traditional values of No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC), among others. While this is a population level endpoint easy to compare, due to its vast use and ease of obtaining, it may only evaluate sporadic aspects (Clotfelter et al., 2004). Furthermore, because it is based on animal death some challenges of ethical and legal nature may exist, so that alternatives must be considered. Additionally, because it involves an unrecoverable generalized failure of organ systems, mortality is of very limited interest to anticipate loss of ecosystem quality in time to elaborate adequate prevention or mitigation actions aiming at maintenance of the good ecological status. Evaluation of sub-lethal effects, these being effects on organisms that do not directly reflect in death due to the tested substance (Desneux et al., 2007), thus give a more complete and useful picture of what is happening since not all the organisms may die from the exposure (Desneux et al., 2007) but may suffer some kind of effect. Several sublethal effects exist including growth, reproduction capabilities, maturation and behaviour related ones, as immobility (Ashauer et al., 2011). To quantify sublethal effects, biomarkers are used. Biomarkers are biological responses measured at individual to sub-individual levels that give information about the exposure and/or effects elicited by to chemical contaminants (Peakall, 1994; Rodrigues and Pardal, 2014).

Behaviour analysis is getting attention as an endpoint and as a biomarker in ecotoxicological assays, mainly due to a potential greater sensitivity than mortality, ranging from 10 to 1000 times (Hellou et al., 2008). The major advantage of using behavioural endpoints is the capacity of observing sub-individual effects, without the need of invasive methodologies. This is possible because an organism's behaviour is dependent on the circumstances that it is experiencing. Behaviour presents a variety of possible endpoints, such as: balance, respiration, locomotion, mating, fear reactions and righting ability (Hellou, 2011). These are all measurable at sublethal exposure concentrations, with impact at population level, and thus, can be considered as early warning signals for the onset of toxicity (Hellou, 2011). Moreover, alterations of these Behaviours can be caused by different types of substances, belonging to diverse chemical types. Behavioural changes have been noted in both vertebrate and invertebrate organisms (Hellou et al., 2005), so behavioural endpoints can be used in a wide variety of organisms (Behrend and Rypstra, 2017; Broly and Deneubourg, 2015; Endo et al., 2016; Hirazawa et al., 2017).

Behavioural monitoring is a useful facet of using behaviour as a biomarker. This type of evaluation uses responses of keystone species to their environment (Xia et al., 2018). This approach has been proved efficient and can detect the effects of a wide variety of contaminants, as it is not limited by specificity to any particular compound or exposure time, in contrast with many traditional physicochemical sensors (Xia et al., 2018). Behaviour analysis has been utilized since the 1900's, some studies reporting on the respiratory exchanges of some organisms (Keys, 1930) and the opercular rates of fish (Belding, 1929). Alongside the evolution of overall technology and particularly of computing systems, these analyses attained higher sensitivity and higher volumes of data could be gathered constantly (Cairns Jr et al., 1970), aiding the significance of the statistical analysis of the collected data. In particular, locomotor behaviour, which is also at the basis of many other vital animal behaviours (e.g. prey finding, predator escaping, mating) has been successfully used to develop Biological Early Warning Systems (BEWS) useful to evaluate water quality and diagnose ecosystem health (Amorim et al., 2017a; Fernandes et al., 2016; Oliva Teles et al., 2015).

1.5 Video-tracking for behaviour analysis

Behaviour recording has been used for years and has evolved during that time, allowing for development of sensitive BEWS. Initially, the methods used were based on direct observation, with the researcher taking notes that could be considered somewhat subjective. With new and enhanced technology, novel methodologies were created, such as fully automated systems (Crispim Junior et al., 2012), which present advantages that must be considered when choosing the adequate method to be used. Automated systems are more versatile and allow the study of a variety of different behaviours, that are otherwise impossible to analyse; namely the study of rare behaviours happening briefly or of the behaviour of organisms that have long periods of inactivity. On the other hand, some behaviours happen during long hours so that spatial measurements can't be adequately done with simple direct observation (Spink et al., 2001). Moreover, automated systems eliminate the subjectivity that comes with human observation and the observer fatigue, both aspects that can negatively affect the results obtained.

Automated systems usually detect behaviours indirectly, through infrared sensors or image processing as video-tracking analysis (Rousseau et al., 2000; Spruijt and DeVisser, 2006). The latter offers a range of information about the locomotion of the recorded organism, such as velocity (Shijun and Yao, 2012) and the animal's position, among others (Crispim Junior et al., 2012). Automated video-tracking systems started to be used in the 1990s, and their utilization has been increasing since then (Endo et al., 2016). Also, many of them are of relatively inexpensive implementation, which is an advantage for routine use. Another point of major importance in behaviour analysis is transforming the data obtained in a quantitative manner, so several methods have been used to quantify behaviours, including telemetry (Westcott and Graham, 2000), direct observation (Teather et al., 2001) and computerized digitization into X,Y coordinates (Beauvais et al., 2000). This data can then be used to evaluate different endpoints in a systematic and objective manner.

1.6 Neuronal networks

Artificial neuronal networks are computational models based on working principles of the animal central nervous system. They estimate non-linear mathematical functions that require a high number of input data (Cheng and Titterington, 1994). Usually, these are systems that have simple processing units, the “neurons”, interconnected, able to relate inputs and recognize patterns. These “neurons” are connected, forming a network that resembles an animal brain due to an unclear division of labour between the various artificial neurons while working in parallel. The development of these networks changed the landscape of artificial intelligence, fostering the transition from systems concentrating the whole available knowledge in its code to dynamic learning systems. They are successfully used when previous knowledge is lacking, as they are capable of recognizing subtle relations in the data, even if they are difficult to predict or even unknown. Usually, they are more flexible and sensitive than classical statistical methods, and are able to improve their performance (Zhang et al., 1998).

1.7 Toxicants

Over the years chemicals belonging to several different groups have been found to impact aquatic systems, including metals, various pesticides and organotin compounds. Owing to their persistent nature and potential detrimental effects in aquatic organisms many of them have been classified as priority substances within the European legislation (European Union, 2013) or as emerging compounds of concern. Throughout this study, a set of five different toxicants belong to such groups were used: tributyltin (TBT), lindane, formaldehyde, mercury chloride, and bleach were chosen in order to perform the behavioral assays. Moreover, these chemicals have been used in previous studies with different species, thus, allowing for an interspecific comparison analysis. Additionally, cadmium and fenitrothion were selected for preliminary mortality tests due to their rising interest as emerging compounds.

1.7.1 Tributyltin (TBT)

Tributyltin, commonly referred to as TBT, is a class of organotin compounds that contains three C_4H_9 groups, one tin (Sn) group, plus a variable group that is frequently an oxide. These substances were regularly used in antifouling paint, due to their biocide capacities, in order to prevent the development of marine organisms on boats and other floating structures. This application was necessary as an accumulation of organisms on the immersed sections of those structures could lead to increased weight, higher fuel consumptions, and reduced speed. The concentration of 20 parts per trillion was shown as effective for its function. However, as TBT is a nonspecific biocide, many non-target organisms were being affected, even at low concentrations (Wong, 1991). The mode of action of this toxicant mainly involves the inhibition of oxidative phosphorylation, having an effect on the structure and function of mitochondria, leading to some toxic effects including mortality, growth retardation, immobilization and *imposex* (Wong, 1991). As a consequence, the use of TBT was banned in 2008.

