

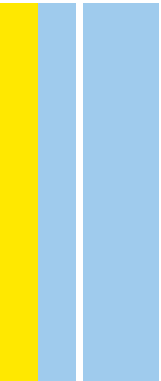
Mestrado

Toxicologia e contaminação ambientais

Effects of TBT on gene expression,
locomotory behaviour and protein
affinity prediction of *Triops
longicaudatus*

Adriano Romário Correia Chessa

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2021



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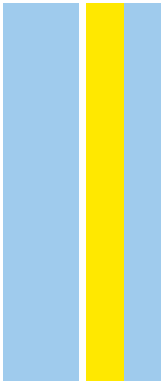
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M.ICBAS 2021

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Abstract

One of the biggest challenges faced by humankind is finding the balance between the usage of profit-enhancing chemicals (e.g. biocides, pesticides, fertilizers) and environmental health. These chemicals are often utilised to remove unwanted or harmful organisms, but unfortunately, very often, non-target species are also critically affected. It becomes crucial to assess sub-lethal effects as any given contaminant may only manifest severe toxicity after chronic exposures.

Freshwater crustaceans such as *Triops longicaudatus* are present in ephemeral ponds across the world and possess high ecotoxicological relevance, as they are located in intermediate positions in the food chain while also feeding on disease-spreading organisms. Additionally, this species contains various properties that classify it as a good model for ecotoxicological experiments.

This work assessed the effects of environmentally relevant concentrations (0; 0.075; 0.25; 1.54µg/L) of the biocide Tributyltin (TBT) on the genetic expression and behavioural pattern of juvenile *T. longicaudatus* acutely exposed. Regarding the impact at a molecular level, key genes in pathways associated to neurotoxicity, cytotoxicity, endocrine disruption and genotoxicity were impacted in different intensities throughout the treatments. The behavioural analysis revealed changes to the natural behavioural pattern and swimming activity even at the lowest concentration of 0.075µg/L, since all treatments had a different behavioural type than the one attributed to the control group. The results from the behavioural and gene expression analyses could be linked between each other and associated, for example, to impairments at a neurotransmission level or even to energy impairment. Finally, one of the main targets of TBT - the Retinoid X receptor (RXR) protein – was the focus of bioinformatics analyses. Here the aim was to determine the evolutionary conservation and theoretical sensitivity of this protein towards TBT molecules among different invertebrate species, mainly crustaceans. The study revealed a great level of conservation for the RXR protein and, more specifically, the aminoacids that interact with TBT, even in species evolutionarily distant.

This dissertation provided novel information on the toxicity of TBT, a very persistent contaminant, while also highlighting the potential of *T. longicaudatus*, as a model organism in ecotoxicology. This dissertation is also the first step for the development of future work that can better understand the impact of endocrine disruptors, such as TBT.

Resumo

Um dos maiores desafios enfrentados pela humanidade é encontrar o ponto de equilíbrio entre o uso de produtos químicos rentáveis (e.g. biocidas, pesticidas, fertilizantes) e a saúde ambiental. Estes produtos químicos são frequentemente utilizados para remover organismos indesejados ou nocivos, mas infelizmente muitas vezes espécies não-alvo também são criticamente afetadas. Torna-se então crucial avaliar os efeitos sub-letais, uma vez que contaminantes pode só manifestar toxicidade severa após exposições crônicas.

Crustáceos de água doce, como *Triops longicaudatus*, estão presentes em lagoas temporárias em todo o mundo e possuem uma elevada relevância ecotoxicológica, uma vez que estão localizados em posições intermédias na cadeia alimentar, e alimentam-se de organismos vetores de doenças. Além disso, esta espécie contém várias propriedades que a classificam como um bom modelo de estudo para experiências ecotoxicológicas.

Este trabalho avaliou os efeitos de concentrações ambientalmente relevantes (0; 0,075; 0,25; 1,54 µg/L) do biocida Tributil estanho (TBT) na expressão genética e no padrão comportamental de *T. longicaudatus* juvenis após uma exposição aguda. No que diz respeito ao impacto a nível molecular, genes-chave de vias associadas à neurotoxicidade, citotoxicidade, disrupção endócrina e genotoxicidade foram impactados em diferentes intensidades ao longo dos tratamentos. A análise comportamental revelou alterações ao padrão comportamental natural e à atividade natatória mesmo na menor concentração de 0,075 µg/L, uma vez que todos os tratamentos tinham um tipo comportamental diferente do atribuído ao grupo de controlo. Os resultados das análises comportamentais e de expressão genética podem estar ligados entre si quando associados, por exemplo, a disrupções a nível da neurotransmissão ou mesmo a uma disrupção energética. Por último, um dos principais alvos do TBT - recetores de ácidos retinóides X (RXR) – foi o foco de análises bioinformáticas. O objetivo foi determinar a conservação evolutiva e a sensibilidade desta proteína a moléculas de TBT entre diferentes espécies de invertebrados, principalmente crustáceos. O estudo revelou um grande nível de conservação para a proteína RXR e, mais especificamente, os aminoácidos que interagem com TBT, mesmo em espécies evolutivamente distantes. Esta dissertação forneceu novas informações sobre a toxicidade do TBT, um contaminante de persistência muito elevada, destacando também o potencial de *T. longicaudatus* como um organismo modelo em ecotoxicologia.

Esta dissertação é o primeiro passo para o desenvolvimento de trabalhos futuros que possam compreender melhor o impacto dos disruptores endócrinos, como o TBT.

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Index

Acknowledgments.....	i
Abstract.....	ii
Resumo	iii
Funding Acknowledgments.....	iv
Abbreviation list	vii
Gene abbreviation list.....	ix
Figure index.....	x
Table Index.....	xii
1. Chapter I: Introduction, dissertation structure and objectives.....	1
1.1. Introduction	1
Biocides	1
Tributyltin (TBT)	2
TBT's Adverse Effects in organisms	5
Molecular impact of TBT-based compounds.....	6
Ecotoxicological bioassays	8
Test-organism: <i>Triops longicaudatus</i>	8
Ethical legislation and use	12
1.2. Objectives	13
1.3. Dissertation structure	13
1.4. References.....	16
2. Chapter II: Transcriptomic profile of a living fossil (<i>Triops longicaudatus</i>) exposed to Tributyltin	21
2.1. Introduction	22
2.2. Materials and methods	24
Organisms	24
Bioassay to assess LC ₅₀	24
Toxicological assay.....	24
Sample Collection and RNA Isolation	25
Transcriptome assembly.....	25
Differential gene expression	26
2.3. Results	26
Transcriptome sequencing output and assembly.....	26
Homology search and functional annotation	26
RNA-seq analysis for the 0.075µg/L exposure	28
RNA-seq analysis for the 0.25µg/L exposure	29
RNA-seq analysis for the 1.54µg/L exposure	32
RNA-seq comparison of different exposures	32
2.4. Discussion.....	37
Genetic impact.....	38
2.5. Conclusion	41

2.6. References.....	42
3. Chapter III: Does it move like Jagger: Behavioural changes of <i>Triops longicaudatus</i> exposed to Tributyltin.....	45
3.1. Introduction	46
3.2. Methodology.....	47
Organisms	47
Ecotoxicological assay and Video recording setup	47
Behaviour data analysis.....	49
Statistical analysis	50
3.3. Results	51
3.4. Discussion.....	58
Behaviour characterisation	58
TBT in other species.....	59
Gene expression.....	60
3.5. Conclusion	61
3.6. References.....	62
4. Chapter IV: Toxic relationships: Bioinformatic prediction of TBTs affinity to the RXR-α of <i>Triops longicaudatus</i>.	65
4.1. Introduction	66
4.2. Materials and Methods.....	67
Toxicity data.....	67
Protein sequence extraction	68
Sequence alignment and Phylogenetic analysis.....	69
Sequence treatment and Protein Modelling	70
4.3. Results	71
Species Sensitivity Distribution curve (SSD).....	71
Sequence alignments	73
Phylogenetic analysis	76
Protein Modelling	78
Protein structure comparison	80
4.4. Discussion.....	81
Evolutionary relationship.....	82
Protein superposition	82
4.5. Conclusion.....	83
4.6. References.....	85
5. Chapter V: Discussion and conclusion	88
5.1. References.....	92
6. Supplementary data (SD)	93

Abbreviation list

3Rs	The principles of the 3Rs (Reduce, Refine, Replace)
AA	Aminoacid
ANN	Artificial Neural Networks
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BCF	Bioconcentration factor
BUSCO	Benchmarking Universal Single-Copy Orthologs
Ca ²⁺	Calcium ions
DEHP	Bis(2-ethylhexyl)phthalate
DINCH	1,2-Cyclohexane dicarboxylic acid diisononyl ester
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
EU	European Union
GMQE	Global Model Quality Estimate
GO	Gene Ontology
HC ₀₅	Hazardous Concentration to 5% of the species
IDDT	all-atom local distance difference test
IMO	International Maritime Organization
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC ₅₀	Mean lethal concentration 50%
Log2FC	Logarithm base 2 Fold Change
MUSCLE	MUltiple Sequence Comparison by Log- Expectation
NCBI	National Center for Biotechnology Information
ORF	Open reading frame
PVC	Polyvinyl chloride
QMEAN	Qualitative Model Energy ANalysis
QSQE	Quaternary Structure Quality Estimate
Refseq_rna	Reference RNA sequences
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
SOFM	Self-Organizing Feature Maps
SRA	Sequence read archive

SD	Supplementary data
SSD	Species sensitivity distribution curve
TBT	Tributyltin/ Tributyl estanho
TBTO	Tributyltin Oxide
TSA	Transcriptome Shotgun Assemblies

Gene abbreviation list

ABCC5	Multidrug resistance-associated protein 5
ARHGEF7	Rho guanine nucleotide exchange factor 7
ASCC2	Activating signal cointegrator 1 complex subunit 2 homolog
CELA2A	Putative Chymotrypsin elastase family member 2A
CP7	Cuticle protein 7
cprE	Cysteine proteinase 5 precursor
DHRS4	Dehydrogenase/reductase SDR family member 4-like
DMXL2	dmX-like protein 2
Dscam	Down syndrome cell adhesion molecule
Dscam2	Down syndrome cell adhesion molecule-like protein Dscam2
EcR	Ecdysteroid hormone receptors
EIF5A	Eukaryotic translation initiation factor 5A
ESG2	Endocuticle structural glycoprotein SgAbd-2
FXR	Farnesol X receptor
H2AC11	Putative histone H2A
HSPA1A	Heat shock protein 70
LRH-1	liver receptor homolog-1
LXR	Liver X receptors
MAG	Myelin-associated glycoprotein
Nach	Sodium channel protein Nach-like
PPAR	Peroxisome proliferator-activated receptor
RXR	Retinoid X receptor
SOD	Superoxide dismutase
SUOX	Sulfite oxidase, mitochondrial
UBA52	Ubiquitin-60S ribosomal protein L40
USP	Ultraspiracle protein
VTG1	Vitellogenin fused with superoxide dismutase

Figure index

Figure 1.1- The molecular structure of the most common TBT compounds (From Sigma-Aldrich).	2
Figure 1.2- Schematic representation of the steps leading to hydroxylation of TBTO by an oxygenase system and resulting metabolite (From Lee, 1985 – created using BioRender.com).	4
Figure 1.3- Theoretical Adverse Outcome Pathways for TBT toxicity (From Lagadic <i>et al.</i> , 2017).	6
Figure 1.4- RXR/EcR transcriptional activation in the cell nucleus. I- Binding of PPAR and RXR ligands; II- Changes in conformation; III- Activation of the complex and gene expression. (From Rigano <i>et al.</i> , 2017).	7
Figure 1.5- Diagram of the external anatomy of a female <i>Triops longicaudatus</i> . Right- ventral view. Left- dorsal view.	9
Figure 1.6- Life cycle of <i>Triops longicaudatus</i> . In the left section is represented the asexual life cycle where the population is mainly made of female or hermaphrodite individuals. To the right, the sexual reproduction cycle that usually happens under poor environmental conditions where genetic diversity provides a greater chance of survival. Cysts usually pass through a desiccation phase and hatch after rehydration.	11
Figure 2.1- Resulting distribution of taxa homologues to the <i>Triops longicaudatus</i> transcripts. A- BLAST hits for Metazoa phyla. B- Hits for Plantae, Virus, Fungi, Bacteria, Protozoa and other non-specified taxonomic groups.	27
Figure 2.2- <i>Triops longicaudatus</i> transcript distribution through the Arthropoda phylum with special attention to the Crustacea sub-phylum. Number of transcripts shown beneath each separation, final count regards the class of the species. In red square is the Order to which <i>T. longicaudatus</i> belongs. The BLAST hits were with $1e^{-5}$ e-value within the NCBI database.	28
Figure 2.3- Distribution of Gene Ontology (GO) classifications of <i>Triops longicaudatus</i> into to three general categories: Molecular function, Biological process, and Cellular components (central) and their respective subcategories (periphery).	29
Figure 2.4- Percentage of GO annotation of transcripts of <i>Triops longicaudatus</i> regarding 10-fold up and down-regulated genes into three functional categories: Biological process, Cellular component and Molecular function and their respective subcategories. A- 0.075µg/L; B- 0.25µg/L and C- 1.54µg/L.	31
Figure 2.5- Venn diagrams showing the differentially expressed transcripts both unique and common between treatments. Down-regulated (left) and upregulated (right) transcripts for <i>Triops longicaudatus</i> exposed to TBTO at 0.075; 0.25 and 1.54µg/L.	33
Figure 3.1- Circular arenas containing exposed organisms. Each area contained 12 circular arenas to which the different treatments were randomly assigned. In each area all treatments were tested in triplicate.	49
Figure 3.2- Automatic tracking of the behaviour of 12 independent <i>Triops longicaudatus</i> in the circular arenas. Each treatment is depicted at the upper left corner of the circle.	51

Figure 3.3- Behaviour variables exhibiting significant differences among TBTO treatments. Different letters identify statistically significant differences between groups as indicated by the Tukey HSD test.	52
Figure 3.4- Differences in the locomotion variables found for each behaviour type identified. Different letters identify statistically significant differences among groups as indicated by the Tukey HSD test.	55
Figure 3.5- Correspondence analysis carried out with the behaviour types identified for the <i>T. longicaudatus</i> and the four treatments tested. Three significant dimensions were extracted. The plot shows the discrimination of the treatments, and their associated behaviours, by the first and second dimensions. The blue discontinuous squares indicate the clusters of similar behaviours identified. The histograms show the significant differences in the frequency of the six behaviours found among groups as indicated by the Chi-square test, with pairwise comparisons adjusted with the Bonferroni correction for the family-wise error rate.....	57
Figure 4.1- Species Sensitivity Distribution curve (SSD) based on the LC ₅₀ values for 96h of aquatic vertebrate and invertebrate species to tributyltin-based compounds and correspondent HC ₀₅ . Black arrow indicates the position of <i>T. longicaudatus</i>	72
Figure 4.2- MUSCLE sequence alignment of the RXR- α isoform of <i>H. sapiens</i> and <i>T. longicaudatus</i> . Colour code shows aligned sequences that are possess the same aminoacid indicating conservation in the sequence. Gray coloration demonstrates non-conserved residues. Dark blue labels next to the consensus sequence indicates customized annotation of interaction residues.....	74
Figure 4.3- Neighbour-joining phylogenetic tree based on Jukes-Cantor model regarding RXR- α protein sequences. Bootstrap values represented at the beginning of each branch. Genetic distance can be assessed trough branch lengths. Different colour circles represent changes in the interaction aminoacids when compared to the <i>H. sapiens</i> sequence.	77
Figure 4.4- Predicted 3D structure of the RXR protein and related information regarding its quality. A- Predicted 3D structure of the RXR protein. B- Global quality of the RXR protein model. C- Local quality estimate of the RXR protein model...	79
Figure 4.5- RXR- α ligand binding domain visualization. A- <i>T. longicaudatus</i> sequence (Red) superposed to <i>H. sapiens</i> sequence (Green). B- <i>T. longicaudatus</i> sequence (Red) superposed to <i>D. magna</i> sequence (Blue).....	80
Figure 4.6- Representation of interaction AAs for each of the sequences of interest. AA in red is indicative of a substitution when compared to the <i>H. sapiens</i> structure.	81
Figure 5.1- Theoretical adverse outcome pathway for TBT toxicity based on the obtained results	91

Table Index

Table 1.1- Concentrations of TBT in freshwater of several countries.....	3
Table 2.1- Most relevant up-regulated genes. Protein column refers to the name of protein followed by the abbreviation followed by the Log2FC value observed in each treatment.	34
Table 2.2- Most relevant down-regulated genes. Protein column refers to the name of protein followed by the abbreviation followed by the Log2FC value observed in each treatment.	35
Table 2.3- Impacted KEGG pathways consequent of down-regulated genes (A) and up-regulated genes (B) for each treatment indicated by the “X” in the respective column.....	36
Table 3.1- Results of the One-way ANOVA performed to investigate the effects of TBT on each of the locomotion variables analysed.	52
Table 3.2 - Contribution of each locomotion variable for the six behaviour types identified, as indicated by the results of the Correspondence Analysis. The intensity of the shade relates to a higher (green) or lower (white) contribution. The six types of behaviour were identified by a Cluster Analysis based on the Kohonen algorithm.	53
Table 4.1- Accession number and corresponding database of the sequences of RXR- α obtained for each species of interest.	69
Table 4.2- Section of the MUSCLE alignment showing in greater detail the conservation of interaction residues. Cystein responsible for the covalent bind to the tin atom of the TBT molecule is highlighted in red.....	76

1. Chapter I: Introduction, dissertation structure and objectives

1.1. Introduction

Biocides

The European legislation defines biocides as chemicals whose main objective is to destroy, deter, neutralise, or control a given organism (European Parliament, 2012). The main difference between these chemicals and pesticides is the purpose to which they are used. Biocides are applied for non-food related purposes, such as disinfectants, antifouling products, or industrial repellents. On the other hand, pesticides are used to control pests and plant pathogens, thereby increasing agricultural yields and handling insect-borne diseases such as dengue (Abhilash & Singh, 2009). Since biocides primary purpose is to eliminate living organisms, they should receive a significant amount of attention as they pose a threat to both human and environmental health (Konstantinou & Albanis, 2004). Nonetheless, the demand for these chemicals has been increasing in the last decades (Acmite Market intelligence, 2012). Biocides have proven to be effective, thereby allowing more profitable industrial work despite the unpredicted environmental consequences (Mcneil, 2018). Fortunately, many countries have already restricted the use of several of these compounds. This is the case of the European Union (EU), which implemented legislation to ban organotin antifouling biocides, such as cybutryne, for amateur use due to its effects on non-target species and the possibility of being biomagnified through the food chain (European comission, 2016). Although some chemicals are banned or restricted, they usually get quickly replaced by others whose effects may not be fully understood. Bis(2-ethylhexyl)phthalate (DEHP), a compound used mainly to confer flexibility to rigid plastics, was quickly banned after several reports of reproduction toxicity and endocrine disruption (Arcadi *et al.*, 1998). Still, the commercial demand remained for a chemical with these properties, which lead to its substitution by 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH), which is nowadays one of the main substitutes for phthalates (Crespo *et al.*, 2007). Unfortunately, although several of the DEHP endpoints were avoided, DINCH leads to haemorrhages in mice, proving that the substitution was not flawless (Nardelli *et al.*, 2017).