Although it has been banned, TBT could still be found in the environment for a long time. In seawater, its half-life could vary from days to weeks, depending on the conditions. However, given its low solubility and high density, TBT can deposit towards the sediment zone. Here, the half-life is longer, going from 1.5 years to possibly over 19 years (Adelman et al., 1990; de Mora et al., 1995). More recently, a study showed that the levels of TBT and *imposex*, a disorder where females develop male sexual characters (Smith, 1971), have been decreasing over the last 15 years along the Portuguese coast supporting the success of the restricted use of the substance (Laranjeiro et al., 2018).

1.7.2 Formaldehyde

Formaldehyde (CH_2O) is an organic compound, that was used as a precursor of several materials and chemicals, and thus employed in a large number of industries. Its uses include being a precursor of various resins, plastics and even used as a finisher in the textile industry. Formaldehyde is a natural compound, so it is also produced by living organisms. Formaldehyde is normally a gas, but some aqueous solutions exist, one of which includes up to 15% methanol, to prevent the creation of polymers, becoming known as formalin. Methanol in formalin should not present toxic potential, due to its low concentration and high Median Lethal Concentration (LC50) values when isolated (Hohreiter and Rigg, 2001). Formalin (HCHO) is used to control populations of fungi, protozoans, and ectoparasites in aquaculture facilities. However, its use has already been banned in Europe because of its classification as a human carcinogen (Baan et al., 2009).

1.7.3. Mercury

Mercury (Hg) is a heavy metal that has had a variety of applications throughout human history. It has been used as a pigment, as medicine to treating diseases such as syphilis, and in a variety of other products. This substance has different forms, each presenting different effects on exposed organisms. All forms of mercury affect proteins by altering its quaternary and tertiary structures, leading to changes in cellular function. Therefore, it is possible that mercury can cause damage in any organ or subcellular structure. However, in its vapor form, the major target is the brain, although effects in other organs can also be observed, such as renal and/or immune functions, as well as some types of dermatitis (Bernhoft, 2012). If a massive exposure happens, it can lead to respiratory failure due to erosive bronchitis (Bernhoft, 2012). The kidneys and gastrointestinal tract are the main affected organs in an exposure to mercuric salts. This can lead to vomits, bloody diarrhea and possible gut mucosa necrosis (Bernhoft, 2012). Regarding mercuric salts, acute poisoning is more common than the chronic exposure. Effects on the kidneys are also possible, including necrosis of the renal tubes and autoimmune glomerulonephritis, even simultaneously (Bernhoft, 2012)**.** Another possibility is immune system impacts, including asthma, dermatitis and alteration of lymphocyte populations, specifically natural killer cells (Bernhoft, 2012). Other effects exist, such as spermatogenesis inhibition, capillary damage and muscle atrophy in the thigh (Bernhoft, 2012).

In its organic form, methyl mercury (CH_3Hg) can obstruct the synthesis of proteins and DNA transcription (Bernhoft, 2012)**.** This form can also reduce the activity of natural killer cells and can lead to autoimmunity (Bernhoft, 2012). Even as a priority pollutant, high concentrations can still be found in the environment such as the 20 µg/L reported in Spain (Nevado et al., 2003).

1.7.4 Lindane

Lindane ($C_6H_6Cl_6$) is how the gamma isomeric form of Hexachlorocyclohexane is commercially known. Since the 1940s, it was heavily used as an insecticide all over the globe.

Alongside this use, it was also utilized in seed and crop treatment, as a wood preservative and as an ectoparasite treatment. However, due to its effects on non-target organisms and persistence in the environment the use of this substance was internationally banned in 2009.

Lindane is considered a Persistent Organic Pollutant (POP) and classified as an organochloride. It can be transported atmospherically to any part of the globe, being already detected in the artic region (Marini et al., 2012). This is a very stable compound, resistant to various types of degradation and, due to its lipophilic nature, it can bioaccumulate and even biomagnified (Albanis et al., 1996).

This substance has neurotoxic effects, interfering with gamma-Aminobutyric acid (GABA), with the main role of reducing neuronal excitability in the central nervous system. Lindane interacts with the GABA receptor (GABAAR), blocking the ionic channel and prevents the connection between GABAAR and its endogenous ligand. This leads to continuous nervous pulses that hyperstimulate neurons and muscles, possibly causing paralysis and death (Islam and Lynch, 2012; Nolan et al., 2012). Other effects can be observed, such as cytotoxic and endocrine disruption (Briz et al., 2011; Vale et al., 1998).

Acute exposures to lindane can lead to neurologic effects, as shaking, convulsions, ataxia, tremors and possibly death. A chronic exposure can create major systemic effects, including heart arrythmia, decreased liver function, and necrosis of blood vessels that can cause lower blood volumes in the lungs, kidneys and brain (Nolan et al., 2012).

1.7.5 Sodium hypochlorite

Sodium hypochlorite (NaOCl), more commonly known as bleach, is one of the most used compounds at a global level, in a variety of settings, from industrial to domestic, as a consequence of its disinfecting capabilities and low cost of production (Magalhães et al., 2007).

This substance can be absorbed through the skin, ingested or inhaled and can cause a variety of nefarious effects, including burning, diarrhea and skin inflammation, (Nimkerdphol and Nakagawa, 2008). Furthermore, in some organisms, carcinogenic capabilities were observed, leading to increased numbers of tumours in various locations, including the testis, pancreas and uterus (Kurokawa et al., 1986). Depression effects, lower body weight and reduced feed intake have also been noted in Japanese quails exposed to this contaminant (Hamdullah et al., 2010; Khan et al., 2008). In warmer habitats, NaOCl can be more dangerous, as the higher temperatures can thermally decompose it and form corrosive gases (Nimkerdphol and Nakagawa, 2008). Some different modes of action have been reported, such as reduction of ATP levels possibly lowering them to undetectable levels (Hidalgo et al., 2002). Human dermal fibroblast cell viability also decreases in a dose-dependent curve, but they can recover from this exposure after 24h of incubation (Hidalgo et al., 2002).