Such lack of information makes it more difficult to perform risk assessments, crucial to protect more susceptible or fragile environments (Konstantinou & Albanis, 2004).

Therefore, it is vital to improve the amount of knowledge and use of the precautionary principle in order to avoid unredeemable damage (Hernández-moreno *et al.*, 2019).

Tributyltin (TBT)

Organotin compounds are generally described as compounds based on a tin atom with alkyl substituents covalently bound. The alkyl substituents can be methyl, ethyl, butyl or phenyl (Müller *et al.*, 2013). Tributyltin (TBT) refers to a variety of TBT-derivate compounds (**Figure 1.1**). The main difference resides in the nature of the fourth group linked to the tin atom. The base chemical formula is $(n\text{-C}_4\text{H}_9)_3\text{Sn-X}$, where X can be attributed to various anions or covalently linked groups and the chemical properties, such as solubility and melting point, vary accordingly (Müller *et al.*, 2013). Although several of these TBT-based compounds were extensively used in industry, not much information is available (Müller *et al.*, 2013).

These compounds were extensively used since the early '60s and are known to have a strong broad-spectrum biocidal effect on a great variety of aquatic organisms (Caseri, 2014).

This molecule's physical and chemical properties lead to strong adsorption to particles suspended in the water column, significantly increasing its persistence. Predictions

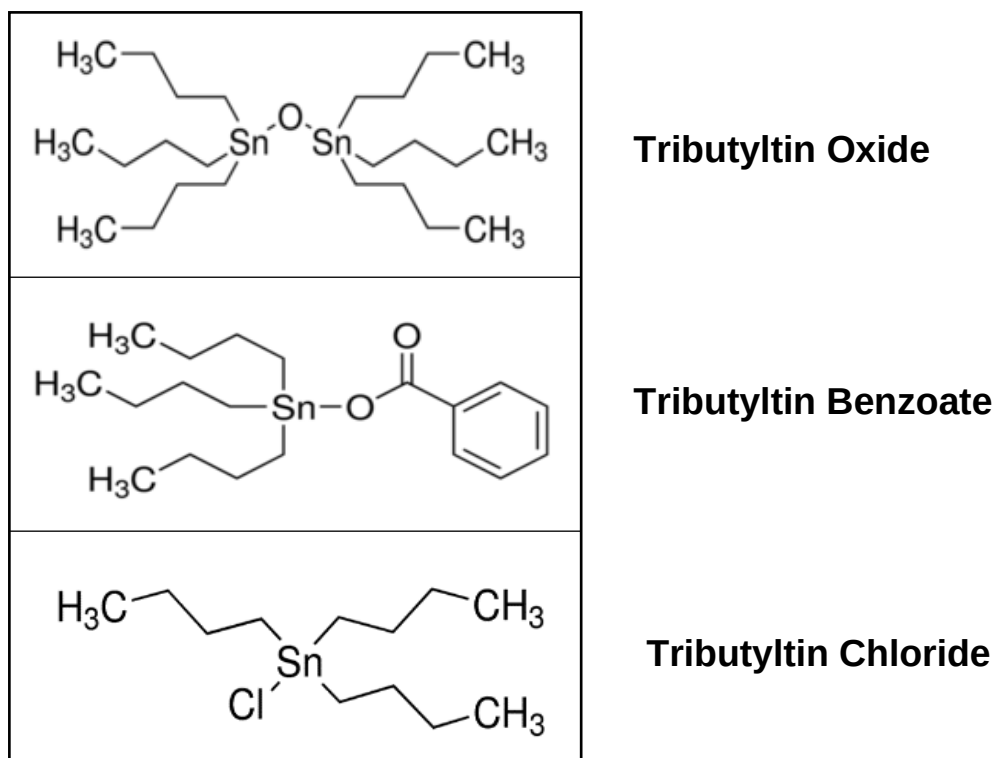


Figure 1.1-The molecular structure of the most common TBT compounds (From Sigma-Aldrich).

point that up to 90% of TBT molecules suffer this adsorption (ATSDR, 2005). TBT half-life varies mainly due to salinity, ranging from 4 to 19 days in seawater and increasing in freshwater from weeks to several months (Champ & Seligman, 1996). A very important fact since TBT has been found in freshwater environments in harmful concentrations (Sayer, Hoare, Simpson, Henderson, & Liptrot, 2006). In **Table 1.1** it is possible to observe TBT found in the environment across the globe.

Table 1.1- Concentrations of TBT in freshwater of several countries.

Country	Location	Environment	Concentration (ng/L)	Reference
South Korea	Jinhae	Bay	26.9	Kim <i>et al.</i> , 2014
Brazil	Todos os Santos	Bay	262	Artifon <i>et al.</i> , 2016
United Kingdom	Wroxham Broad	Freshwater body	898	Sayer <i>et al.</i> , 2006
Argentina	Paraná	River	1350	Martínez <i>et al.</i> , 2017
United Kingdom	Bure	River	1540	Sayer <i>et al.</i> , 2006
Canada	Ontario	Lake	5760	Grinwis <i>et al.</i> , 1998

TBT-based compounds may spontaneously degrade via hydrolysis at a low pH forming cationic forms, and at higher pH values, a neutral hydroxide will start to appear (ATSDR, 2005). Still, since these conditions rarely occur in the environment, this process is almost not observed (Müller *et al.*, 2013). The biodegradation of TBT-based compounds is mainly done by microorganisms (bacteria, microalgae and fungi). The formation of intermediate hydroxylated derivatives is achieved by breaking the tin-carbon bond (Müller *et al.*, 2013). More complex organisms are also capable of metabolising TBT molecules. For Tributyltin oxide (TBTO), this metabolism is known to happen through hydroxylation by oxygenase systems of several fish and crustaceans (Lee, 1985). After binding the oxidised cytochrome P450 (Fe^{+3}), the complex undergoes reduction to P450 (Fe^{+2}), which then interacts with oxygen resulting in a hydroxylated substance and a water molecule as represented in **Figure 1.2** (Lee, 1985).

The biodegradation rate strongly varies according to water conditions such as temperature, pH, luminosity, turbidity, or oxygen. Degradation of TBT-based compounds' can occur both in aerobic and anaerobic conditions (Müller *et al.*, 2013).

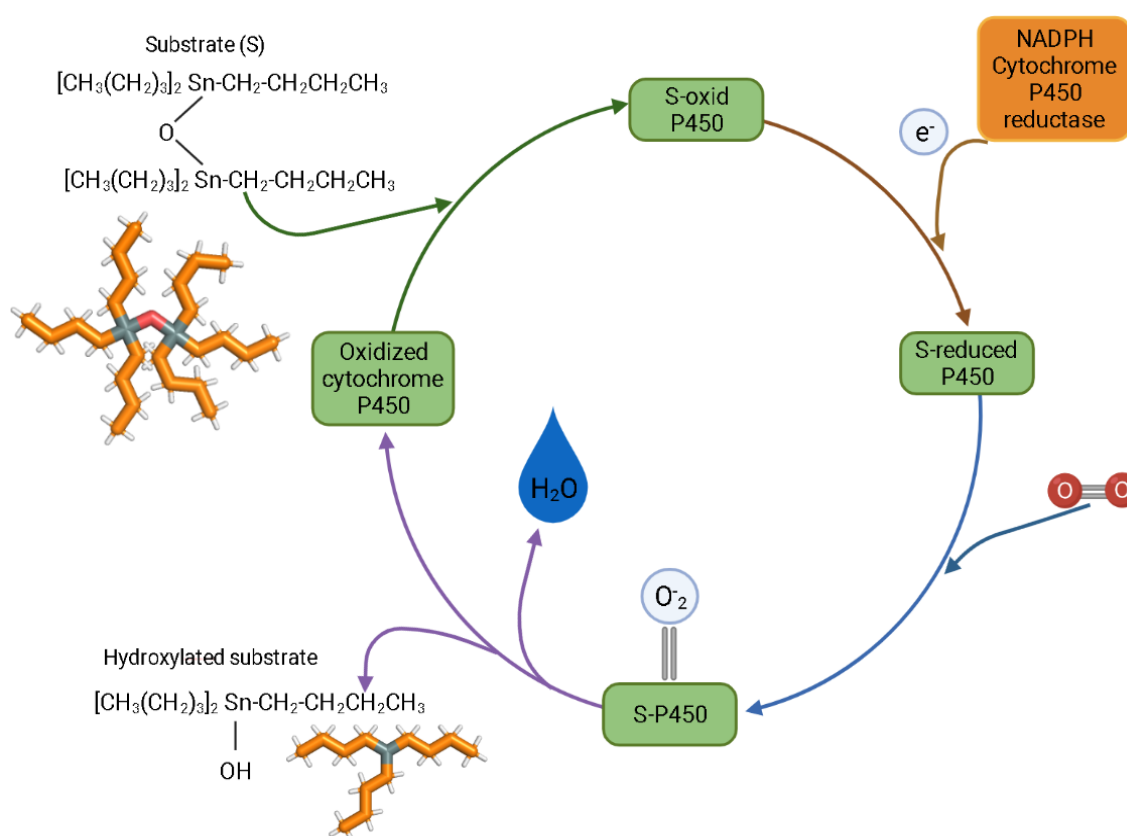


Figure 1.2- Schematic representation of the steps leading to hydroxylation of TBTO by an oxygenase system and resulting metabolite (From Lee, 1985 – created using [BioRender.com](https://www.biorender.com)).

The primary use of TBT was to protect the outer layer of boats from organisms such as algae, molluscs or barnacles, that could colonise and increase the traction of the vessel (Scianni & Georgiades, 2019). Colonised vessels lead to increased water resistance thus making the vessels less hydrodynamic. This would lead to an increased fuel cost and time spent on dry docks for maintenance, thereby reducing the transportation profit (IMO, 2002). These chemicals were added to the paint, turning them into a non-selective anti-fouling agent or stabilisers (Müller *et al.*, 2013). Since these compounds are human-made, TBT found in the environment is always a direct consequence of anthropogenic activity. Due to negative impacts on organisms, TBT-based compounds are regulated internationally since 1990. Many countries banned TBT from vessels under 25 meters, aiming to reduce the paint in amateur or recreational vessels. In 2001, the International Maritime Organization (IMO), together with the Marine Environment Protection Committee (MEPC), proposed the complete ban of TBT for all ships (Konstantinou & Albanis, 2004). At the Rotterdam Convention, held in 2003, TBT compounds were classified as chemicals with unacceptable risk to non-target species and high persistence in the environment and therefore, in the EU, all paints and products containing this chemical were banned from being applied to vessels

or equipment totally or partially submerged (UNEP/FAO, 2015). A variety of countries adopted this policy, but unfortunately, in countries with poor enforcement policies, the use of TBT-based chemicals is still active (Castro *et al.*, 2018).

TBT's Adverse Effects in organisms

TBT's relatively high octanol-water partition coefficient ($\log K_{ow} > 4.7$) gives this chemical a high biomagnification potential throughout the trophic chain (Franke, 1996). TBT is also known to bioaccumulate in aquatic organisms mainly due to its high lipid solubility and poor or limited metabolic capacity of the organism (Cao *et al.*, 2009). This high bioaccumulation could also reach Humans since many aquatic organisms, such as molluscs, pelagic fish and crustaceans, are incorporated into the Human diet (Müller *et al.*, 2013). As a result, bioconcentration factor (BCF) as high as 1,280 was found for zebrafish larvae, 5,000 for common mussels and 67,258 for Baltic clams (López-Serrano Oliver *et al.*, 2011; Coelho, 2004; Filipkowska & Lubecki, 2016). TBT enters the organisms mainly through oral intake followed by rapid distribution onto different tissues (Filipkowska & Lubecki, 2016).

In **Figure 1.3** are represented the most common TBT pathways described by Lagadic *et al.* (2017). Documented adverse effects show that low concentrations can induce imposex in marine gastropods leading to masculinisation of the females (Anastasiou *et al.*, 2016b) and shell deformation in bivalves (Batista *et al.*, 2016). In mammals, TBT can impact the immune metabolic activity and reproduction mechanisms (Batista *et al.*, 2016; Boyer, 1989). Another well-documented mode of action of TBT is the induction of cell death. This endpoint is mainly reached by mechanisms that elevate intracellular Ca^{2+} concentration, inhibition of substrate uptake and Adenosine triphosphate (ATP) synthesis by the mitochondria (Müller *et al.*, 2013). Activation of the vertebrate retinoid X receptor/peroxisome proliferator-activated receptor heterodimer (RXR/PPAR) and the RXR homodimer is also frequent, which can interfere with the metabolism of adipose tissue (Müller *et al.*, 2013). TBT administered orally to rats led to suppression of neural ATPases and the occurrence of seizures and tremors, while in rainbow trout, TBT exposure led to ultrastructural lesions in the optic nerves (Müller *et al.*, 2013; Yu *et al.*, 2013). Other studies done with sticklebacks showed that TBT exposure led to spatial unawareness, longer response times, and decreased expression of neurotransmitter receptors leading to abnormal behaviour (Wibe *et al.*, 2001). Exposed daphnids suffered disruptions in lipid metabolism and impaired transfer of triacylglycerols to eggs. This impairment can reduce the fitness of the adults while also impacting reproduction since the viability of newly formed eggs would be at risk (Jordão *et al.*, 2015).

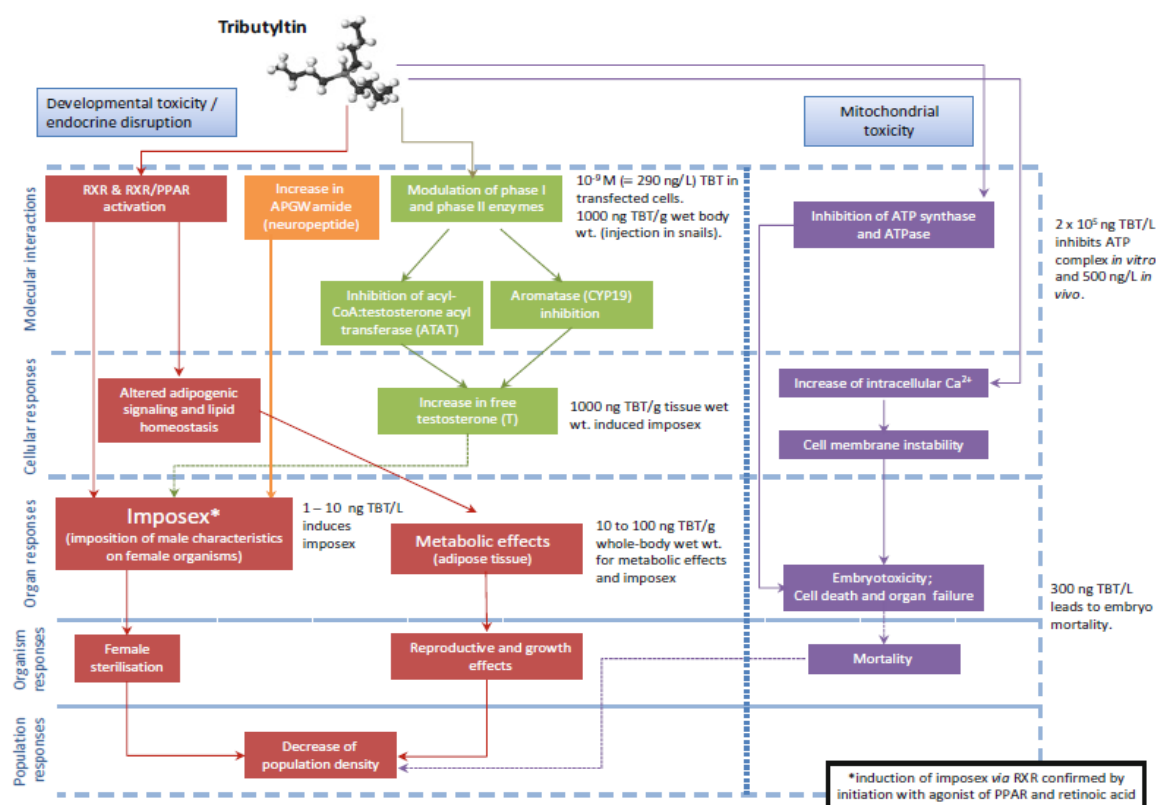


Figure 1.3- Theoretical Adverse Outcome Pathways for TBT toxicity (From Lagadic *et al.*, 2017).

Molecular impact of TBT-based compounds

Ecdysteroid hormones are essential elements for the correct moulting process typical of organisms from the Ecdysozoa clade, with the most emblematic examples being insects and crustaceans. In addition to moulting, ecdysteroid hormones and correspondent ecdysteroid hormone receptors (EcR) intervene in other biological processes such as development, growth and even reproduction. EcRs are part of the nuclear receptor family alongside other ligand-activated transcription factors such as liver X receptors (LXRs), liver receptor homolog-1 (LRH-1) or farnesol X receptor (FXR - Francis *et al.*, 2003). In order to induce the expression of target genes, EcRs must interact with nuclear response elements that usually are located in the target gene promoter. This feature turns EcR particularly sensitive to exogenous or artificial ligands such as pharmaceuticals or pesticides (Verhaegen *et al.*, 2011). Interestingly, from an evolutionary point of view EcRs are present not only in animals but also in plants and fungi, being absent in vertebrates where they are replaced by PPARs. This is indicative that the ecdysteroid regulatory circuit is highly conserved and an evolutionarily successful strategy existing for at least 500 million years (Spindler *et al.*, 2009).

The EcRs are also known to form obligate heterodimers with RXR (**Figure 1.4**) in order to operate as transactivators, which will work as a control measure for the transcription of specific genes related to moulting, growth and development (Verhaegen *et al.*, 2011). RXR are also nuclear receptors responsible for mediating the effect of retinoids, such as

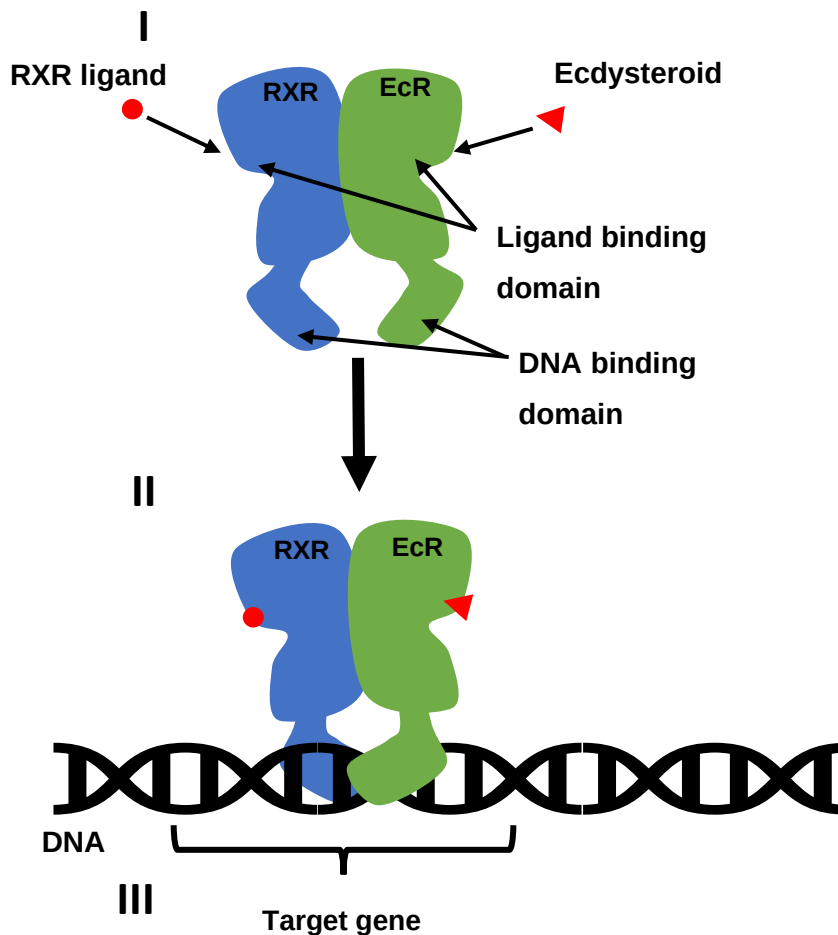


Figure 1.4- RXR/EcR transcriptional activation in the cell nucleus. **I-** Binding of PPAR and RXR ligands; **II-** Changes in conformation; **III-** Activation of the complex and gene expression. (From Rigano *et al.*, 2017).

vitamin D. This mediation occurs mainly by regulating (both as homodimer or heterodimer) the transcription of specific gene sequences by binding to the promotor regions of those target genes (Ziouzenkova & Plutzky, 2008). TBT is known to be one of the most effective RXR ligands as micromolar concentrations have showed to activate the Human α isoform (Le Maire *et al.*, 2009). Furthermore, by covalently binding to a central cysteine, TBT occupies a portion of the RXR's ligand-binding domain enough to change its conformation into a stable activated form, thereby having an agonistic activity (Le Maire *et al.*, 2009). Due to the high conservation of the sequence and composition of this molecule between species it is safe to assume that a similar interaction will occur in organisms belonging to the Arthropoda taxa (Fonseca *et al.*, 2020).

Ecotoxicological bioassays

Ecotoxicology is a field of study that essentially combines toxicology and ecology, resulting in progressively more comprehensive knowledge regarding the effects of chemicals and toxicants that end up in the environment (Zhang *et al.*, 2018). A toxicant can affect several levels of biological organisation, such as population or ecosystems, but also lower levels such as cellular and molecular. Only when this information is combined is it possible for the scientific community to understand the full extent of environmental contamination. One of the most common evaluated endpoints in ecotoxicology is mortality, and although effective at providing irrefutable results, it leaves gaps in sub-lethal and long-term effects (Garcia-Reyero & Murphy, 2018). Sub-lethal effects are of great interest as they could lead to the reduction of fitness or survival capacities that might lead to the death of the organism through indirect ways such as starvation or predation. Besides this, mortality assays may raise ethical concerns, and therefore, alternatives such as sensitive biomarkers could be considered more appropriate.

Behaviour analysis nowadays is becoming more frequent due to its ability to provide more sensitive results when compared to the conventional median lethal concentration endpoint (LC₅₀ - Robinson, 2009). The evaluation is made essentially by integrating the real conditions to which the organism is subjected and the exposure time, culminating in an acute cumulative effect (Hellou, 2011). Behaviour analysis also sheds light on subtle changes that are imperceptible to sporadic observations allowing to discover the emergence of unusual patterns in a non-evasive way (Hellou, 2011). Assessing the gene transcription of organisms under stressful conditions through technics such as RNA sequencing can provide information regarding the molecular effects of toxicants and give additional information on its mode of action (Ferreira *et al.*, 2019). This type of biomarker allied to progressively more advanced technologies will enrich the possibilities of animal testing by allowing the analysis of more data simultaneously and more precisely both in vertebrates and invertebrates.

Test-organism: *Triops longicaudatus*

Triops longicaudatus, commonly known as tadpole shrimps, are freshwater crustaceans of the Notostraca Order and Branchiopoda Class. They can be found in several areas such as North and South America, throughout most of the Pacific islands and Japan, making them one of the most widely distributed species of their Order (Sassaman *et al.*, 1997). They can often be found in ephemeral and semi-permanent aquatic habitats like

ponds, swamps or small pools with temperatures ranging from 25 to 30°C (Sassaman *et al.*, 1997). Tadpole shrimps tend to occupy the benthonic region of these habitats but can move freely in the water column with the aid of dorsal appendages called thoracopods (**Figure 1.5**- Fryer, 1988). The dorsal dome-like carapace is continuous, with the integument serving as protection for most trunk appendages (since this species swims mostly with the ventral surface facing down), ending in a row of sclerotin spines. On top of the carapace, they present two compound eyes and a single ocellus providing sensory function. The head integument develops a particular angle that facilitates burying into the sediment. The thickened structure continues ventrally until the mandibles and labrum, which itself consists of a hard cuticle. The first pair of limbs are denominated endites, and the main use of these structures is for sensorial purposes. The segmented trunk ends at the telson, which houses two very long furcal rami that increase hydrodynamic and movement efficiency. After the first two pairs of limbs, the limbs start to change their shape. The limbs are leaf-shaped, and the main purpose is to propel the animal forward. The size of the limbs gradually decreases until the last pair reaches approximately 2/3 of the trunk (**Figure 1.5**).

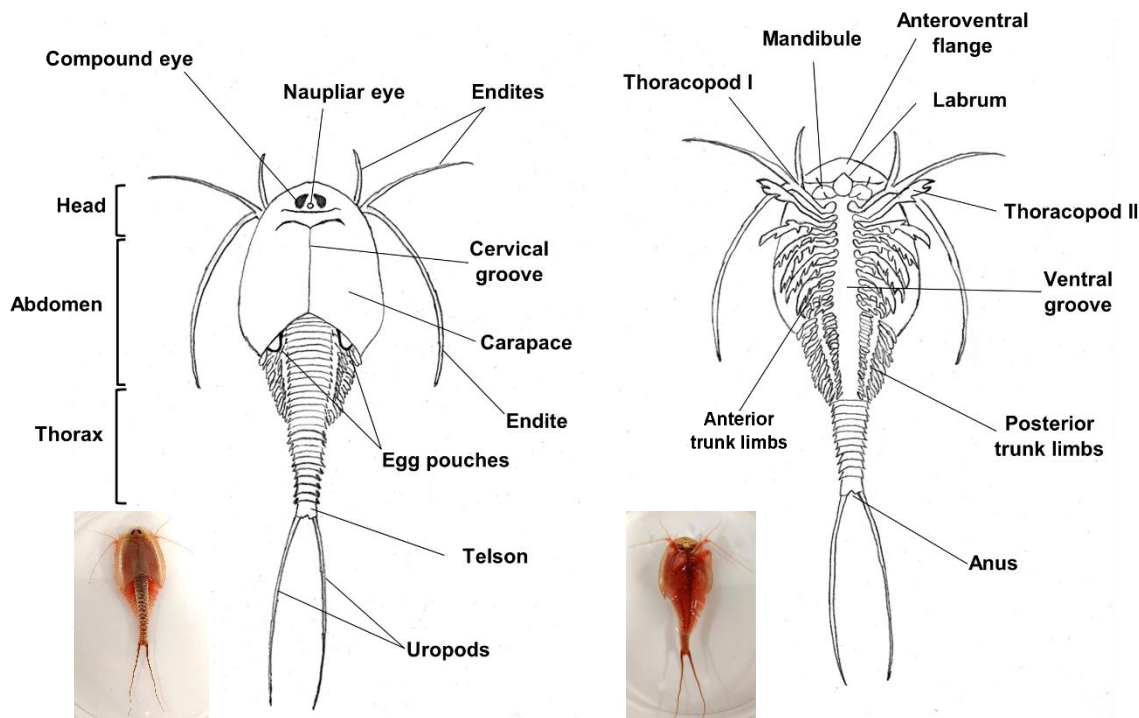


Figure 1.5- Diagram of the external anatomy of a female *Triops longicaudatus*. **Right-** ventral view. **Left-** dorsal view.

The *Triops* genus prevails for 300 million years, and the *T. longicaudatus* species is one of the known examples of morphological stasis, as the fundamental morphology of the species has not changed in over 170 million years, while also being considered a living

fossils (Suno-uchi, 1997). This species is also defined as being cryptic since morphologically, there are few differences between the several species of the genus despite having independent evolutionary histories (Korn & Hundsdoerfer, 2006; Smith *et al.*, 2011). This species tends to live relatively solitary, a strategy acquired to reduce predation that tends to rise when large groups of these organisms are formed (Hasbun, 2014). These small crustaceans are primarily omnivorous scavengers since they feed on both vegetal and animal detritus found on the bottom of ponds. Predation behaviour is also observed (Hasbun, 2014). As an example, *T. longicaudatus* can hunt and eat everything that presents a smaller size than itself, including zooplankton, small fish, insect larvae and adults, and, in extreme conditions, even smaller members of their species (Waterkeyn *et al.*, 2011). The predation behaviour turns this species environmental function crucial since they present a particular preference for mosquito larvae, including larvae from the *Culex* genera (Fryer, 1988). Feeding on these larvae or preventing their oviposition is particularly important since it controls the mosquito population that can become vectors of several diseases (Fry *et al.*, 1994).

T. longicaudatus is also used as a biological agent for aquatic weed control. Their feeding and oviposition behaviour stirs up the soil surface, preventing young seedlings from laying roots. The consequent disturbance suspends particles responsible for turning the water muddy or preventing sunlight from passing through it (Takahashi, 1994). Thereby, they are considered helpful for rice farmers throughout Japan and are consequently protected (Takahashi, 1994). Unfortunately, if a population becomes too large, it can have a negative impact, as *T. longicaudatus* can achieve adulthood before the rice plants leading to an unrooting of the plants and a need to reseed the field (Bloese *et al.*, 2020).

In 2004 the Ministry of Environment of South Korea registered this species as endangered due to the drastic reduction of their population. However, due to subsequent conservation efforts, the numbers have risen again, resulting in their removal from the list in 2012. **Figure 1.6**, it is possible to see a schematic representation of *T. longicaudatus* life cycle. Although cysts have been observed to hatch without a dry period, the survival of the hatchlings is extremely low in the presence of larger organisms. Therefore, the depicted strategy is the most successful for developing a new generation. These reproduction strategies are known to increase inbreeding and reduce genetic diversity and the size of effective populations leaving the population with fewer genomic resources to face environmental changes (Charlesworth & Wright, 2001). Nonetheless, these strategies ensure reproductive success since there is no need to find a sexual partner, a more frequent

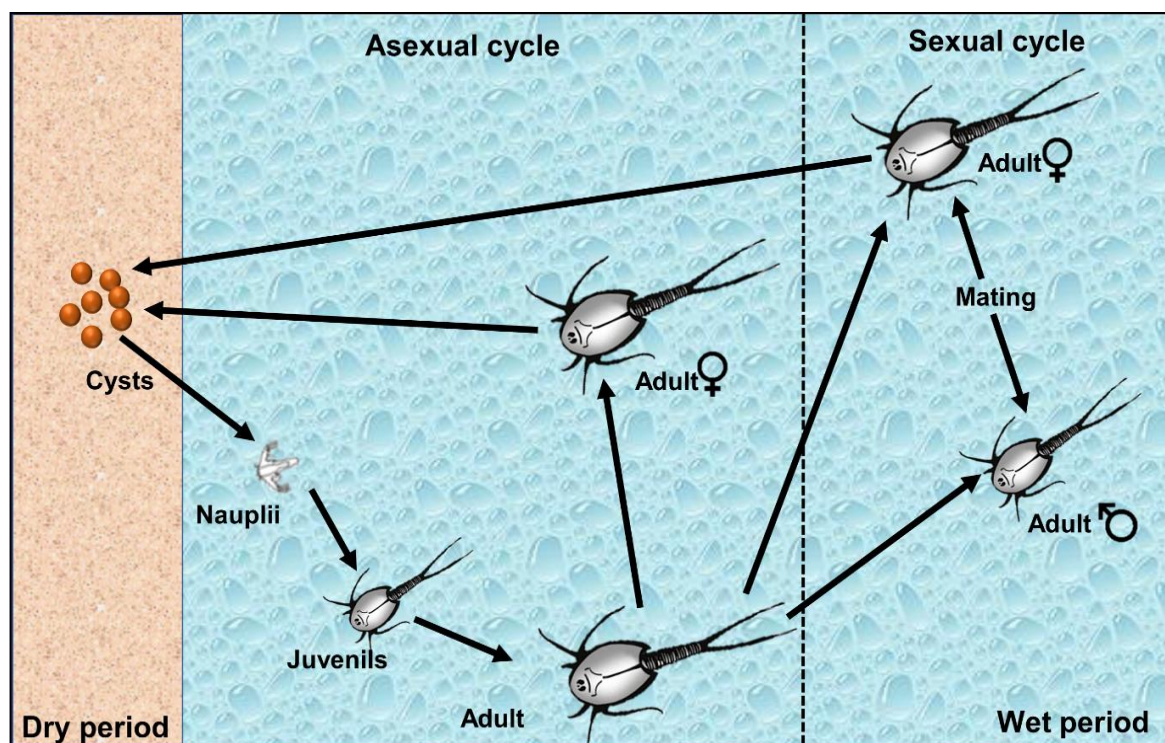


Figure 1.6- Life cycle of *Triops longicaudatus*. In the left section is represented the asexual life cycle where the population is mainly made of female or hermaphrodite individuals. To the right, the sexual reproduction cycle that usually happens under poor environmental conditions where genetic diversity provides a greater chance of survival. Cysts usually pass through a desiccation phase and hatch after rehydration.

transmission of a favourable combination of genes and the possibility to colonise a new habitat with only one individual. This last feature allows for some populations to be considered clonal (Otto, 2009). Reproduction in this species is an almost continuous event since females gradually bury their cysts in the sediment as they mature, protecting them from predators. This species can also quickly release all the cysts when the individuals find themselves in shallow or drying water, an indicator of the end of the wet period.