1.7.6 Fenitrothion

Fenitrothion (O,O-dimethyl O-(4-nitro-m-tolyl)-phosphorotioate) is an organophosphorus (OP) pesticide, used to overcome a variety of pests (Abdel-Ghany et al., 2016). Pesticides are used worldwide to increase crop production, by protecting them against pests that would decrease the quality and/or the quantity of the crops (Derbalah et al., 2019). Additionally, they can also be used in a public health setting, by controlling disease vectors (Walker et al., 2016), such as its use as a control agent of mosquito populations in Thailand. Heavy usage of this compound results in it being present, along with its metabolites, in natural water sources, as lakes and rivers, as well as in soils. Consequently, it can be found in food products, becoming another route of exposure alongside occupational exposure (Taib et al., 2013). Given the non-specific nature of fenitrothion, its use and existence in nature has been reported as harmful to non-target organisms (Derbalah et al., 2004). This pesticide, however, acts as an inhibitor of acetylcholinesterase (AChE; EC 3.1.1.7) activity (Forsyth and Martin, 1993). This inhibition leads to a build-up of acetylcholine (ACh), a neurotransmitter, and to a considerable overstimulation of postsynaptic cholinergic receptors, altering the function of postsynaptic cells and, consequently, causing cholinergic toxicity (Abdel-Ghany et al., 2016). Furthermore, it can also generate morphological and functional effects in animal tissues (Abdel-Ghany et al., 2016; Elhalwagy et al., 2008; Taib et al., 2013). Another possible mechanism of action is the creation of oxidative stress, with variation of the activity of antioxidant enzymes (Abdel-Ghany et al., 2016; Elhalwagy et al., 2008; Taib et al., 2013). It has been reported that fenitrothion can present higher ecotoxicological risk than other OP pesticides (Derbalah et al., 2019).

1.7.7 Cadmium

Cadmium (Cd) is a widespread metal, used in many industrial settings, including metallurgical, electroplating and paints that presents many human and environmental health concerns (Newairy et al., 2007). Like other heavy metals, it has carcinogenic, mutagenic and teratogenic effects (Galán et al., 2000). It can also induce stress responses, marked by the increase of heat-shock proteins (Galán et al., 2000) and affects the antioxidant defence system (Newairy et al., 2007). Among reported effects are renal and hepatic damages, both functional and morphological (Dehn et al., 2004; El-Sharaky et al., 2007; Newairy et al., 2007), biochemical and morphological changes in the gastrointestinal tract and in the lungs (Newairy et al., 2007) and some reproductive hazards (Choe et al., 2003). These effects may be explained by an increase in lipid peroxidation (Calderoni et al., 2005; Manca et al., 1991; Newairy et al., 2007) that can be related to alterations in the antioxidant defence system (Newairy et al., 2007; Ognjanovic et al., 1995).

Cadmium chloride is a water-soluble salt, therefore, reaching and able to accumulate in a variety of tissues, like brain, kidney, and heart amongst others.

1.8 Main objectives

The more noticeable climate change scenario and the continuous use of various types of chemical substances cause great stress on all the habitats in the world. Small and isolated environments tend to be the ones affected in the first place, and in a harsher form. These vulnerable habitats, like temporary ponds, need to be protected in order to maintain the existing biodiversity and the ecosystem services.

The main aims of this study were twofold: i) to develop a reliable rearing and maintenance method of *Triops longicaudatus* in a laboratory setting and assess its possible use as a test species in ecotoxicological assays; and ii) to develop a BEWS system based on the TPS locomotor behaviour. The following chapters present the materials and methodology employed and the results obtained. The results section is subdivided in two; one addressing *T. longicaudatus* laboratory culture and toxicity testing and the other dedicated to the video-tracking of the locomotion behaviour of *T. longicaudatus* under exposure to different toxicants for BEWS development. These are followed by a general discussion and main conclusions of the work.

2. Materials and methods

2.1 Triops longicaudatus rearing

Triops longicaudatus individuals were hatched from commercial cysts (Triopsking), and rearing followed the general procedures described in FryOBrien and Mulla, (1996), however, with modifications aimed at improving survivability, which was quite low initially. Because the information available about controlled laboratory conditions for maintenance of tadpole shrimps is scarce, particularly in regard to conditions providing adequate animals for ecotoxicological assays, several parameters were first investigated; namely the culture medium, substrate type, temperature and feeding. The culture media tested were deionized water, dechlorinated water, bottled water with low or high mineralisation. As feeding diet four possibilities were investigated: live algae, dried Spirulina (Triopsking), granulate mixture of 3 algae (3-Algae Granulat from Ropical), fish granulate (Vitality and Color Granulat from Tropical) and yeast (Condi).

2.2 Chemicals

Sodium hypochlorite (NaClO – 5%, CAS 7681-52-9) was acquired from PanReac AppliChem. Tributyltin (TBTO – 96%, CAS 56-35-9), formaldehyde (HCHO – 36.5-38%, CAS 50-00-0), mercury ($HgCl_2$ ≥ 99.5%, CAS 7487-94-7), lindane (γ-BHC – 97%, CAS 58-89-9), cadmium (CdCl2 - 99.99%, CAS 10108-64-2) and fenitrothion (C9H12NO5PS - ≥ 95%, CAS 122-14-5) were obtained from Sigma-Aldrich.

2.3 Ecotoxicological assays

Pilot ecotoxicological assays, based on lethality as endpoint, were carried out with two model compounds, cadmium and fenitrothion. Test conditions (test medium, substrate, feeding, light/day cycle and temperature) were established according to the results of the initial investigations for culture maintenance (see results section). Six or seven treatments, including a negative control, were tested per chemical substance. The exposure was done in 200 mL polyethylene vessels. A total of 10 animals aged 7 days were exposed per treatment in replicates of 5 animals per test vessel. Test concentrations were 0.1,1,10,100,1000 and 10000 µg/L for cadmium and 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 µg/L for fenitrothion. Animals were fed daily at a dose of 1 granulate per individual. The test solutions were changed daily.

The test duration was seven days. Mortality was checked daily and dead animals were removed immediately.

2.4 Behaviour assays

The behavioural assays were carried out with sodium hypochlorite, tributyltin, formaldehyde, mercury and lindane. The test concentrations were chosen according to Amorim et al., (2017b) in order to allow for an interspecific comparison analysis with zebrafish data (Table 1). The assays were done in circular arenas suited for video recording, filled with 100 mL of either control or toxicant solution. The control and the five toxicants were tested simultaneously. The sodium hypochlorite and formaldehyde solutions were added last due to their volatile nature.

The animals were transferred to the arenas (one per arena) and allowed a non-recording acclimatization period of 1 hour. The recording started from this point onward and lasted for 30 minutes, in a total assay period of one and half hours. Organisms were previously selected and removed from their culture aquarium into another recipient to ease the process of transferring them to the exposure/recording arenas.

2.4.1 Preparation of test-solutions

Stock solutions for TBT, mercury, and lindane were initially prepared by dissolving the chemicals in deionized water for 2 hours, in a magnetic stirrer (VWR®-VMS-A S40). These stock solutions were of a higher concentration than the exposure concentration, necessitating a dilution before usage.

Table 1 – Concentrations tested of tributyltin, mercury, lindane, sodium hypochlorite and formaldehyde according to Amorim et al., (2017).