Another important feature of this species is the ability to produce resistant cysts that can withstand heat and desiccation throughout several years. These cysts must pass through a dry period where the pool they were laid dries out. Afterwards, cysts are rehydrated, either by a rainy season or flood and hatch. In the wild, these conditions indicate the beginning of a new wet session where environmental changes tend to be more favourable for survival. When the cysts hatch, the organisms are called metanauplius, and at this stage, the larvae are considered planktonic (Hasbun, 2014). After $\pm$ 20h, the larvae enter the metamorphosis stage by shedding its exoskeleton and start looking like a miniature version of the adult with essentially anamorphic development. The organism will then start to forage for any food particles and grows rapidly in the days that succeed (Hasbun, 2014). Growing rate is achieved through regular moulting events (that can happen every day) reflecting the density of the population, volume of the habitat, food availability and temperature.

Regarding its laboratory relevance, the selection of this species as a test organism was motivated by various factors making it a suitable choice for ecotoxicological tests. Additionally, small crustaceans are particularly important as they represent intermedium positions in the food chain, thus providing early information about chemical bioaccumulation (Scholnick, 1995).

Among its favourable set of characteristics, the most important to classify these organisms as an ecotoxicological model species are:

- Small size (up to 60mm);
- Rapid external development;
- High fecundity (up to 250 cysts per day at peak maturity);
- Short life cycle and generation time (around 14 days to reach a full adult stage);
- Rapid sexual maturity (around ten days for the first release of cysts);
- Asexual reproduction;
- Simple maintenance;

Ethical legislation and use

According to the European Food Safety Authority (EFSA), these crustaceans fit into category II, which defines organisms unable to experience pain or distress either directly or by analogy with organisms in the same taxonomic group (European Food Safety Authority, 2005). This classification is mainly due to the less complex nervous system of *T.*

longicaudatus and is consequently considered a non-protected species. Therefore, the legislation regarding testing with these organisms does not require ethical approval.

This model gains additional importance nowadays since ethical issues are being taken into great consideration and the search for these alternative models is an ever-rising priority (Ibrahim, 2006). Consequently, this project is within the 3 R's (Reduce, Refine, Replace) initiative that promotes the use and development of alternative experiment models to improve scientific discoveries with the least repercussion (Ibrahim, 2006).

1.2. Objectives

The objective of this work was to evaluate the acute effects of TBT exposure on the transcriptomic expression of *T. longicaudatus*, with particular attention to genes related to pathways known to be disrupted by this chemical. The second objective is to understand locomotory behavioural and swimming patterns of organisms exposed to TBT under environmentally relevant concentrations. Finally, the last objective is to assess the sensitivity of different species towards TBT's main target – the retinoid X receptors (RXR) using bioinformatics tools,

1.3. Dissertation structure

Conceptual framework

The overall objective of this dissertation was to evaluate and understand the effects of the biocide Tributyltin at several organisational levels. The genetic expression will provide valuable information on all impacted biological processes. Behavioural will elucidate the sub-lethal effects of TBT, and its impact at a population level. Finally, bioinformatics analysis of the molecular interaction between TBT and its main target (RXR protein) will assess RXR-TBT affinity among different species and their possible toxicity. All the previous analyses will result from exposures to environmentally relevant TBT concentrations. Additionally, these sub-lethal concentrations will provide novel insight into the impact of endocrine disruptors, specifically in *T. longicaudatus*, that show high sensitivity for TBT.

The results of this study will provide a better understanding of the chemical's mode of action towards crustaceans and their overall toxicity. In the final chapter of the dissertation, the data was integrated to present a summary of all the endpoints that were found during our analysis. Our results could be used as building blocks for other studies

since the information gathered could provide the initial steps for several other analyses (e.g. biomarkers or tissue-specific transcriptomics).

In order to achieve the main goals presented, several approaches were carried out, which can be divided into the five chapters listed below:

- **Chapter 1- Introduction, main objectives, and dissertation structure.**
- **Chapter 2- Evaluation of TBTs impact on gene expression of *Triops longicaudatus* using RNA-seq analyses.** After acute exposure to four different concentrations of TBT, a whole-body RNA extraction was performed, followed by transcriptome assembly. Here the base gene expression was compared to the treatment groups, and significantly up and down-regulated genes were evaluated and linked to the correspondent pathway. This provided insight into TBT's mode of action and the general response mechanism taken by the organism to cope with the contaminant. The observed responses indicate that TBT exerts cytotoxicity, genotoxicity, neurotoxicity, and endocrine disruption.
- **Chapter 3- Effects of TBT on the locomotory behaviour of *Triops longicaudatus*.** Juvenile organisms exposed to TBT were individually recorded. The recordings were analysed to assess any behavioural changes against the 'normal behaviour'. The analyses revealed six distinct behaviours, one closely associated with the control treatment and the remaining distributed through the TBT treatments. These results showed that TBT contamination affects the organism even at low concentrations. Using the transcriptomic data with behaviour data was possible to link impacts at a neurotransmission level that could lead to motor discoordination and spatial unawareness along with a reduction on the organism's overall fitness, which could indirectly lead to its death.
- **Chapter 4- Comparative genomics and bioinformatic prediction of RXR/EcR sensitivity to TBT.** The main objective of this chapter was, therefore, to understand how TBT molecules interact with one of the main proteins of the ecdysone pathway (RXR protein). Initially, a species sensitivity distribution curve was developed in order to understand the sensitivity of different organisms from different taxa. Additionally, a phylogenetic analysis was performed to assess the conservation of the protein and, in more detail, the binding pocket to the TBT molecule. Although the protein was highly conserved in almost all evaluated species, no correlation was made between aminoacid conservation and overall sensitivity to TBT.

- **Chapter 5- Discussion and conclusion.** In this Chapter, an integration of the overall results was done, presenting the most updated adverse outcome pathway for crustaceans until the present moment.

Relevance of the dissertation

The usage of *T. longicaudatus* in this study revealed its potential for a suitable model organism for freshwater ecotoxicological experiments. Additionally, it provides a novel option since crustaceans are scarcely studied (with the exception of daphnids). While evaluation and screening of temporary environments are often not seen as a priority, results obtained with this species could be extrapolated to other studies, increasing the impact ray. As a novel model organism in ecotoxicology, there is a substantial lack of protocol and assay-standardisation for *T. longicaudatus*. In this sense, this work presents standard procedures that can be used in further studies.

Regarding TBT, since several countries still allow its usage, it is probable that contamination of water bodies will continue to occur. As so, it becomes crucial to understand the lethal and sub-lethal effects of this chemical to minimise its impact. While this work provides essential information on the mode of action, several complementary analyses could be performed, including but not restricted to biomarkers, energy reserves allocations and tissue-specific sequencing.

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2. Chapter II: Transcriptomic profile of a living fossil (*Triops longicaudatus*) exposed to Tributyltin

Abstract:

Tributyltin (TBT) is a biocide extensively used in the 60's. It was added to antifouling paints in order to prevent ship hull colonisation by sessile organisms. Despite its ban by several countries in 2008, measurable concentrations are still found in the environment. Due to its toxic nature, it became part of the EU priority list of compounds to control, especially in coastal and freshwater ecosystems. Besides TBTs lethal effects, much has yet to be revealed regarding sub-lethal effects on non-target organisms.

This study focuses on the potential of RNA-seq to determine changes in transcriptional gene expression of *Triops longicaudatus* exposed to TBT. Besides the significant lack of genetic information on this species, it possesses ecological importance and provides additional information on the somewhat neglected Crustacea sub-phylum. A reference whole-body transcriptome was generated, followed by its annotation.

Differential gene expression was determined across the four treatments, 0; 0.075; 0.25 and 1.54µg/L and used to infer impacts on the most notorious molecular processes and pathways. Several genes were found to be significantly up- and down-regulated, and in some cases, dose-response dependency was found. The results indicated that, besides the most documented endocrine disruptive potential, TBT induces cytotoxicity, neurotoxicity and genotoxicity on *T. longicaudatus*. This work contributed with more detailed information on the mode of action of TBT molecules which continue to be a threat to environmental health.

Keywords: Tributyltin oxide, Crustacea, Endocrine Disruption, RNA-seq.

2.1. Introduction

Environmental contamination with chemicals is a rising concern worldwide. Several factors such as climate change, population growth or even outdated legislation are the main reasons for these increasing contamination levels (Lim *et al.*, 2017). The past uncertainty of pollutants levels due to technological limitations are now being overcome. Advances in nanoscale imaging or lower costs for state-of-the-art techniques such as 'omics', greatly improve the amount and specificity of acquired data (Wang *et al.*, 2018). This additional information could facilitate the biomonitoring of ecosystems and help correct or mitigate the adverse impacts of environmental contamination.

Transcriptomics was developed to aid with the complex task of understanding the full transcriptional differences of organisms under normal and stressful or pathological situations and pinpoint the most crucial differences (Kalisky *et al.*, 2018). Therefore the information given by a transcriptome can be used to assess gene expression profiling, genome annotation or quantification and even the discovery of unknown non-coding RNA (Morozova & Marra, 2008). Transcriptomes provide crucial information of a given organism, tissue or single cell at a certain point in time, reflecting the organism's basal gene expression or a response to new conditions (Lowe *et al.*, 2017). With a transcriptomic analysis, it is possible to understand how genes are actively being expressed and in what proportion. This information is crucial to understand the main functional biology of the organism and altered states induced by diseases, but also the molecular effect of specific compounds such as endocrine disruptors (Lowe *et al.*, 2017).

Endocrine disruptors are emergent pollutants whose presence in the environment recently suffered a rapid increase (Khan *et al.*, 2020). This increase occurs mainly due to improper discard and deficient removal from wastewater (Muller *et al.*, 2010). Due to their capacity to interfere with the normal activity and regulation of natural hormones, endocrine disruptors are responsible for various deleterious effects in physiological systems (*e.g.* immune, metabolic or neurological - Huang *et al.*, 2018). Tributyltin-based products (TBT) are an example of endocrine disruptors. TBT are organotins mainly used as paint stabilisers to prevent organisms from colonising the submerged hull of vessels (Sayer, Hoare, Simpson, Henderson, & Liptrot, 2006). Although highly effective towards the primary target species, several effects were observed on non-target organisms even at low concentrations (Alzieu, 2000). In vertebrates, such as mice, TBT concentrations of 5 µg/kg were found to induce insulin dysregulation and disturb overall glucose homeostasis (Zuo *et al.*, 2014). TBT concentrations of 100 ng/L led to a decrease in thyroid hormones in zebrafish (Li & Li, 2020). In invertebrates, such as oysters, concentrations as low as 2 ng/L lead to shell malformation due to deficient calcification and imposex in gastropods besides impacting several other

pathways (e.g. inhibition of segmentation, slow growth/development or trochophore malformations - Alzieu, 2000; Anastasiou *et al.*, 2016). TBT can also impact less observable pathways by changing gene expression or abnormal energy allocations related to moulting and lipid homeostasis (Jordão *et al.*, 2015).

Despite its importance in scientific research and the aquaculture industry, the biology and genomic information of the Crustacea sub-phylum remains vague. A possible reason may be the significant similarity it shares with the insect taxon, which is preferred by the scientific community due, for example, to a large number of model organisms such as the *Drosophila* genus or scientific initiatives such as the 5000 insect genome project (Nong *et al.*, 2020). *Triops longicaudatus* is a freshwater crustacean that inhabits ephemeral or semi-permanent ponds worldwide and is considered a cryptic species and a living fossil due to its fundamentally unchanged morphological traits for more than 170 million years (Sunouchi, 1997). Most populations consist of female individuals, so reproduction is mainly asexual, leading to clonal individuals and ultimately clonal populations (Otto, 2009). Equally important is their small size, short generational time, simple maintenance, and easy manipulation, making *T. longicaudatus* a suitable model-organisms for ecotoxicological experiments. Unfortunately, repository data on *T. longicaudatus* is very scarce. A brief analysis of NCBI shows one sequence (Schwentner *et al.*, 2018), a mitochondrial genome (Cook *et al.*, 2005) and several individual sequences related to barcoding, mitochondrial genes and microsatellites development (Regier *et al.*, 2008). The inclusion of more data on this species would significantly improve its use in ecotoxicological assays and other research areas, such as phylogeny, where data from a 'living fossil' can be used as a connection link to other species. In this sense, RNA-seq data of *T. longicaudatus* exposed to TBT can provide insight at the genetic level by quantifying the expression of core genes and species-specific genes, which could help discover changes in the organism as a result of the induced stress (Nong *et al.*, 2020). In this sense, this study's main objective is to evaluate changes in the gene expression of *T. longicaudatus* exposed to different concentrations of TBT oxide (TBTO) and its impact on key metabolic pathways and strategies of this species to cope with TBT's toxicity.

2.2. Materials and methods

Organisms

Triops longicaudatus were hatched from commercial resistance cysts obtained through Triopsking™ (Germany). After hatching, a single individual was isolated to ensure asexual reproduction and initiate a clonal population. This procedure was done three more times in subsequent generations.

The rearing process is explained in detail in **Supplementary data 1 (SD1)** and was a progressive task since information on this topic is limited. Conditions were optimised in order to improve the lifespan and reproduction rate of the organisms.

The individuals were maintained in a large aquarium (40L) with natural sand from SwellReptiles (United Kingdom) and kept at $25 \pm 1^\circ\text{C}$, with a 12h:12h (light:dark) photoperiod. Nauplii were fed *ad libitum* with crushed highly rich chlorophyll flakes (Tropical®) and adults fed daily with fish granulate (Vitality & Color from Tropical®). Water was changed once a week, and adults were transferred to a new aquarium once a month depending on the need to recover any laid cysts. The sand was then air-dried to allow desiccation of the resistance cyst and was stored at room temperature in a dry place. To start a new culture or test, cysts were carefully selected under a magnifier from the sand and rehydrated.

Bioassay to assess LC₅₀

For the mortality test, seven concentrations of TBTO were selected (0, 0.001, 0.002, 0.004, 0.008, 0.016, 0.032, 0.064, 0.128 and 0.256µg/L). To avoid any absorbance to the walls of the polyethylene containers (36.13 x 4cm), and thereby changing the real concentration, solutions were prepared 24h prior to the exposure and replaced the day after. Each container contained 220mL of the solution and five organisms with seven days of age.

To calculate the LC₅₀ value, data was imported into SigmaPlot®, using a four parameters logistic curve.

Toxicological assay

T. longicaudatus cysts were rehydrated with 250mL dechlorinated water inside polyethylene containers. Four concentrations of tributyltin oxide (TBTO, 96%, CAS 7487-94-7) were selected: 0; 0.075; 0.25; 1.54µg/L.

The 0.075µg/L concentration was the value obtained in a mortality test previously performed (data not shown), 0.25µg/L was selected due to its usage in a previous work performed by our group (Ribeiro, 2019) and the 1.54µg/L was the highest concentration

found in the environment (Sayer, Hoare, Simpson, Henderson, & Liptrot, 2006).

Solutions were prepared from a stock solution of the highest concentration, and serial dilutions were performed to achieve the remaining desired concentrations. A solution without TBTO was used as the control group.

A volume of 220mL of each solution was put into 250mL polyethylene pots for 24h prior to the exposure to avoid any adsorption of TBTO molecules to the walls of the container and thus becoming unavailable in the solution. Five juveniles, with seven days, were selected and put into the polyethylene pots inside an incubator with the same conditions as the cultures: 25 ± 1 °C, 12h:12h (ligh:dark) photoperiod. Organisms of similar sizes were selected for the experiment. Three replicates, each containing five organisms, were performed for each concentration. The organisms were exposed for 90min, and afterwards, the organisms were immediately snap-frozen in liquid nitrogen and stored at -80°C in 500µL of Trizol reagent (ALFAGENE®).

Sample Collection and RNA Isolation

A whole-body RNA extraction was performed by initially stripping the organisms of any chitinous structures such as the carapace, antenna, and caudal fulcra to avoid hard structures that would interfere with a good RNA extraction. The adult whole-body of *T. longicaudatus* was homogenised individually, and mRNA was extracted using the protocol described by Ferreira *et al.*, (2019). After extraction RNA purity and concentration were measured using a Nanodrop machine, and its integrity was determined using a Tapestation. Following these analyses, the purified RNA was later stored at -80°C.

Transcriptome assembly

The transcriptome used in this study was assembled according to the methodology previously elaborated by Ferreira *et al.*, (2019). To evaluate the quality of the sequencing, the FastQC (v.0.11.9) software was used. After this step Trimmomatic (v.0.39) was used to remove low-quality bases and to remove sequence adaptors (Bolger *et al.*, 2014), followed by a *de novo* assembly with Trinity (v.2.11.0 - Grabherr *et al.*, 2013), this program was designed for constructing full-length transcriptomes without a reference genome.

In order to validate non-redundant contigs, the EvidentialGene package was used (arthropods.eugenex.org/EvidentialGene/) and with the resulting information, a final assembly was constructed, which was used as a Base Assembly. To understand the completeness of the transcriptome, a BUSCO analysis was performed providing information regarding complete, fragmented and missing “Core” genes (Simão *et al.*, 2015).

Differential gene expression

The resulting sequences were mapped against the Base Assembly using the Omicsbox (www.biobam.com/omicsbox), followed by reads counting and normalisation. Gene transcription was compared between treatments and against the control.

Only genes with a greater or equal Logarithm base2 Fold Change (Log2FC) of 3.3 were considered to be significantly up-regulated and genes with a lower or equal Log2FC of -3.3 were considered to be significantly down-regulated. This more conserved approach was based on the work of Dalman *et al.* (2012), where they show that the chosen arbitrary cut-off of fold-change > 2 can provide valuable information not obtained using only *p* values. Since in this work a pool of organisms were performed for each treatment the use of cut-offs is the most appropriate approach for RNA-seq analysis.

2.3. Results

LC₅₀

The LC₅₀ value obtained for *T. longicaudatus* when exposed to TBT for a period of 96h was 0.072 [0.046-0.139] µg/L.

Transcriptome sequencing output and assembly

The high throughput sequencing produced 868,218,068 reads, each containing 150 bp, which represents approximately 130.2Gb of sequence data. After the different pipeline analysis, the Base Assembly had a total of 28,681 unique transcripts, with a mean of 1211bp, and a N50 of 1896bp (**SD 2**).

BUSCO analysis was performed using the Arthropoda lineage. A total of 1013 highly conserved fundamental genes (core genes) were identified, from which 98.22% of the BUSCO groups have complete gene representation (single-copy or duplicated), while 0.79% are only partially recovered, and 0.99% are missing indicating that the obtained transcriptome contained an almost complete set of core eukaryotic genes. Our results, shown in **SD 3**, indicate a high level of completeness superior to 90% in all cases.

Homology search and functional annotation

To assess the primary function of the genes, a Blastx was performed with OmicsBox (v.1.4.11) with an e-value cutoff of 1.0e⁻⁵ against a non-redundant metazoan protein database. Transcripts with lengths under 200bp were removed from the analysis as their small size leads to non-specific hits.

The top hit species distribution showed representation throughout the Animal Kingdom (**Figure 2.1**). Even though the majority of hits (9835 out of 11482), as expected, were identified against hits from organisms belonging to the Arthropoda phylum, a number of hits were allocated to other taxa (**Figure 2.1**). More detail was given to hits belonging to the Crustacea clade, as shown in **Figure 2.2**, using the Integrated Taxonomic Information System classification (ITIS - www.itis.gov).

Regarding the biological pathways, to assess the function of the identified genes two different types of analysis were performed. The first was a homology Gene Ontology classification (GO). GO terms were assigned to 19,044 out of 28,680 transcripts (66.4% - **Figure 2.3**). GO refers to a standardisation of the gene functional classification system and it comprises three central categories: molecular function, biological process, and cellular components (**Figure 2.4**).

Afterwards, to understand the biological pathways throughout the transcriptome, a KEGG metabolic pathway analysis was performed. In total 2703 transcripts were associated with 44 KEGG pathways and the number of transcripts associated with each pathway ranged from a single gene up to 486 genes. The impacted pathways with the highest transcript numbers for each treatment are shown in **Table 2.3**.

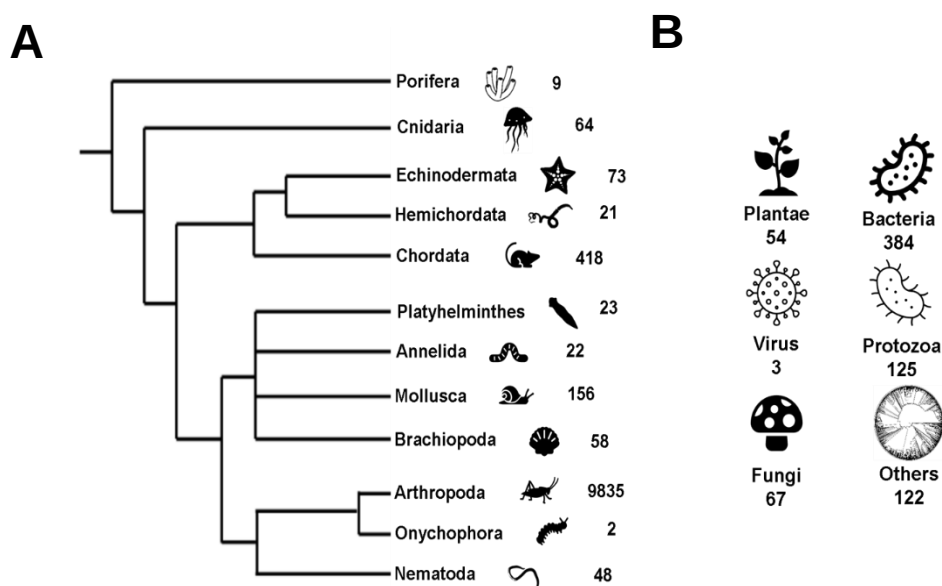


Figure 2.1- Resulting distribution of taxa homologues to the *Triops longicaudatus* transcripts. **A-** BLAST hits for Metazoa phyla. **B-** Hits for Plantae, Virus, Fungi, Bacteria, Protozoa and other non-specified taxonomic groups.

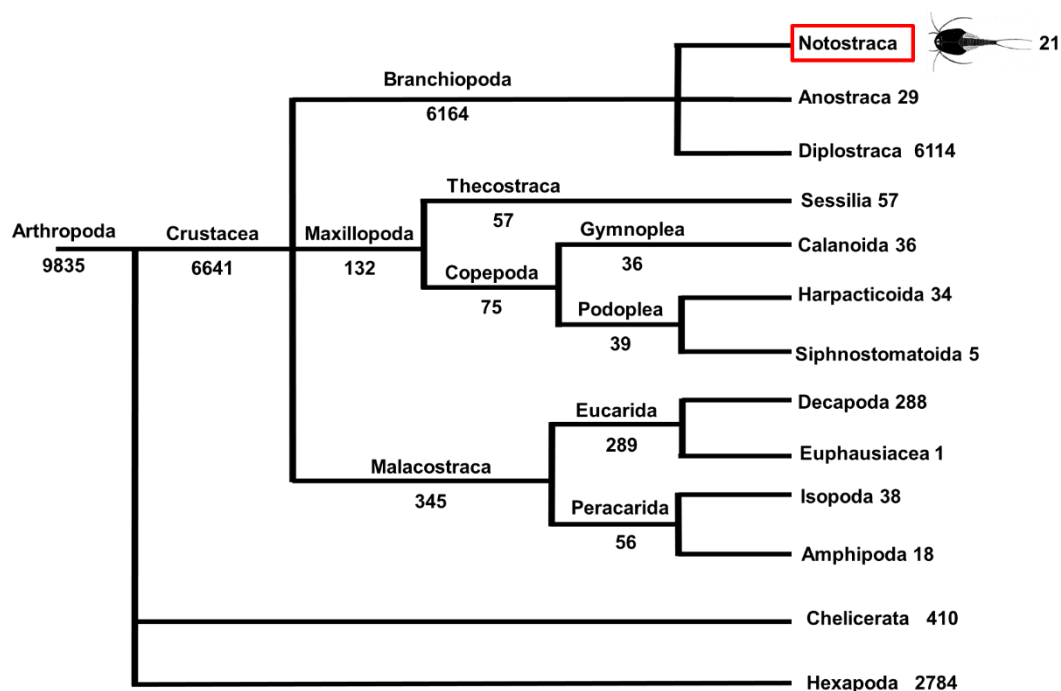


Figure 2.2-*Triops longicaudatus* transcript distribution through the Arthropoda phylum with special attention to the Crustacea sub-phylum. Number of transcripts shown beneath each separation, final count regards the class of the species. In red square is the Order to which *T. longicaudatus* belongs. The BLAST hits were with $1e^{-5}$ e-value within the NCBI database.

RNA-seq analysis for the 0.075µg/L exposure

At this concentration, no mortality was observed during the exposure, which probably is related to the short exposure time of the assay, especially taking into consideration that this concentration is near this species 96h-LC₅₀ for TBT. The most up-regulated gene identified had a Log₂FC= 9.8 and corresponded to the Cuticle protein 7 gene, which is a structural constituent of the cuticle. The most down-regulated identified gene, with a Log₂FC= -4.9 was the chymotrypsin elastase family member 2A. As expected, five out of the ten following up-regulated genes were also related to the structural constituents of cuticle.

Regarding the KEGG pathway analysis, it revealed that the up-regulated genes for this concentration were related to eight different pathways. In comparison, down-regulated were related to seven pathways, as shown in **Table 2.3**. Both the considered down-regulated and up-regulated genes showed a minimum of 10-fold decrease and increase, to the control. The results were divided into the respective GO classifications as shown **Figure 2.4A**.

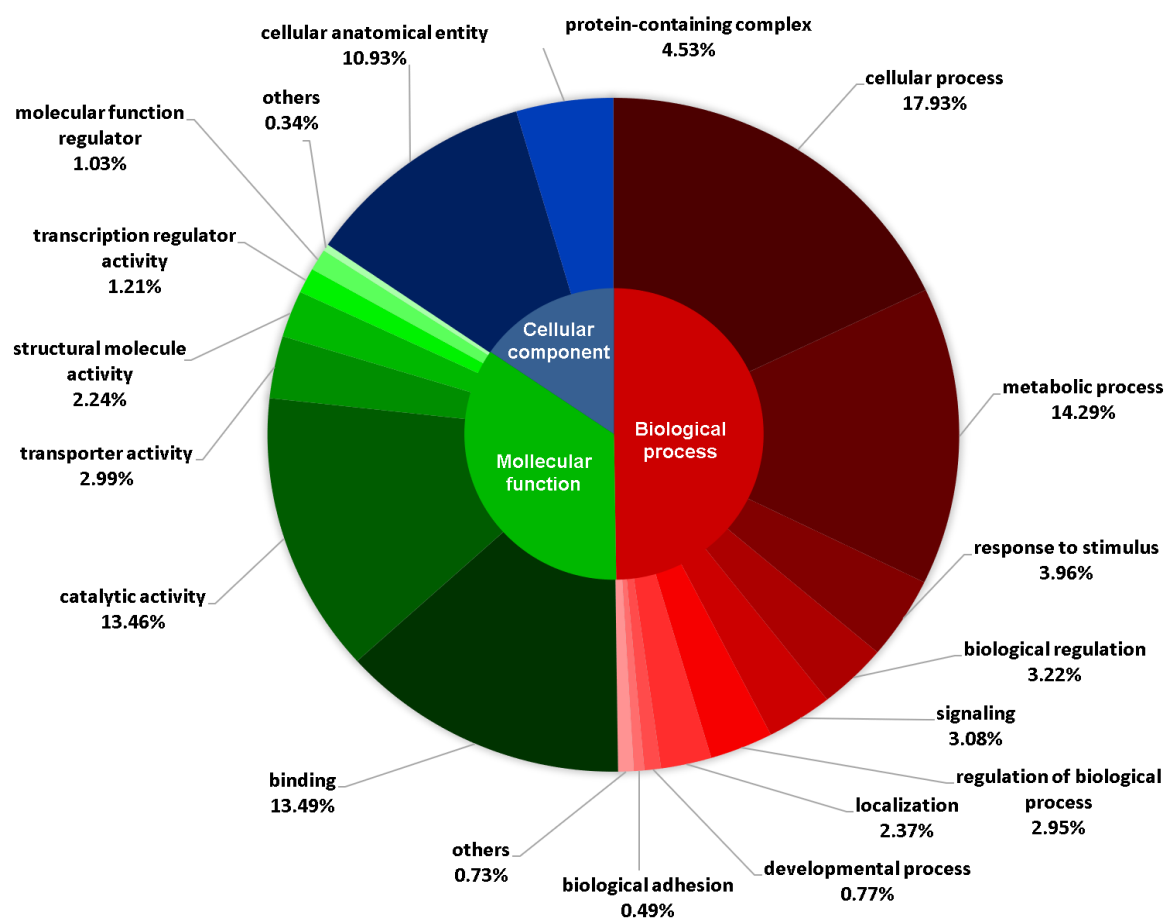


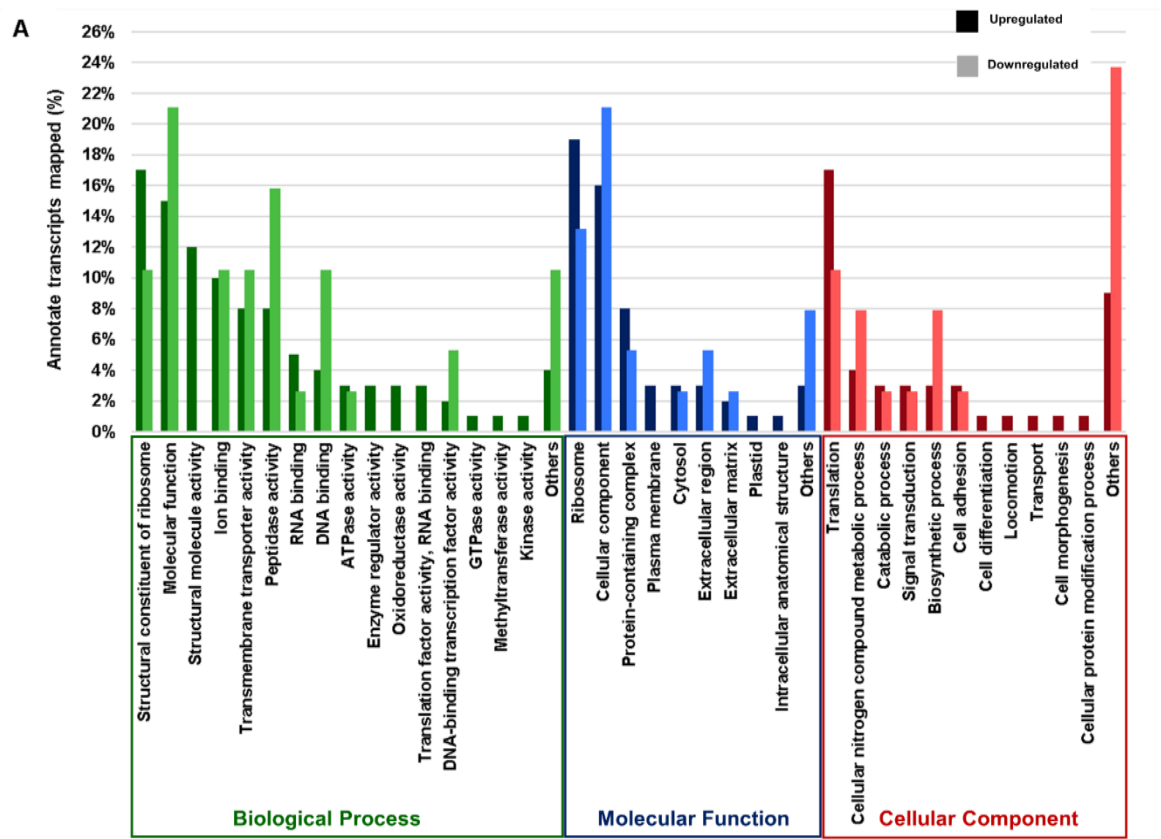
Figure 2.3- Distribution of Gene Ontology (GO) classifications of *Triops longicaudatus* into three general categories: Molecular function, Biological process, and Cellular components (central) and their respective subcategories (periphery).

RNA-seq analysis for the 0.25µg/L exposure

As in the previous treatment, no mortality was observed. In this treatment, the most up-regulated gene was once again Cuticle protein 7 with a slightly lower Log2FC= 7.19. Regarding the most down-regulated gene, it was the Serine protease 30 with a Log2FC= -5.57, a gene known to encode a protein responsible for cleaving trypsin substrates.

Regarding the KEGG analysis, this treatment presented major differences when compared to the 0.075µg/L treatment. The up-regulated genes were related to four pathways, while the down-regulated ones were related to two different pathways. Same as before, only genes with a differential expression of 10-fold were considered, which are presented in more detail **Table 2.1** and **Table 2.2**. Interestingly, several impacted pathways observed in the previous treatment are absent in this treatment, mainly related to the

biosynthesis of specific amino acids (Table 2.3). The results were divided into the respective GO classifications, as shown Figure 2.4B.