Toxicant	Concentration
Tributyltin	0.243 µg/L
Mercury	4.5 µg/L
Lindane	10 µg/L
Sodium hypochlorite	0.5 mg/L

Formaldehyde	3.69 mg/L

2.5 Video-tracking settings and recording

The recording area was previously established, consisting of 4 cameras installed above the arenas, alongside the illumination system. The recording area was isolated with cork plaques in order to reduce possible effects of exterior noise, light and vibration on the test organisms.

A single recording involved the 4 cameras at the same time, with each camera filming 12 arenas, in a total of 48 arenas per recording. Furthermore, each tested condition (*e.g.*, control group, 0.243 µg(TBT)/L, 4.5 µg(Hg)/L, …) was distributed randomly and evenly, being represented twice in each camera and, hence, 8x per recording. This recording procedure was replicated 6 times throughout the study.

2.6 Behaviour data analysis

The algorithm *Biotracker* (Matlab software 2017a), adapted and improved for use with the above video-tracking assay system, was used to automatically track and analyse the trajectories and several other parameters of *T. longicaudatus* behavior, such as mean velocity. To analyse the provided videos, the algorithm run through three phases, each responsible for different but equally important steps in making the video easier to analyse. This algorithm works by differential brightness between the organism of interest and the background, *i.e.*, it follows a dark object in a bright background.

The first phase, known as pre-processing, corrects various aspects of the provided video. Here the fishbowl effect is removed, diminishing the distortion observed in the extremities. Furthermore, the contrast between the object and background is increased, making it an almost black and white image.

The second phase is the trajectory detection. Here the algorithm detects the number of circular arenas, delimits them and then detects the trajectories created by the moving organisms. This tracking is done by the varying intensity of colour frame by frame. This approach requires stable zoom and position while recording and correct lighting to ensure the best possible contrast between the background and the organism.

The last phase is the post-processing. Here, the detected trajectories are analysed, making it possible to obtain the values associated with them. A variety of data can be obtained such as: trajectory, travelled distance, position in the arena and mean velocity. This step essentially transforms visual information into analysable data, removing many subjective evaluations. The obtained data is exported as Excel files.

Additionally, two video files are also available: one created by the first step for checking the correct detection of the arenas and a verification video. The latter is extremely important since it shows the trajectories detected by *Biotracker*, thus, allowing for the validation of the algorithm analysis.

2.7 Statistical analysis

Initially, a *Cluster Analysis*, using the artificial neuronal network algorithm, *Kohonen*, was performed to describe the main behavioural patterns recorded. This analysis groups the data by their similarity, meaning that variables in the same group have similar data. As such, the provided data endpoints were: Y coordinate, the position in the Y axis of the organism; X coordinate, the position of the organism in the X axis; mean velocity (mm/s), mean angular velocity (degrees/s); degree formed by movement vectors, instantaneous velocity (mm/s); square root of the standard deviations of X and Y (dispersion measure); mean meander (degrees/mm) and instantaneous acceleration (mm/s^2). One-way Analysis of Variance with the Tukey HSD was used to investigate differences among behavioural types.

A Correspondence Analysis (CA) was subsequently carried out to investigate possible behaviour differences among test toxicants. Data entered in the Correspondence Analysis were the behavioural types defined by the Cluster Analysis based on an artificial neural network algorithm against the test toxicants. Homogeneous groups among toxicants were investigated using the Chi-square test with the Bonferroni Correction. All statistical analyses were performed using *Statistica* version 13.4.0.14.

3 Results

3.1 Rearing and ecotoxicological assays

Investigation about adequate conditions for maintenance and preliminary ecotoxicological testing with *T. longicaudatus* started with a serious of pilot assays to select suitable temperature, photoperiod, test media, substrate and feeding for further testing. Dried cysts were hatched in aquaria with fine sand (0.1-0.3 mm) and the different types of water tested. In these trials, high mortality (~90%) was observed within less than a week when the animals were maintained in deionized water and high-mineralized bottled water, despite food was provided. Utilizing low-mineralized bottled water, the animals were capable of surviving much longer than 1 week, allowing for maturation that previously was impossible. When dechlorinated tap water was used no differences in survival (>80%) and development were observed, compared to the low-mineralized water. Due to this and the easiness to obtaining dechlorinated tap water at a more effective cost, this medium was used for all following rearing trials. This usage allowed the animals to complete their 2 to 3-month life cycle, starting with the cysts hatching. In these pilot trials a temperature of 26ºC ± 1ºC and a photoperiod of 16h light to 8h dark were found suitable for survival and growth.

Of the feeds tested (live algae, freeze-dried *Spirulina* sp., a granulate mixture of 3 algae named GMA_3, fish granulate and yeast) different diets were found to produced improved growth according to the species life cycle. From preliminary experiments freeze-dried *Spirulina* sp. and GMA_3 dissolved in dechlorinated water were found to improve survival, development and growth of hatched *nauplii* and juveniles. Table 2 presents length measurements obtained after one week of feeding of *T. longicaudatus* juveniles with either dried *Spirulina* sp. or GMA_3 (n=9 per diet). On average, the *Spirulina* sp. fed animals had a length of 8.1±1.74mm while the granulate fed animals had an average length of 10.9±1.50mm. The increased and more consistent size is helpful as a bigger body increase the effectiveness of the tracking algorithm, so the granulate was chosen as preferred feed. The organisms fed with the GMA_3 diet also presented a better coloration, when compared to the other diets. Nevertheless, when organisms reach adulthood, they are mostly omnivorous and require a new base diet. Therefore, a non-ground fish granulate was incorporated into their feeding habits.

Table 2 Length (in mm) of *T. longicaudatus* at the end of one week feeding with either freeze-dried *Spirulina* sp. of a granulate mixture of 3 algae (GMA_3) (N=18).

***Spirulina* sp.**	**GMA_3**
8.4	14.2
10.1	10.0
6.8	9.3
9.0	10.5
7.0	11.6
6.6	9.8
5.4	11.7
10.1	11.0
9.9	9.6

These conditions were further tested in the ecotoxicological assays with cadmium (six test concentrations ranging from 0.1 to 10000 µg/L in orders of magnitude) and fenitrothion (seven concentrations ranging from 0.0001 to 100 µg/L in orders of magnitude). A wide range of concentrations was used to obtain some information on the sensitivity of the species while and the test conditions selected. The exposures were carried out at 26.0 ± 1.0 °C and a light cycle of 16h:8h (light:dark). The test solutions were prepared by dilution of the respective stocks in dechlorinated water. The test vessels contained 200 mL of each test solutions or only dechlorinated water for the controls. A solvent control with DMSO was also prepared for the fenitrothion assay. Animals were fed daily at a dose of 1 granulate of GMA_3 per individual. The test solutions were changed daily. For cadmium mortality reached 100% in the two highest test concentrations (1000 and 10000 µg/L) and 90% at 10 and 100 µg/L. No mortality was observed in the two lowest concentrations tested. Mortality in the control condition was 20% at the end of the seven days of exposure. The data indicates the 7-day LC_{50} should be between 1 and 10 µg/L. For fenitrothion, 100% mortality was observed at the three highest concentrations tested (100, 10 and 1µg/L) and 90% in the two following concentrations. Mortality in the three lowest concentrations ranged from 30% to 60%.