(Caption on the next page)

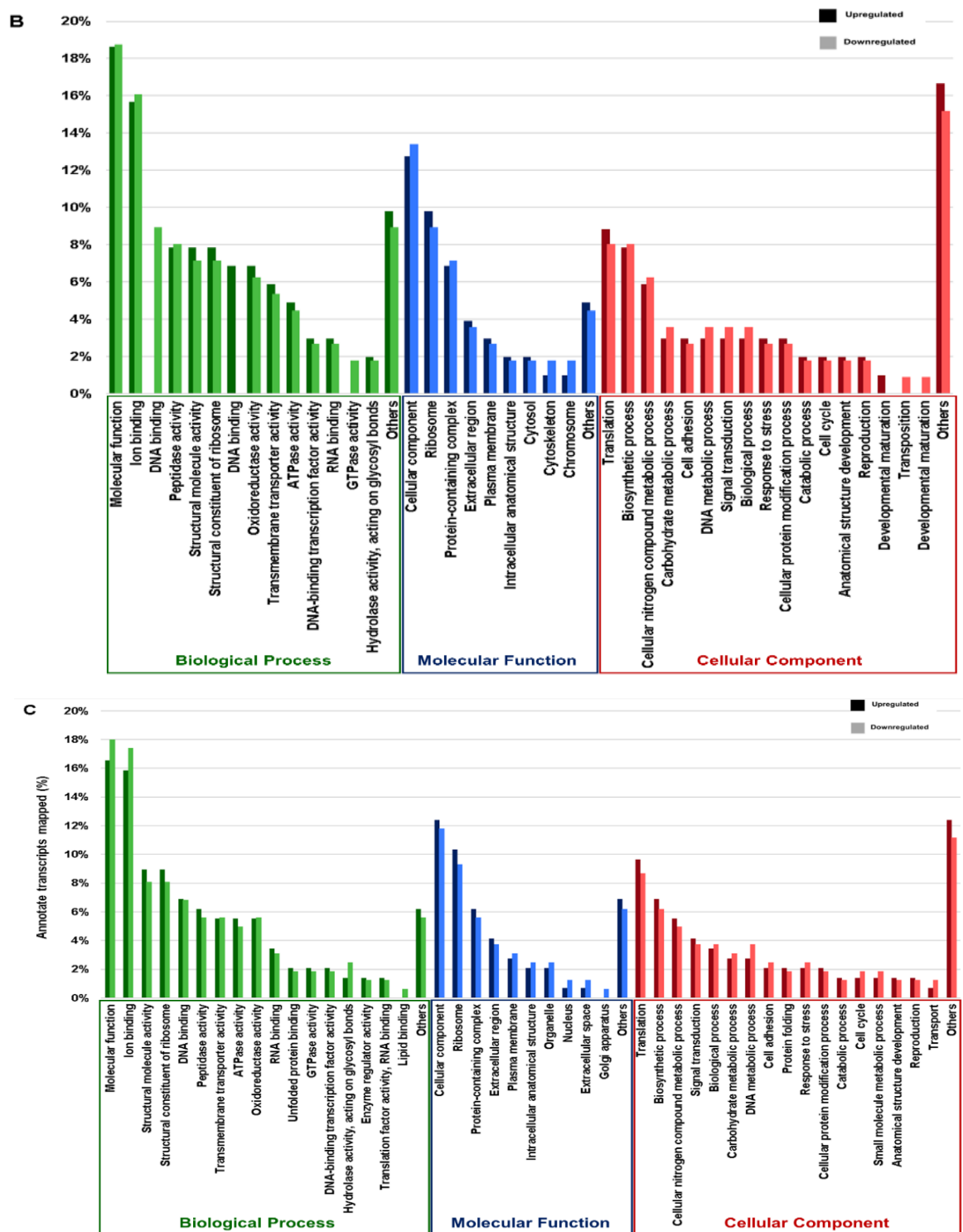


Figure 2.4- Percentage of GO annotation of transcripts of *Triops longicaudatus* regarding 10-fold up and down-regulated genes into three functional categories: Biological process, Cellular component and Molecular function and their respective subcategories. **A-** 0.075µg/L; **B-** 0.25µg/L and **C-** 1.54µg/L.

RNA-seq analysis for the 1.54µg/L exposure

As with the previous treatments, no mortality was observed. Due to the high concentration of this treatment (1.54µg/L) it is safe to assume that the exposure time was not enough to lead to lethal effects since this value, although ecologically relevant, is more than twice the value determined for the 96h LC₅₀. The most up-regulated gene for this treatment was also related to the organism's cuticle. The cuticle protein CP14.6 gene had a Log2FC= 6.24. Regarding the downregulated genes, histone H4 gene showed a Log2FC=-4.93 and Putative neural-cadherin 2 gene, responsible for encoding calcium-dependent cell adhesion proteins, with a Log2FC= -4.33.

Compared to the last two treatments, although having a greater number of differentially expressed transcripts, only one pathway was impacted for the down-regulated analysis and two pathways for the up-regulated ones. Purine and Thiamine metabolism remained common for the up-regulated section in all three concentrations. Regarding the down-regulation, only one pathway related to glycan degradation was impacted (**Table 2.3**). The results were divided into the respective GO classifications, as shown in **Figure 2.4C**.

RNA-seq comparison of different exposures

The most up-regulated genes of the three treatments were cuticle related and had the same associated GO term, meaning that all three had essentially the same function - structural composition of the cuticle of the organism. The down-regulated genes were less specific, with the most down-regulated gene of the 0.075µg/L being associated with a GO terms related to peptidase activity, the one from 0.25µg/L with serine-type endopeptidase and the one from 1.54µg/L related to DNA binding.

Overlapping differentially expressed genes between the three different exposures is shown in **Figure 2.5**. An accentuated increase of differentially up-regulated genes (Log2FC $\geq$ 3.3) was noted in the 0.25µg/L treatment having a total of 586 when compared to the 0.075µg/L treatment, which had only 426 and to the highest concentration, 1.54µg/L, which had a lower count of 386 genes from which the most relevant ones are presented in **Table 2.1**. For the under-expressed genes (Log2FC $\leq$ -3.3) the 0.075µg/L treatment had 460 genes, the 0.25µg/L treatment had 360, and the 1.54µg/L treatment had 482 genes from

which the most relevant ones are presented in **Table 2.2**. Regarding the attributed GO term for the impacted genes the 0.075µg/L treatment had a total of 52, the 0.25µg/L treatment had 72 and the 1.54µg/L treatment had the highest number with a total of 76, which could indicate a dose-dependency between TBTO concentrations and impacted genes

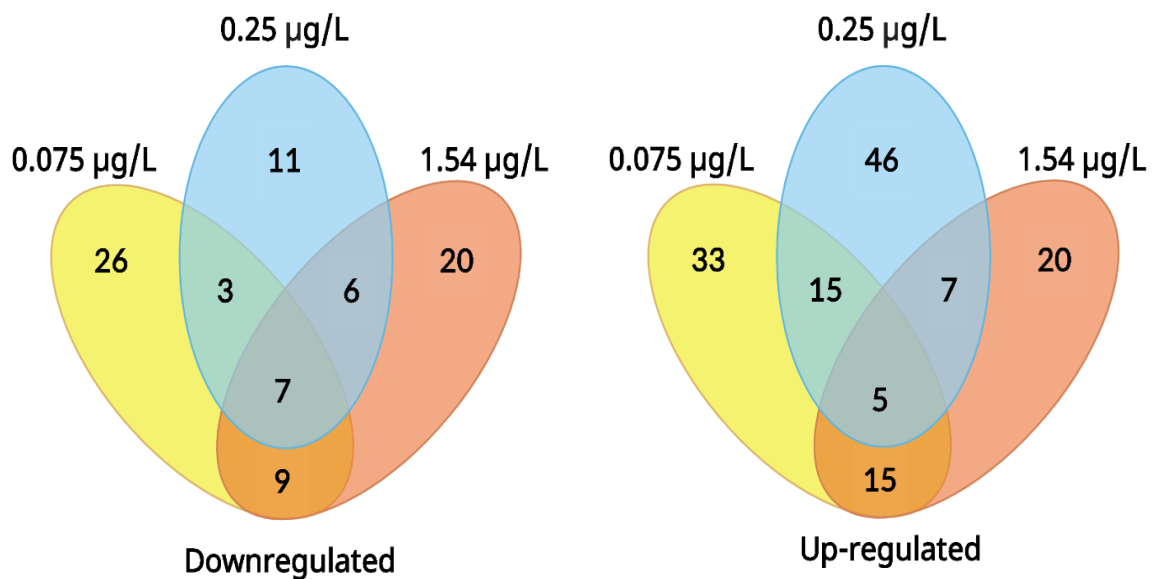


Figure 2.5- Venn diagrams showing the differentially expressed transcripts both unique and common between treatments. Down-regulated (left) and upregulated (right) transcripts for *Triops longicaudatus* exposed to TBTO at 0.075; 0.25 and 1.54µg/L.

Table 2.1- Most relevant up-regulated genes. Protein column refers to the name of protein followed by the abbreviation followed by the Log2FC value observed in each treatment.

Gene description	Acronym	Log2FC		
		0.075µg/L	0.25µg/L	1.54µg/L
Cuticle protein 7	CP7	9.826	6.610	3.664
Endocuticle structural glycoprotein SgAbd-2	ESG2	6.917	7.013	4.449
Eukaryotic translation initiation factor 5A	EIF5A	4.224	4.915	5.154
Myelin-associated glycoprotein	MAG	3.680	5.140	4.815
Rho guanine nucleotide exchange factor 7 isoform X1	ARHGEF7	3.621	4.324	3.878
Activating signal cointegrator 1 complex subunit 2 homolog	ASCC2	7.964	7.132	1.732
dmX-like protein 2	DMXL2	3.274	3.509	4.983
Down syndrome cell adhesion molecule-like protein	Dscam2	3.683	4.444	4.983
Sodium channel protein Nach-like	Nach	3.848	2.680	3.285
Dehydrogenase/reductase SDR family member 4-like	DHRS4	4.700	3.469	3.295
Multidrug resistance-associated protein 5	ABCC5	3.652	3.801	2.762
Heat shock protein 70	HSP70	3.300	3.128	3.573
Sulfite oxidase, mitochondrial	SUOX	4.395	2.623	4.696

Table 2.2- Most relevant down-regulated genes. Protein column refers to the name of protein followed by the abbreviation followed by the Log2FC value observed in each treatment.

Gene description	Acronym	Log2FC		
		0.075µg/L	0.25µg/L	1.54µg/L
Putative histone H2A	H2AC11	-1.168	-4.962	-4.865
Down syndrome cell adhesion molecule	Dscam	-3.835	-3.973	-3.879
Putative Chymotrypsin elastase family member 2A	CELA2A	-4.942	-0.417	-1.887
ubiquitin-60S ribosomal protein L40	UBA52	-4.317	-4.457	-4.362
cysteine proteinase 5 precursor	cprE	-3.428	-3.562	-3.471
vitellogenin fused with superoxide dismutase	VTG1	0.791	0.753	-3.710

Table 2.3- Impacted KEGG pathways consequent of down-regulated genes **(A)** and up-regulated genes **(B)** for each treatment indicated by the “X” in the respective column.

A

Down-regulated

	0.075µg/L	0.25µg/L	1.54µg/L
Purine metabolism	X	X	
Thiamine metabolism	X	X	
Cysteine and methionine metabolism	X		
Glycine, serine, and threonine metabolism	X		
Valine, leucine, and isoleucine biosynthesis	X		
Other glycan degradation			X

B

Up-regulated

	0.075µg/L	0.25µg/L	1.54µg/L
Purine metabolism	X	X	X
Thiamine metabolism	X	X	X
Glyoxylate and dicarboxylate metabolism	X		
Tryptophan metabolism	X		
Glycerolipid metabolism		X	
Drug metabolism - other enzymes		X	

2.4. Discussion

The short exposure time of this study allowed us to understand and determine expression changes that occurred at high concentrations. This approach provides information that would normally be unavailable since the high concentrations will lead to the death of the organism and with it the possibility to assess the response of the organism to the chemical. The main objective of this study was to understand the transcriptomic changes consequent of TBT exposure. Both abnormal gene expression and toxic effects have already been documented for other species of the Crustacea subphylum, although the focus was more directed to lipid homeostasis and detoxification proteins (Jordão *et al.*, 2015; Oberdörster *et al.*, 1998). Although the main mode of action of TBT is related to endocrine disruption, several other toxicological impacts take place and have not yet been fully elucidated. In this work, evidence was shown of several other pathways directly and indirectly impacted by TBT, such as impaired cellular transport and neuronal and visual development.

All three treatments shared genes between each other, and a portion was common to all three, possibly indicative of the more common responses to stressful situations such as translation initiation factor, ribosomal protein subunits and guanine nucleotide exchange factor for the up-regulated genes. Although another transcriptome analysis was performed for *T. longicaudatus*, to the best of our knowledge, none obtained a representation above 95% of the core genes for the Arthropoda phylum. An explanation for the majority of the transcripts top-hits being associated with the Diplostraca Order instead of the Notostraca Order (to which *T. longicaudatus* belongs) could be the superior representation that species in this order possess. Belonging to the Diplostraca Order is the *Daphnia* genus which is for decades one of the most used genera for model-organism in aquatic ecotoxicological experiments. This popularity increases the amount of information and entries in databases which consequently will lead to a greater amount of similarity. Even so, this significant similarity can lead to homology comparison between these species which in turn could lead to a better understanding on a toxicological level for freshwater crustaceans in general. The resulting reference transcriptome includes more information than other already published transcriptomes from the genus *Triops* (Seong *et al.*, 2016).

Genetic impact

Up-regulated genes

As it is well documented, TBT is a known agonist of the retinoid X receptor (RXR - Le Maire et al., 2009). Although the ecdysone receptor (EcR) is not directly activated by TBT, its heterodimerisation with RXR could lead to an indirect activation (although this would only be possible in the presence of ecdysteroid). In the study of Abdullah-Zawawi *et al.* (2021) the group pointed out that the activation of this transcriptional factor played a central role in the moulting process leading, among others, to upregulation of genes regulating cuticle release. This could explain the fact that the most up-regulated genes in all treatments were associated or related to cuticle proteins such as cuticle protein 7, cuticle protein CP14.6 or endocuticle structural glycoprotein SgAbd-2-like, indicating a disruption in the moulting process which is corroborated by the increased number of moults found during a preliminary mortality test (data not shown). Although no significant upregulation was found for either receptor, an upregulation of the Dehydrogenase/reductase SDR family member 4 gene (DHRS4) was found. This protein is of utmost importance as its main function is the reduction of all-trans-retinal and 9-cis retinal molecules, the latter being one of the most well documented natural ligands of RXR. Therefore the upregulation of this gene could be in response to an intracellular excess of the natural ligand as these molecules have the highest affinity to the RXR protein (R. M. Evans & Mangelsdorf, 2014). This increase in DHRS4 could also translate into a specific response of the cell to the TBT contamination since its binding pocket is the same as the natural ligand, this way the cell could be trying to reduce the quantity of active RXR. Due to the different chemical properties of the two molecules, it is safe to assume that the reduction or oxidation of TBT by DHRS4 is probably less efficient.

The myelin-associated glycoprotein (Mag) gene was also found up-regulated in all three treatments. This protein has an important function in protecting axons from myelin degeneration and preventing apoptosis of neuronal cells, so an increase of these molecules might indicate neurological damage consequent of the exposure. Lagadic *et al.* (2017) hypothesised that the neurotoxicant potential of TBT could be associated to abnormal secretion of the neuropeptide APGWamide in molluscs. Although no direct evidence of an increase of this neuropeptide was found in our study, a slight upregulation (Log2FC of 0.884) of a similar neuropeptide receptor, neuropeptide CCHamide-1 receptor was found. Also related to neural events is the Down syndrome cell adhesion molecule-like protein (Dscam2) gene. The expression of this gene could be correlated with the concentration of TBT as the Log2FC started at 3.683 with the 0.075µg/L,

increased to 4.444 in the 0.25µg/L treatment and increased even more in the final concentration reaching a Log2FC of 4.983. This cell-surface protein is expressed in the nervous system of several species and this isoform is particularly known for maintaining axon tiling between the same cell type such as the visual system (Millard *et al.*, 2007). Unfortunately, since a whole-body RNA extraction was performed, it becomes nearly impossible to pinpoint what type of cell produced the upregulation of this gene. In line with effects to neurons is the upregulation of Sodium channel protein gene (Nach). These proteins are responsible for the regulation of neuronal excitability and, therefore, the correct electric impulse transmission, thereby an imbalance in these proteins could translate into inefficient neurotransmission throughout the organism (Hwang *et al.*, 2011). The upregulation of Multidrug resistance-associated protein 5 (ABCC5) could be linked with cellular detoxification, as proteins from this family have been shown to mediate the efflux of TBT molecules (Jha *et al.*, 2000).

The down-regulation observed in the highest concentration could be related to a high level of cytotoxicity that reduced protein biosynthesis in general since the same trend is observed for several other genes. The occurrence of genotoxicity consequent of TBT exposure has already been documented for insects and molluscs (Jha *et al.*, 2000; Morales *et al.*, 2013). The activating signal cointegrator 1 complex subunit 2 gene (ASCC2) was significantly up-regulated in the 0.075µg/L (Log2FC= 7.964) and 0.25µg/L (Log2FC= 7.1322) treatments. The same gene was found up-regulated in the 1.54µg/L treatment, although not in the same proportion (Log2FC= 1.79). ASCC2 is known to be an important component of the ASCC complex, which in turn is crucial in the repair of alkylated DNA (Soll *et al.*, 2018). TBT's genotoxic potential has already been reported in crustacea spermatozoa (Rani *et al.*, 2015). In this previous work, genotoxicity was mainly associated with oxidative stress damage. In our study, the upregulation of ASCC2 could be indicative of genotoxicity resulting from DNA subjected to alkylating agents.

The E3 ubiquitin-protein ligase (HERC2) specialises in regulating ubiquitin-dependent repair proteins that target damaged chromosomes and presented a 22.5-fold. Interestingly, this gene appeared up-regulated only in the 0.25µg/L treatment.