Based on the above results an overall protocol was finally established for each rearing stage (Annex 1) and subsequently adopted for the behavioural experiments. Briefly, *nauplii* were hatched in plastic containers containing fine grain sand and dechlorinated water, under constant conditions of temperature (26.0 ± 1.0 °C) and photoperiod (16h:8h light:dark). After hatching, *nauplii* were fed, twice a day, with GMA_3 previously grounded and dissolved in dechlorinated water (1:1, m/v). Each container was provided with 20 mL of this solution. Only half the water volume was renewed, daily, until day 7. From day 8 to day 15 juveniles were fed, twice a day, with fish granulate (Vitality and Color Granulat from Tropical), with one granulate (~1mg) per individual added to each container. Complete water renewal started to be performed daily. After day 15, organisms were transferred to full sized aquariums with sand, air enrichment and dechlorinated water. Medium renewal continued to be performed daily and feeding (*ad libitum*) was done two times a day.

3.2 Locomotory behaviour

Primarily, a preliminary behaviour assay was designed since this was the first work using *Triops longicaudatus* as a model organism for behavioural studies. Thus, to better understand how to manipulate *T*. *longicaudatus* individuals and the limitations and issues that could arise from the recording settings, two small test recordings were performed. First, smaller arenas (a set of 5 cm in diameter each) imbued in an acrylic plaque (Figure 1 left) the second recording, larger isolated arenas (8 cm in diameter each) were used (Figure 1 right) with two-week old individuals. The easier manipulation of the organisms, less time necessary until the assay is possible and lower volumes of test solutions necessary make utilizing 1-week old organisms preferential. The final behaviour data was then recorded with seven-day old juveniles.

Figure 1 Types of arenas employed in the preliminary behavioral tests: a block of 12 arenas of 5 cm in diameter each (left) and the isolated arenas of 8 cm in diameter each (right).

In each arena, 100 mL of dechlorinated water was added and immediately contaminated with either TBT, mercury or lindane. On the other hand, 100 mL of sodium hypochlorite and formaldehyde solutions were added directly into the arenas from a previously prepared solution. These two solutions were added last due to their volatile nature. After the non-recording acclimatization period of one hour the recordings were started.

After processing the videos with the *Biotracker* algorithm the data obtained was analysed for reduction of variables. A first cluster analysis based on a dissimilarity distance (1-Pearson *r*) was thus carried out to identify variables showing highly similar patterns of response and thus redundant in the subsequent analysis. The eight variables recorded initially were found to be arranged in five clusters (Figure 2).

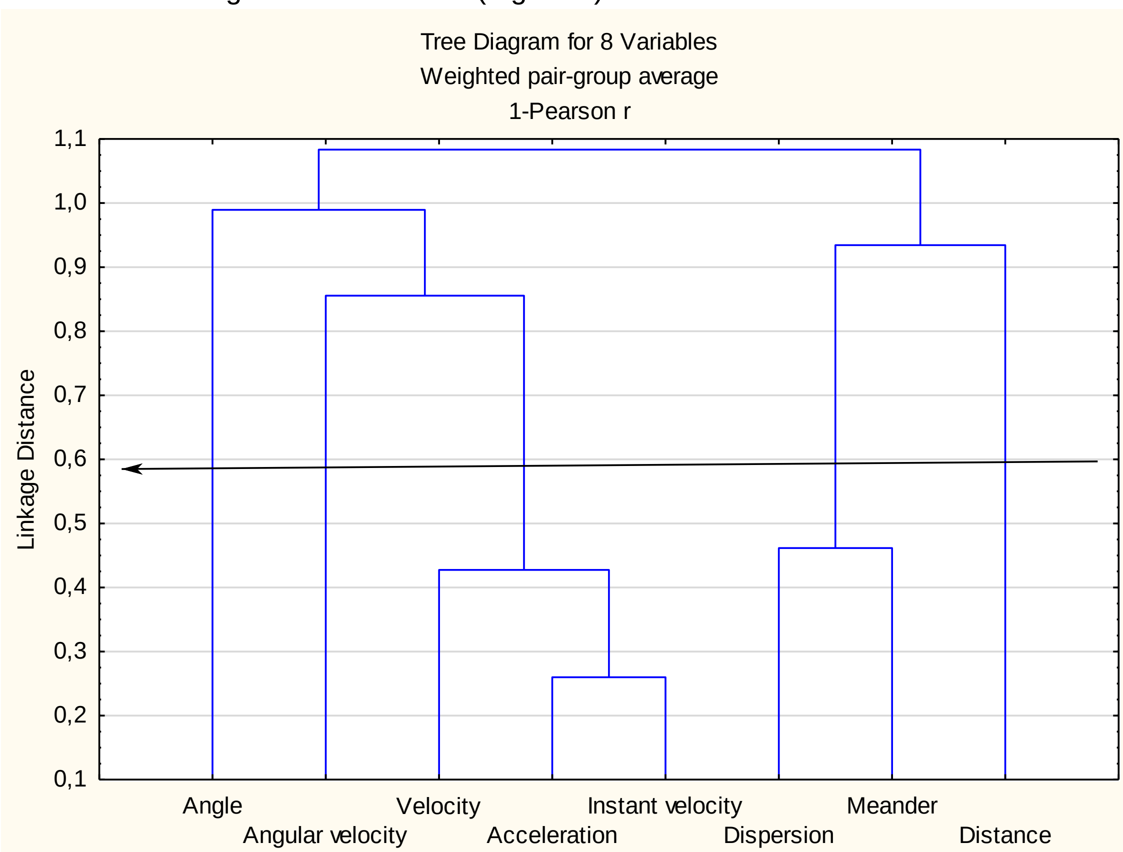

Figure 2 Results of the cluster analysis performed to reduce the behavioural variables measured. Angle - angle formed by movement vectors (degrees), Angular velocity – mean angular velocity (degrees/s); Velocity – mean velocity (mm/s); Instant velocity – linear instant velocity (mm/s); Acceleration – Instant acceleration (mm/s^2); Dispersion – spatial dispersion; Meander - mean meander (degrees/mm); Distance – distance to the centre of the arena (mm).

A big number of variables can be cumbersome to work with and some may even show high similarity in pattern, presenting matching information. The cluster analysis classifies a group of behaviour variables, based on the characteristics of movement, disregarding any information about experimental conditions. In the graph of Figure 2 it is possible to observed, for example, that velocity and acceleration are closely related, meaning that the information gained from each of them is similar. If one of these is discarded, the loss of information would be reduced, allowing for easier analysis going forward. To decide on the variables to maintain or discard a one-way ANOVA was then carried out for each of the eight variables, using the treatments as factor, to investigate which of them had higher contribution to discriminate among them. Based on these results, the variable from each cluster showing the highest *F* statistics (Table 3) was retained for subsequent analysis.

Table 3 Results of the one-way ANOVAs carried out to select informative variables to retain for behaviour analysis. The selection was based on the F statistics. Variables retained are indicated in bold. Angle - angle formed by movement vectors (degrees), Angular velocity – mean angular velocity (degrees/s); Velocity – mean velocity (mm/s); Instant velocity – linear instant velocity (mm/s); Acceleration – Instant acceleration (mm/s2); Dispersion – spatial dispersion; Meander - mean meander (degrees/mm); Distance – distance to the center of the arena (mm).

Cluster	Dependent Variable	*F*
a	Angle	**3**
b	Angular velocity	**1061**
c	Velocity	**2672**
c	Instant velocity	1252
c	Acceleration	645
d	Meander	**4864**
d	Dispersion	1307
e	Distance	**37**

The five variables selected were then employed to perform a cluster analysis based on an artificial neural network algorithm to identify the main behavioural types exhibited by the tadpole shrimp. The cluster analysis defined six main types of behaviours indicated as A to F in Table 4. These represented a gradient of variation where the five variables measured have different contributions to each behaviour type as indicated in Table 5. For instance, animals exhibiting behaviour type A are mostly characterized by swimming in the periphery of the arena as opposed to animals exhibiting behavioural type B, in which velocity (faster swimmers) and the angle (small) are the most prominent characteristics, or animals with behavioural type F characterized especially by swimming fast in the periphery of the arena (Tables 4 and 5).

Table 4 Mean values of each variable in each behaviour type identified through the cluster analysis based on the artificial neural network algorithm. The different shadings represent the groups identified by the cluster analysis. Angle in degrees, angular velocity in degrees/s, velocity in mm/s, meander in degrees/mm and distance in mm

	Behaviour type					
	A	B	C	D	E	F
Distance	335^{f}	161^{c}	154^{a}	158^{b}	322^{e}	319^{d}
Velocity	0.8^{a}	1.1^{b}	1.1^{b}	$1.1^{c,d}$	1.1^{d}	$1.1^{b,c}$
Angle	-0.6^{c}	0.9^{d}	-120^{a}	123^{f}	114^{e}	-116^{b}
Angular velocity	9.7^{a}	9.8^{b}	11.1^{e}	11.2^{f}	10.9^{d}	10.8^{c}
Meander	22.8^{e}	13.7^{a}	$15.0^{b,c}$	14.7^{b}	$15.6^{c,d}$	15.7^{d}

Table 5 Contribution (weight) of each variable for the different behaviours identified, as indicated by the results of the cluster analysis based on the artificial neural network algorithm. The different shadings represent the groups identified by the cluster analysis. Angle in degrees, angular velocity in degrees/s, velocity in mm/s, meander in degrees/mm and distance in mm.

	Behaviour type					
Variable	A	B	C	D	E	F
Distance	0.62	0.16	0.12	0.10	0.16	0.62
Velocity	0.11	0.84	0.21	0.11	0.06	0.66
Angle	0.06	0.80	0.22	0.18	0.07	0.29
Angular velocity	0.09	0.23	0.22	0.51	0.06	0.29
Meander	0.09	0.18	0.63	0.51	0.06	0.31

The Correspondence Analysis, carried out with the behaviour types identified and the test conditions, produced three significant dimensions explaining 99% of the data dispersion (Figure 3). The first two dimensions clearly discriminated animals exposed to mercury, sodium hypochlorite and the controls from each other and from the remaining toxicants (Figure 3 top). The second and third dimensions show a clear distension between the control and TBT conditions Animals exposed to mercury tended to exhibit behaviour type B (fast swimmers with a small angle), animals exposed to sodium hypochlorite tended to exhibit behaviour type A(swimming in the periphery of the arena) and control animals were fast swimmers preferring the periphery of the arena (behaviour types C trough F). Table 6 indicates the proportion of time spent on each behaviour type for each test condition. The homogeneous subsets indicated by the Chi-square test are indicated in the table. The first and the third dimensions extracted discriminated the control, TBT and sodium hypochlorite from each other and from the remaining treatments. Animals exposed to TBT tended to display a mixture of behaviours (F, Figure 3 bottom left and Table 6) suggestive of discoordination or disruptive control. The second and the third dimensions extracted opposed mercury, TBT and the control (Figure 3 bottom right and Table 6). In contrast, lindane and formaldehyde elicited similar proportions of time spent in all behaviour types although it is possible to differentiate from the remain treatments.

Table 6 Each cell represents the proportion of time (in percentage) animals exposed to a certain condition exhibit a specific behaviour. The homogenous groups are indicated by small letters; brown indicates positive changes relative to the average and blue represent negative changes.

Categories	Control	Mercury	Sodium Hypochlorite	TBT	Formaldehyde	Lindane
A	12^{a}	14^{b}	30^{e}	$15^{c,e}$	17^{d}	$16^{c,d}$
B	14^{a}	22^{d}	16^{b}	19^{c}	18^{c}	18^{c}
C	15^{c}	16^{c}	11^{a}	13^{b}	15^{c}	14^{c}
D	18^{d}	16^{c}	12^{a}	14^{b}	$15^{b,c}$	$15^{b,c}$
E	20^{d}	$16^{a,b}$	16^{a}	20^{d}	$17^{b,c}$	18^{b}
F	20^{c}	16^{a}	15^{a}	$20^{b,c}$	18^{b}	$19^{b,c}$