Although not much information has been revealed regarding the DmX-like protein 2 (DMXL2) it is known that it plays an important role in notch signalling primarily by regulating acidification of intracellular components (Faronato *et al.*, 2015). The acidification is usually achieved by increasing levels of Ca²⁺, which can lead to the collapse of the cellular membrane potential and overall viability. Since in our study, the up-regulation of this gene suffered a gradual increase with the concentration (**Table 2.1**) it is safe to assume that increased concentrations of TBT could be related to increased intracellular Ca²⁺, which may cause cell membrane instability (Lagadic *et al.*, 2017).

The remaining genes represented in **Table 2.1** are most likely associated with generic proteins produced during stressful events, such as heat shock protein 70 (HSPA1A), cytochrome P450 or sulfite oxidase.

Downregulated genes

It becomes evident that the down-regulated genes were in lower number than the up-regulated ones. The most down-regulated gene was serine protease 30-like (Prss30), which had a Log2FC= -5.572 equivalent of a fold change of 47.5, which is dwarfed next to the highest up-regulated gene, which had a fold change of 907.51 (Log2FC= 9.826). The Histone H2A gene (H2AC11) was significantly down-regulated in the two higher treatments, although in the first treatment, the Log2FC was only -1.168 it decreased drastically in the 0.25µg/L treatment demonstrating a Log2FC= -4.962 and keeping a similar value of -4.865 in the 1.54µg/L treatment. This group of histones represent the core components of nucleosomes and are known to mark damaged DNA sites while also recruiting proteins responsible for the repair of those sites. These proteins are thereby one of the quickest repair responses to double-strand DNA breaks (Yuan *et al.*, 2010). A reduction of the production of these proteins could thereby be related to an increase in DNA damage mainly through double-strand breaks.

Also related to genotoxicity is the excessive presence of reactive oxygen species. As mentioned before, Rani *et al.* (2015) observed an increase of oxidative damage in DNA mainly caused due to a reduction of the activity of superoxide dismutase (SOD). In their work, the reduction, although not significant, started at 1mg/L and significantly declined with higher concentrations. Our work demonstrated that TBT reduces the expression of the Vitellogenin fused with superoxide dismutase (VTG1) at the 1.54µg/L concentration, which is 1000x lower than the one used by Rani *et al.* (2015). This reduction of the number of superoxide dismutase associated to a genetic down-regulation could cripple the immune system of the organism leaving it vulnerable to oxidative damage.

The expression of the chymotrypsin-like elastase family member 2A gene (CELA2A) was also significantly decreased in the 0.075µg/L treatment presenting a Log2FC= -4.942, the expression increased slightly in the other treatments being -0.417 and -1.887 for 0.25µg/L and 1.54µg/L, respectively. Since the main function of this protein is to secrete and degrade insulin, a down-regulation might be associated with abnormal glucose metabolism and energy allocation, which could have major impacts on a variety of biological processes such as development, growth and/or reproduction.

Down syndrome cell adhesion molecule gene (Dscam) was down-regulated in all three treatments with a similar value of Log2FC (-3.835 for 0.075µg/L; -3.973 for 0.25µg/L; -3.879 for 1.54µg/L), unlike the Dscam2 isoform mentioned in the section above, Dscam proteins have a more generalised function being responsible for self-avoidance of dendritic arborisation ensuring that no obstruction happens within ramifications of the same cell (Millard & Zipursky, 2008). The down-regulation of this gene could translate into neuronal disorganisation and less distributed dendritic arborisation, which in turn affects the efficiency of synapses and neurotransmission.

Worth mentioning is also the down-regulation of the Cysteine proteinase 5 gene (cprE). The down-regulation of cprE was similar in all three treatments showing a Log2FC variation from -3.562 to -3.4278 (**Table 2.2**). Since the main function of this protein is intracellular proteolysis assigned to immune response, it is possible to infer that exposure to TBT leaves the cell more susceptible to cytotoxicity since unwanted molecules such as defective proteins are not metabolised (Combaret *et al.*, 2016).

2.5. Conclusion

This work provides genetic information which could be considered valuable insight for further research on the often-neglected Crustacea sub-phylum. This molecular information could be used to assess genetic diversity and comparative genomics with other species as high levels of similarity were found with other members of this group.

The second objective of this study provided specific information on altered pathways consequent of TBT contamination. The differential gene expression was found to affect different sections of the organism leading to cytotoxicity, neurotoxicity, genotoxicity and the most accentuated one, endocrine disruption. Although no tissue-specific evaluation was performed, our results could prove crucial to bridge molecular changes to major effects on higher organisation levels such as populations.

2.6. References

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3. Chapter III: Does it move like Jagger: Behavioural changes of *Triops longicaudatus* exposed to Tributyltin

Abstract

In the 60's, tributyltin (TBT) was added to the hull paint to prevent biofouling, which would reduce vessels velocity. Due to its toxicity towards aquatic species and high persistence in the environment, TBT was banned in several countries, but measurable concentrations are still found in the environment. Although TBT's lethal effects have been extensively documented, much remains to be studied about its possible sub-lethal and early toxicity effects.

This study presents changes in the behavioural pattern of the freshwater crustacean *Triops longicaudatus* elicited as a consequence of an acute exposure to four different concentrations of TBT-oxide (TBTO), 0; 0.075; 0.25 and 1.54µg/L. The organisms were recorded with a video tracking system, and the recordings were afterwards analysed with *Statistica*. Five different parameters were used for the analysis, and a self-organising feature map detected six distinct behaviours across the four treatments. After analysing the behaviours, it became clear that all three TBT treatments affected the organisms since all the behaviours assigned to the treatments were different from the one assigned to the control. The swimming activity of 0.075µg/L could be described as hyperactivity, 0.25µg/L would be described as hypoactivity, and 1.54µg/L reverted to hyperactivity. Crossing this information with a previous study performed on the genetic expression led to the conclusion that TBT, at least partially, impairs electric impulses, leading to discoordination and loss of awareness.

Keywords: Video tracking, Tributyltin Oxide, Behaviour, Neural network, Crustacea.

3.1. Introduction

Organotins such as tributyltin-based compounds (TBT), although banned in several countries since 2008, are still a problem due to unregulated or illegal use that urgently needs to be dealt with (Anastasiou et al., 2016). This group of organotins was mainly used as paint stabilisers to avoid biofouling of vessel hulls, which reduces its speed and increases transportation costs in maritime trade (S. M. Evans, 1999). The chemical properties of the TBT molecule allow for its release from the paint due to erosion, thus becoming available on the water body and sediments (Berto et al., 2007). As a result, TBT will impact several non-target species in the ecosystems it is introduced (Berto et al., 2007). Examples of the more documented effects of TBT are induction of imposex and shell malformation in invertebrates or impairment of the immune metabolic activity and reproduction in vertebrates, which can eventually lead to the organism's death (Batista et al., 2016; Boyer, 1989). Besides the more obvious mortality endpoint, TBT is able to induce sub-sublethal effects that are often overlooked since these effects may not be directly observed (Hook & Fisher, 2001). Still, sublethal effects should not be disregarded as they could lead to the organism's death through indirect measures such as fitness reduction, predation or starvation (Little & Finger, 1990).

Among sub-lethal endpoints, behaviour is very sensitive and useful to develop biological early warning systems for toxicant exposure (Oliva Teles et al., 2015). Behavioural analysis has a non-evasive nature and can contribute to a more extensive understanding of the chemical's mode of action on a given organism even at residual concentrations (Rodrigues & Pardal, 2014). Furthermore, though behaviour is a response measured in individuals it has an impact in the population (more ecologically relevant), linking these two levels of biological organisation. Locomotion is an example of a vital behavioural response. It is essential for several other behaviours such as predation, evasion and, mating, which are vital for survival of the organisms and species fitness (Oliva Teles et al., 2015). Evaluation of locomotion could also be combined with biochemical and physiological endpoints useful for biomonitoring of chemical toxicity (Bae & Park, 2014). This procedure is used mainly with vertebrates, such as fish however, new species from different taxa have been evaluated as suitable test models (Scott & Sloman, 2004). The small freshwater crustacean *Triops longicaudatus* can become an additional suitable species. *T. longicaudatus* characteristics make it an appropriate model species for ecotoxicological experiments. Namely, their small size, rapid development, asexual reproduction, short life cycle, and intermediate position in the food web (Scholnick, 2016). Additionally, these organisms possess a fast metabolism linked to constant

movement, which may be a key aspect for its sensitivity to toxicants and provides an additional advantage for behavioural analysis.

The present study aimed at understanding the effects of a short-term exposure to TBT on the behaviour of *T. longicaudatus*, evaluating its adequacy as a model species for behavioural tests and increasing the information available about the adverse effects of TBT on aquatic invertebrates and its risk assessment.

3.2. Methodology

Organisms

Triops longicaudatus organisms used in this study were obtained from laboratory cultures kept for several months at Faculdade de Ciências da Universidade do Porto (FCUP-UP). The individuals were maintained in a large aquarium (40L) with approximately 1.5cm of depth of natural sand (SwellReptiles, UK) and kept at 25 ± 1 °C, with a 12h:12h (light:dark) photoperiod. Nauplii were fed *ad libitum* with powdered food rich in chlorophyll (3-Algae flakes from Tropical®) and adults fed daily with fish granulate (Vitality & Color from Tropical®). Water was renewed weekly, and adults were changed into new aquaria when the recovery of laid cysts was required. The sand was then air-dried in a room at 25 ± 1 °C with high relative humidity to allow for desiccation of the resistance cysts. The sand was left humid for at least three days and afterwards stored at room temperature in a dry place.

Ecotoxicological assay and Video recording setup

For the behavioural test, four TBT treatments were selected: 0; 0.075; 0.25 and 1.54µg/L. The concentrations tested were selected based on values found in the literature for environmental samples of water and sediments. The lowest concentration (0.075µg/L) was an approximation of the previously calculated 96h-LC₅₀ value for this species in this life stage. The 0.25µg/L concentration was used in a previous study performed by our group so comparisons could be made with the results obtained for other species. The highest test concentration represents the highest level that has been detected in the environment (Sayer, Hoare, Simpson, Henderson, & Liptrot, 2006)

A TBT-Oxide (TBTO) stock solution of 1.54µg/L concentration was made using dechlorinated water. Dilutions from the stock solution were made to reach the remaining test concentrations (0.075 and 0.25µg/L). To avoid any adsorption to the walls of the

container during the experiment, 220mL of each solution were put into the respective container prior to the experiment, the solution was then renewed before the start of the experiment. The organisms used in the experiment were seven days old with approximately 1.5 ± 0.5 cm length. Ten organisms were transferred to a plastic container filled with 220mL of each test solution and left for 90 minutes. This process was repeated for all treatments. The exposure time was the same used in Chapter II, to allow for an easy comparison with the results previously obtained. The exposure was done in a temperature-controlled environment with $25 \pm 1^{\circ}\text{C}$.

After exposure, the organisms were individually transferred into small circular plastic (PVC) arenas (5 cm in diameter x 5cm in height), with a net, to reduce handling stress where their locomotory behaviour was recorded. The dimensions of the plastic arena allowed for free swim both horizontally and vertically. Each arena was filled with 30 mL of the respective treatment solution. Overall, the video-tracking system employed in the recording of the locomotory behaviour was composed of three cameras (wirelessVelleman Camset W7 cameras) each filming 12 arenas (**Figure 3.1**), and two video recording sessions were done, resulting in a total of 72 arenas and 18 replicates per treatment. Each set of 12 arenas was made of a replicate of each experimental treatment. In each video session, behaviour was recorded for 20 min. The recording area was artificially illuminated with white led lights and arranged to ensure homogeneous dispersion of the light. The cameras transmitted the video to Velleman 2.4 GHz Wireless receivers which in turn stored the data on an Intel®Pentium®Dual CPU (2:00 E2180@2.00 GHz, 1.87 GB RAM) computer with Microsoft Windows XP Home Edition (Service Pack 3, version 2002). The capturing software used was DSS1000 2004 (v 4.7.0041).



Figure 3.1- Circular arenas containing exposed organisms. Each area contained 12 circular arenas to which the different treatments were randomly assigned. In each area all treatments were tested in triplicate.

Behaviour data analysis

For the analysis of the video tracking recordings, an adapted algorithm was used for automatic tracking of locomotory behaviour. The base was the Biotracker[®] algorithm (Leibniz Institute of Freshwater Ecology and Inland Fisheries), which was adapted and refined to the detection of multiple arenas and to automatically calculate and extract into a spreadsheet various locomotory variables for further analysis by Artificial Neural Networks (ANN). This algorithm automatically tracks and displays the trajectory of each organism, its movements and travelled distance, as well as speed and acceleration, within each arena. For the automatic behaviour tracking, the videos were optimised for contrast prior to the analysis, using the adaptations introduced in the algorithm to make sure the organism could be constantly distinguished from the background. Furthermore, the time interval for the detection of the position of the organism was set to 0.8 seconds, and the maximum jump allowed within the detection of the trajectory of the organisms was 20mm. The video analysis is composed of three steps: pre-processing, trajectory detection and post-processing. The first stage corrects aspects of the video such as

distortion, which usually appears in the periphery, and contrast correction prevents the algorithm from detecting shadows. The second stage detects the number of different arenas, defines the boundaries of each arena, and maps the trajectory of the organism, relying on a frame-by-frame colour variation. The final stage compiles the information from the tracking done, in example, trajectory, mean velocity, and travelled distance and converts it into data that can afterwards be exported into a spreadsheet: Y coordinate, the position of the organism in the Y axis; X coordinate, the position of the organism in the X axis; mean velocity (mm/s), distance, total distance, angle, total mean velocity, 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/s²). Further, the algorithm also provides a verification video, which serves for validation of the results of the automatic tracking. Finally, the data obtained is compiled and organised in an Excel sheet to be used in subsequent statistical analysis.

Statistical analysis

To account for an initial adaptation to the arena of the video-tracking system, the first and last five minutes of recording were discarded from the analysis. The data analysis was based only on variables describing the organism's movement. In addition, variables showing 100% correlation (Pearson correlation) with other movement variables were excluded from the analysis (e.g. angle, total distance, mean angular velocity). Variables kept, in the final analysis, were the distance to the centre of the arena (distance to centre, mm), instantaneous velocity (mm/s), instantaneous acceleration (mm/s²), angular velocity (degrees /s) and meander (degree/mm). Analysis of Variance (ANOVA) followed by the Tukey HSD test was first performed to investigate a possible effect of TBT on these variables. A cluster analysis made from an unsupervised artificial neural network (unsupervised learning) based on the Kohonen algorithm, called Self-Organizing Feature Maps (SOFM) or Kohonen Networks, was subsequently done to define six locomotion behaviours from the whole dataset. A Correspondence Analysis (CA) was then performed with the frequency of occurrence of the six behavioural types identified across the TBTO treatments. One-way ANOVAs, followed by the Tukey HSD test, were also used to investigate differences in the means of each locomotion variable across the six behaviour types identified. All statistical analysis were performed using the program Statistica (V.13.4.0.14).

3.3. Results

No mortality was observed during the entire duration of the experiment. The tracking software provides a visual graph for each of the detected arenas and maps the trajectory taken by the organism (**Figure 3.2**). The results of the one-way ANOVAs performed to investigate differences among treatments in the locomotion variables are presented in **Table 3.1**. Significant differences among treatments were found for the variables distance to centre and instantaneous velocity. Instantaneous velocity was significantly higher (+42.3%) in the 0.075µg/L TBTO treatment than in the control group (**Figure 3.3A**). The distance to the centre of the arenas was significantly lower (-8.73%) in organisms exposed to 0.25µg/L TBTO than in the control organisms (**Figure 3.3B**). In the remaining TBTO treatments the distance to the centre was similar to that of controls organisms.

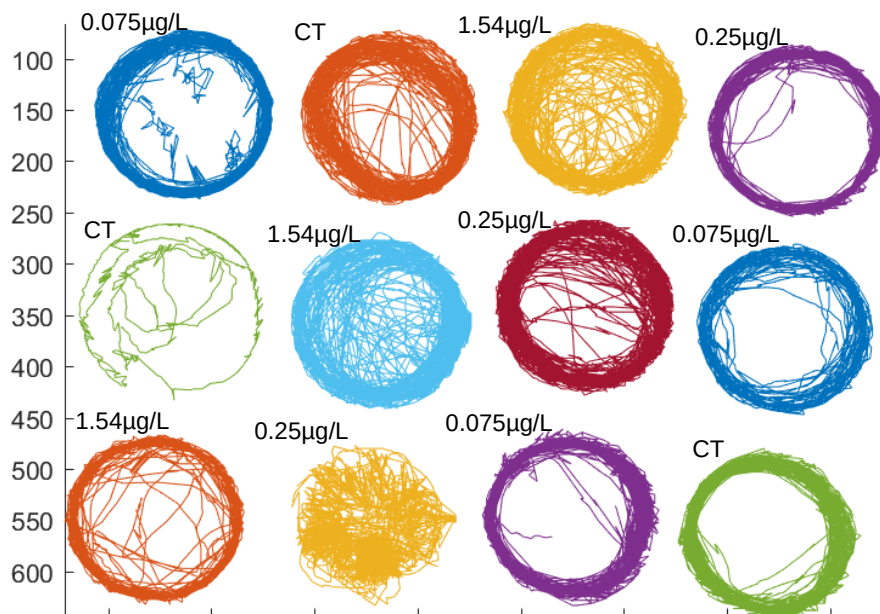


Figure 3.2- Automatic tracking of the behaviour of 12 independent *Triops longicaudatus* in the circular arenas. Each treatment is depicted at the upper left corner of the circle.