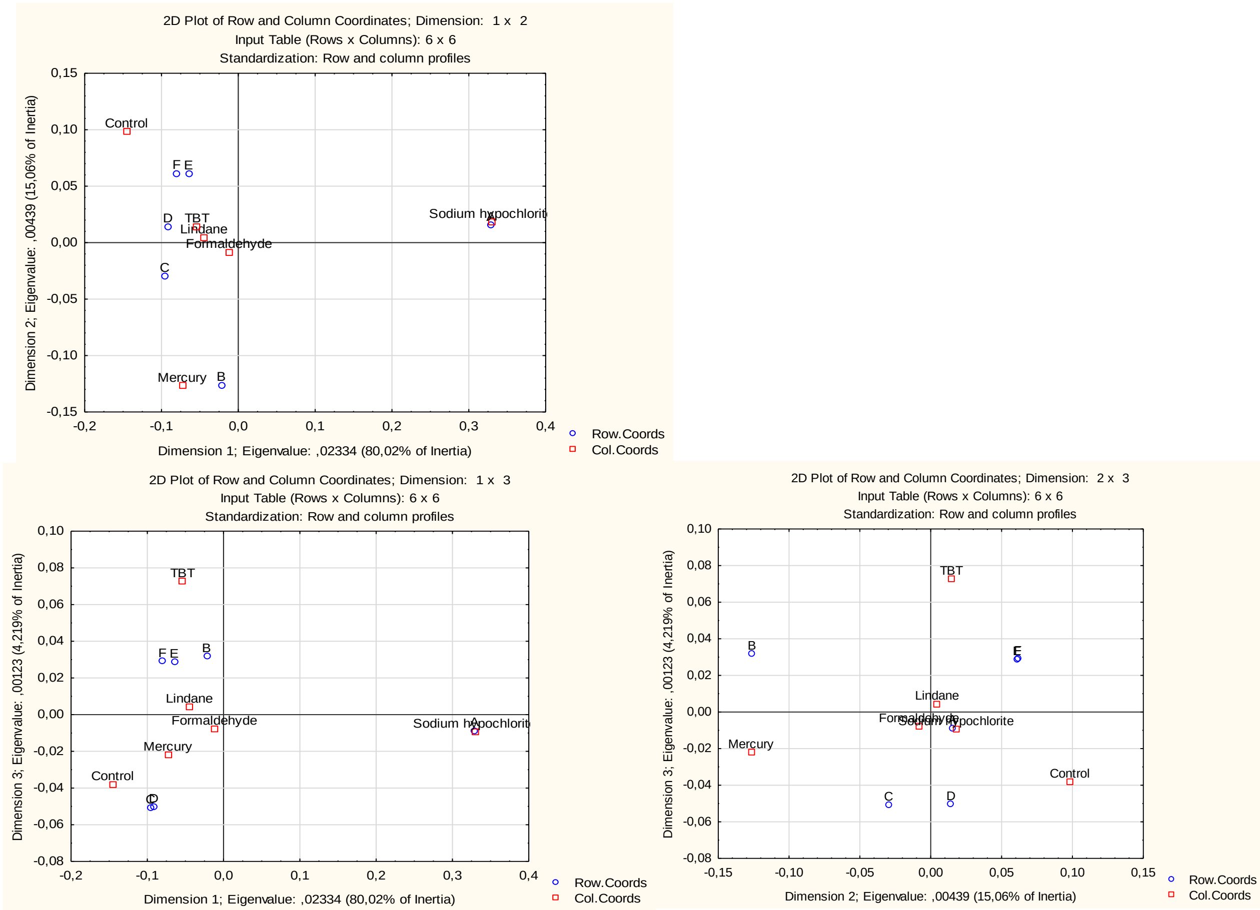

Figure 3 Correspondence analysis carried out with the behaviour types identified for the tadpole shrimp and the six test conditions. Three significant dimensions were extracted. The plots illustrate the separation of the tested conditions and their associated behaviour by the first and second dimensions (top), the first and third dimensions (middle) and the second and third dimensions (bottom)

4. Discussion

The assays related to the rearing of TPS allowed to determine adequate reproducible conditions for laboratory culture, resulting in organisms capable of surviving up to 3 months (their full life cycle). The 3 algae granulate allows an increased and more consist growth, an advantage in the performed assays, as the variability of effects may be influenced by the use of smaller or lighter individuals. The increased size is advantageous in the video analysis as smaller and less coloured animals pose challenges to automated behaviour tracking routines, usually requiring increased amount of time in the verification of the tracking data obtained to avoid confounding or bias due to tracking failure. Increased growth related to an algae-based feed has been previously observed in other species (Mustafa et al., 1995; Shahabuddin et al., 2017). This allowed the creation of a protocol (Annex 1), which was followed during the remaining of the work.

Mortality due to cadmium was observed at the concentration of 100 µg/L, a value close to the LC_{50} at 48h reported for *Daphnia magna* (Mount and Norberg, 1984) and the LC_{50} at 96h reported for *Heterocypris bosniaca* (Aguilar-Alberola and Mesquita-Joanes, 2012), a crustacean native to temporary rock pools. The estimated value for the 7-day LC_{50}, between 1 and 10 µg/L, is within the range of the 10-day LC_{50} of *Tanytarsus dissimilis*, 3.8 µg/L (Anderson et al., 1980). For fenitrothion had mortality values were of 60% at 0.0001 µg/L and 40 % at 0.001 µg/L. These values, along with the estimated 7-day LC_{50} for cadmium (between 1 and 10 µg/L), are lower than the values found in the literature. The lowest value that could be found in the available scientific literature was the LC_{50} at 3h, 0.01 µg/L, for *Anopheles arabiensis* (Alemayehu et al., 2017), a mosquito. This suggests the TPS may be very sensitive to organophosphorous pesticides. Future work to elucidate on this should focus on carrying out ecotoxicological assays with other pesticides belonging to this chemical group. The assays can be complemented with the characterisation of cholinesterases (Rodrigues et al., 2013) present in TPS and the determination of the acetylcholinesterase activity in exposed animals, given that irreversible inhibition of acetylcholinesterase activity is considered the primary mode of action of these compounds, including fenitrothion (Forsyth et al., 1993; Rodrigues et al., 2013).

The behaviour analysis performed in this work showed that this species is sensitive to exposures at low concentrations of TBT, lindane, sodium hypochlorite, formaldehyde and mercury chloride. In fact, behavioural alterations relative to the control were found in animals exposed to low concentrations (within the range found in environmental samples) TBT, sodium hypochlorite, mercury lindane and formaldehyde. Moreover, the multivariate statistical techniques employed allowed to identified six types of behaviours exhibited by TPS. Of the six groups defined, two were associated to substances evaluated, A to sodium hypochlorite and B to mercury. These had the lowest mean angular velocities; A had the highest distance from centre and mean meander and the lowest mean and angular velocity. B had the 3rd lowest mean velocity and distance from centre, the lowest mean meander and largest angle. These results suggest that animals with a typical A behaviour may have a straighter slower movement and those of B type exhibit a faster movement with and a smooth trajectory. Lower meander associated with mercury has been previously reported (Amorim et al., 2017b). This study utilized the same contaminants, lindane, TBT, formaldehyde, sodium hypochlorite and mercury and the same exposure concentrations utilized herein. It differed, however, in the studied species, zebrafish Danio rerio. The system employing the zebrafish was capable of detecting toxicant exposure, as in this study. Furthermore, it allowed discriminating the five exposure contaminants through the analyzed data, mostly correctly (Amorim et al., 2017b). Overall, two main behaviours were identified for zebrafish. The first one reflected and increase in the swimming activity, compared to controls, suggesting a typical escaping behaviour (Amorim et al., 2017b), which was associated to exposure to lindane, mercury, TBT and sodium hypochloride. The second one reflected a lower swimming activity, denoting typically lethargic animals compared to controls, and recognised by the authors as a possible avoidance behaviour to minimise the absorption of the contaminant into the body, which was associated to formaldehyde exposure.

In contrast to findings reported for the teleost model (Amorim et al., 2017b), in the present work, the types of behaviours associated to the control group (C, D, E, F) seem to indicate these animals have higher swimming activities when compared to the remaining types of behaviour identified (A, B) exhibited by animals exposed to test substances. The velocity reduction observed when animals were exposed to sodium hypochlorite may be caused by a reduction in ATP (Hidalgo et al., 2002), related with storing and expending energy (Erecińska and Silver, 1989). This reduction of available energy may cause a reduction of behavioural parameters directly or through disruptions in the central nervous system (Erecińska and Silver, 1989). On the other hand, mercury is a known neurotoxicant, affecting the brain but also possibly the central and peripheral nervous system (Albrecht and Matyja, 1996). This could be causing the also decreased velocities in the behaviours found associated to this toxicant, with the exposure reducing the swimming capabilities of the organism. TBT has been associated with immobilization, explaining the decreased swimming activity observed (Wong, 1991).

Lindane exposure can lead to neurotoxic effects in organisms, possibly leading to decreased swimming (Islam and Lynch, 2012; Nolan et al., 2012), and decreased swimming activity related to formaldehyde exposure has been reported previously in the zebrafish (Amorim et al., 2017b; Mohammed et al., 2012). Such reductions may be associated with increased metabolic costs due to damages to tissues, namely gills (Mohammed et al., 2012), that hamper the organisms' movement capabilities. Despite this, apart from some velocity reduction, it is of note that no typical swimming pattern could be assigned to TPS exposed to these substances in the present work, so that they were not discriminated from each other.

Although the previously pointed characteristics of the used contaminants may explain the observed results, the difference between the response of the zebrafish and the tadpole shrimp must be discussed. In fact, the differing reaction may be related to differences in sensitivity of these species, including differences in xenobiotic metabolism of vertebrates and invertebrates. On the one hand, it is known the relationship between the concentration of exposure and the behavioural responses may not be one of direct proportionality. It has been observed previously that lower concentrations could lead to the increase of some variables, as velocity, while higher concentrations could lead to the opposite response (Little, 1990; Little and Finger, 1990). This may also occur because higher levels of tissue damage can be caused by higher concentrations, affecting both directly and indirectly (through reallocation of energy reallocation to cope with the exposure) the overall animal performance. This means that different types of reactions can be observed after exposure, depending on the species and the exposure level, such as for example a freeze response, where the organisms try to minimize the effects by decreasing their movement and consequently, reduce their metabolism. Another response can be observed is that of avoidance, upon detecting a contamination the organisms attempt to escape from the area, to decrease their exposure and the effects from it (Halappa and David, 2009; Schreck et al., 1997). This response may lead to an increase of some variables, like velocity. Globally, the differences between the results observed here and the previously mentioned can be due to two possibilities: i) the differing reactions can be due to the disparities between the species and their detoxification mechanisms, possibly more relevant since one species is an invertebrate and the other a vertebrate; ii) the concentrations used may not be in the appropriate range to cause effects similar to those that were observed previously in a so short exposure period. For instances, these concentrations could be too high for this species. If the effects of the exposure are too strong, the organisms may be incapable of attempting to escape or try to reduce their metabolism to minimize the consequences of the exposure. The results of the fenitrothion assay suggest this may be the case for some contaminants, as the tested concentrations that were close to multiple LC_{50} values for a variety of species and exposure periods, were too high for the tadpole shrimp, and the 7-day LC_{50} obtained herein is below those that were found in the literature. Future work to investigate this hypothesis focus on performing new behaviour assays whith lower test concentrations of the same toxicants and investigate the tadpole shrimp response to such contamination, be it freeze or avoidance. Based on teh results already obtained for the discrimination of three of the text toxicants employed herein, this will further help refining this video-tracking based system and the implementation of the BEWS to monitoring the status of temporary pools. Furthermore, utilizing a species that is more sensitive than the more commonly used alternatives can help provide more knowledge of what some contaminations may cause in other sensitive species that are not regularly studied due to various motives. Alternatively, the concentrations could be too low. Perhaps the concentrations used do not cause enough damage in the organisms, and thus no clear escaping is not necessary. These organisms may sense the contamination and try to reduce their metabolic rate to minimize the effects, and thus the observed decrease of the variables as opposed to the increased already reported for the zebrafish. If this is the case, the monitoring of the endangered temporary ponds may still be possible although only higher levels of contamination may be detected. However, the reduced solving capabilities due to the inferior water volume present in these pools may make such contamination levels not very uncommon. Important to mention is the possibility that independently of the hypothesis that may be correct, it may not apply to all the contaminants studied. For example, the response to formaldehyde was similar in both *T. longicautaus* and *D. rerio*, in the sense that although not exhibiting a typical behavioural pattern in the video-tracking analysis some decrease in velocity was also apparent in the TPS, similar to results obtained for lindane. In anycase, future work including the histological analysis of exposed animals will bring further highlight on these aspects.

5. Conclusion

The main objectives proposed were successfully accomplished. Preliminary assays allowed the elaboration of a rearing protocol, used multiple times during this work. The preliminary ecotoxicological assays and the behaviour assays showed that this species can be used in different types of ecotoxicological evaluations and produce results, particularly in the behaviour assay. This shows that *T. longicaudatus* could be used in future assays, opening new possibilities in a laboratory setting and providing information about a habitat that can have long reaching consequences.

Future work should focus on exploring possible age differences in the behaviour patterns exhibited by the species, investigate the sensitivity of this model and behavioral tool to other environmental contaminants and. Different concentrations of the ones already tested, and validate this potential biological early-warning system. Evaluation of molecular, biochemical and histological biomarkers in this species under control and exposure conditions will also bring complementary useful information to gain insight on the modes of action of the contaminants tested on *T. longicaudatus*.

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7. Annexes

Annex 1 – *Triops longicaudatus* rearing protocol

Day 0 – hatching phase

To generate test organisms, 12.5 mL of sand containing cysts are placed into an 8 (Ø) cm plastic vessel containing 200mL of dechlorinated water.

The vessels are maintained at 26.0 (±1.0) °C with a photoperiod of 14h:10h (light:dark), for 48h.

Day 2 – 7

From day 2 until day 7, a specific procedure for feeding and medium renewal start to occur.

The diet consists of 60 mg of grounded granulate mixture of three algae (3- Algae Granulat from Tropical) dissolved in 60 mL of dechlorinated water. Then, each vessel receives 20 mL of this solution, twice a day.

Moreover, approximately 50% of the water volume is carefully removed to avoid damaging the organisms and new dechlorinated water is added, daily.

The vessels are constantly maintained at 26.0 (±1.0) °C with a photoperiod of 14h:10h (light:dark).

Day 8 – 15

From day 8 until day 15, a new procedure for feeding and medium renewal is implemented.

The diet consists of one non-grounded piece of fish granulate (Vitality and Color Granulat from Tropical) per individual, in each vessel.

Additionally, approximately 100% of the water medium is carefully removed to avoid damaging the organisms and new dechlorinated water is added, daily.

The vessels are constantly maintained at 26.0 (±1.0) °C with a photoperiod of 14h:10h (light:dark).

Day 15 – adulthood

From day 15, organisms are moved to full sized aquariums with air enrichment. This allows for an increase in growth, as long as the population density is not very high.

A full water renewal is performed daily.

The diet remains equal although now concentrations are *ad libitum*.