Table 3.1- Results of the One-way ANOVA performed to investigate the effects of TBT on each of the locomotion variables analysed.

Dependent Variable	Degrees of freedom	F-value	P-value
Distance to centre	3, 68	3.238856	0.027389
Instantaneous velocity	3, 68	4.009819	0.010911
Instantaneous acceleration	3, 68	0.607226	0.612585
Angular velocity	3, 68	0.264553	0.850705
Meander	3, 68	1.161043	0.331077

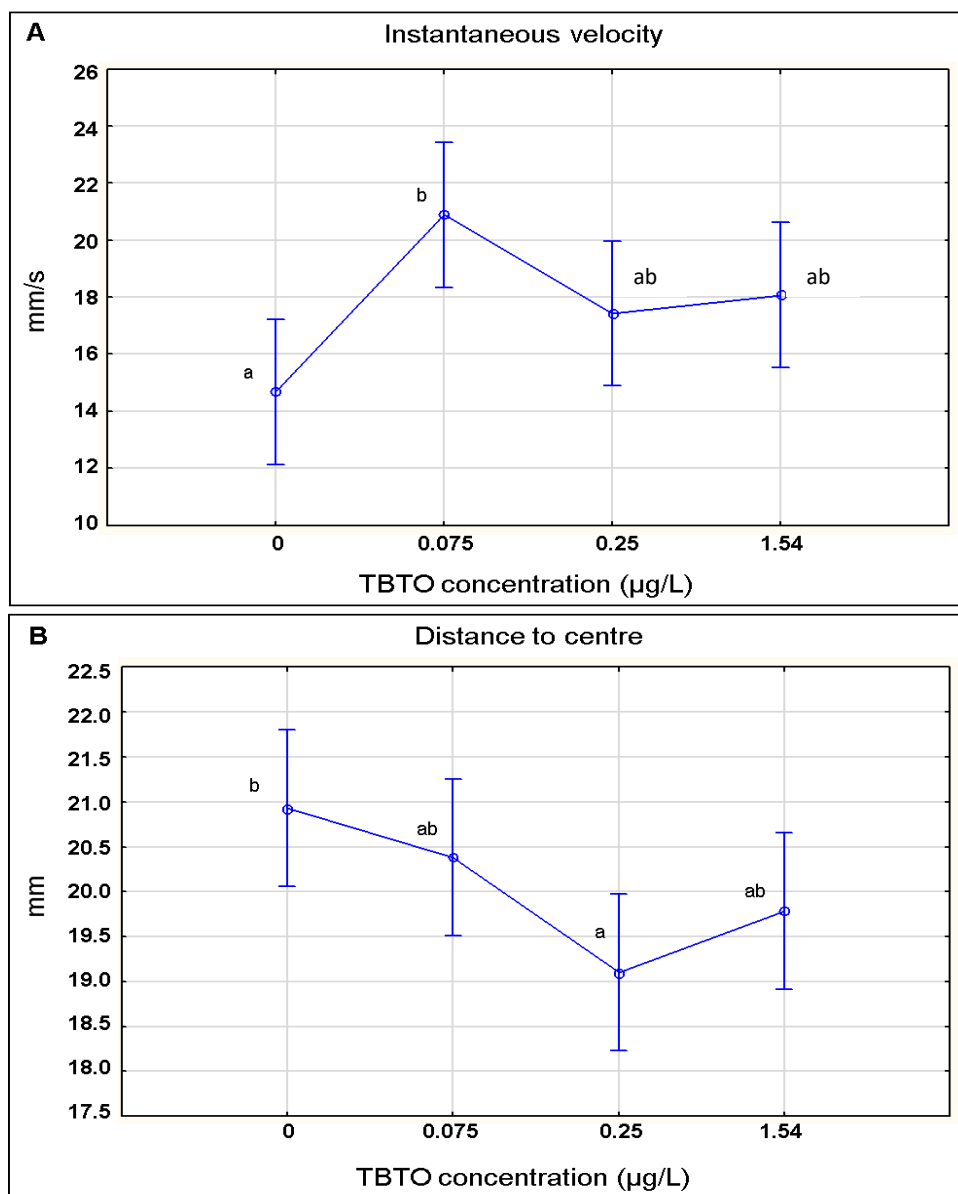
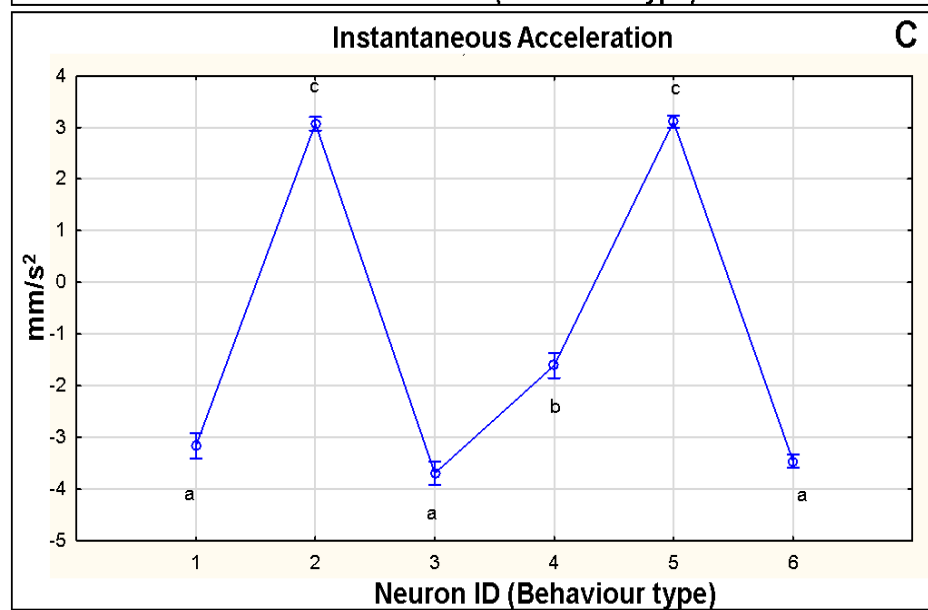
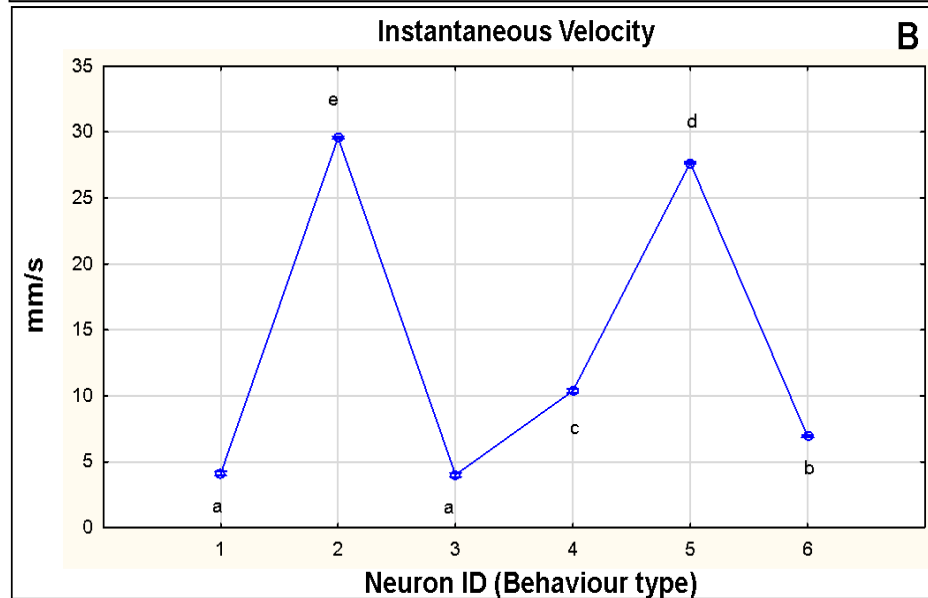
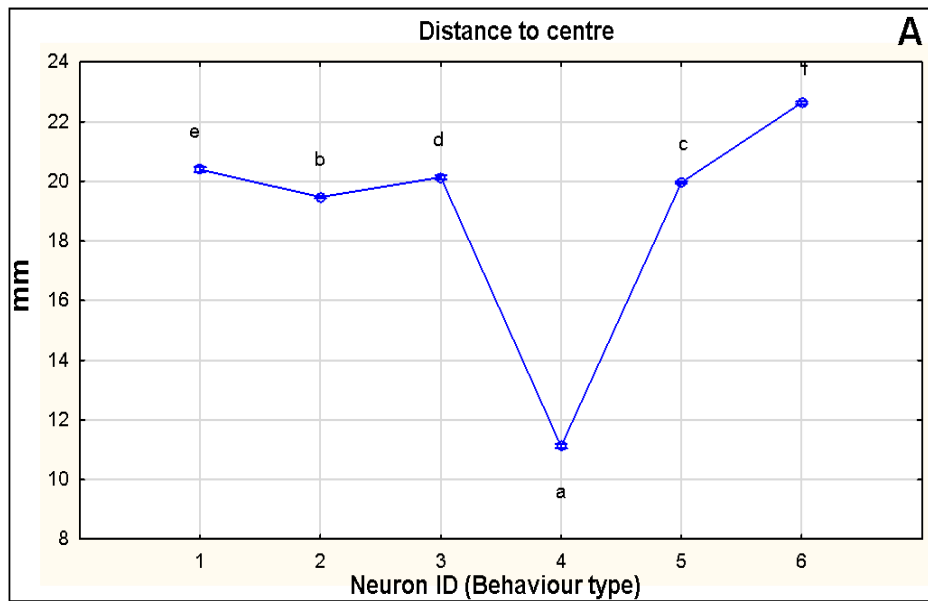


Figure 3.3- Behaviour variables exhibiting significant differences among TBTO treatments. Different letters identify statistically significant differences between groups as indicated by the Tukey HSD test.

Six behaviour types were identified by the Cluster Analysis based on the Kohonen algorithm (**Table 3.2, Figure 3.4**). Significant differences for the contribution of each locomotion variable to the six behaviour types identified were found with the Correspondence Analysis (Chi-square = 6211.5, df (degrees of freedom) = 24, $p < 0.0001$). Differences among behaviour types found for each locomotion variable are shown in **Figure 3.4A-E**. Significant differences in the distance to centre were found for all behaviour types. In particular, organisms exhibiting behaviour type #2 were on average closer to the centre of the arenas, and those exhibiting behaviour #4 were swimming more on the periphery, than those exhibiting the remaining behaviours (**Figure 3.4A**). Organisms exhibiting behaviours #2 and #5 showed highest instant velocity and those exhibiting behaviours #1, #3 and #6 showed the lowest instantaneous velocity. A similar pattern was found for instantaneous acceleration. Organisms exhibiting behaviours #2 and #5 showed the highest values of instantaneous acceleration and those exhibiting behaviours #1, #3 and #6 showed the lowest values, as indicated by the homogeneous groups returned by the Tuckey HSD test (**Figure 3.4C**). Differences in angular velocity were also found among all behaviour types; the highest values were found for behaviours #3 followed by #4 and the lowest values observed in behaviours #1 followed by behaviour #2. The meander also differed significantly among behaviours, with the highest opposing differences observed for behaviours #2 and #3 (**Figure 3.4E**).

Table 3.2 - Contribution of each locomotion variable for the six behaviour types identified, as indicated by the results of the Correspondence Analysis. The intensity of the shade relates to a higher (green) or lower (white) contribution. The six types of behaviour were identified by a Cluster Analysis based on the Kohonen algorithm.

Variables	Behaviour type					
	# 1	# 2	# 3	# 4	# 5	# 6
Distance to centre	0.815	0.858	0.511	0.473	0.824	0.497
Instantaneous velocity	0.659	0.067	0.148	0.572	0.447	0.592
Instantaneous acceleration	0.815	0.607	0.448	0.420	0.717	0.494
Angular velocity	0.422	0.052	0.515	0.193	0.439	0.502
Meander	0.756	0.259	0.445	0.142	0.418	0.495



(Caption on the next page)

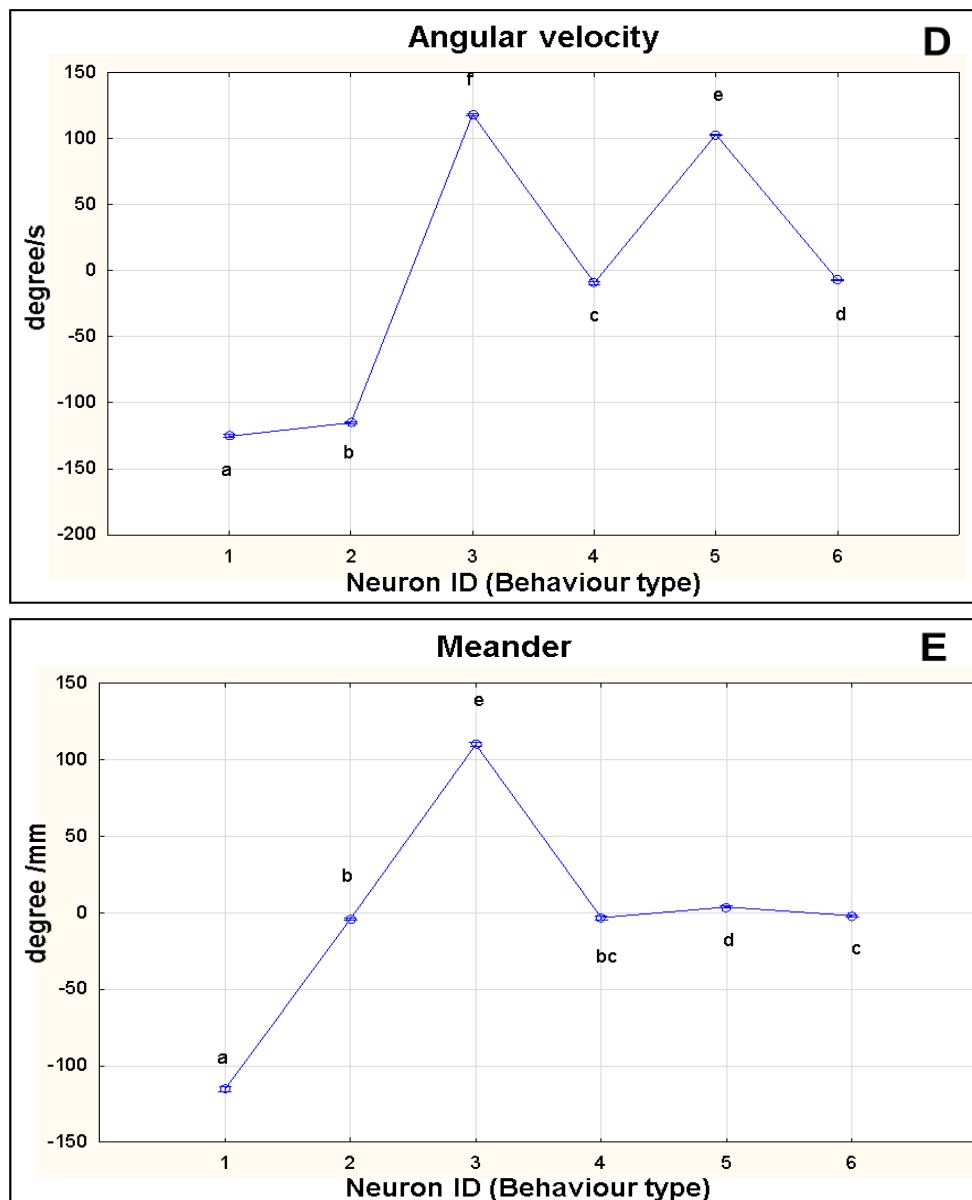


Figure 3.4- Differences in the locomotion variables found for each behaviour type identified. Different letters identify statistically significant differences among groups as indicated by the Tukey HSD test.

Behaviour #6 exhibited the highest distance to the centre, and the meander was close to 0 indicating that the organisms with this type of behaviour had fewer sharp turns. The combination of these two parameters shows that the organisms circulated along the wall for the majority of the time, which again is supported by the concept of thigmotaxis, an expected behaviour from a healthy organism in a similar situation. Behaviour #6 also had one of the lowest values for both instantaneous acceleration and velocity when compared to the other behaviours. Regarding the low acceleration value, this could indicate a more fluid swimming pattern with fewer stops and changes in direction. Behaviours #2 and #5 were more frequent in organisms exposed to 0.075µg/L and 1.54µg/L TBTO, distinguishing these treatments from organisms in the control group and the group exposed to 0.25µg/L. These behaviours were characterised by higher instantaneous velocity and acceleration. The high instantaneous acceleration suggests an increased number of stops and changes of direction possibly associated to motor discoordination. The strongest significant difference between behaviours #2 and #5 was the angular velocity, which was very low in behaviour #2 and very high in behaviour #5. In practical terms, organisms exhibiting #5 behaviour would be expected to show more straight movements than those of exhibiting behaviour #2.

Behaviour #1 and #3 had similar values for distance to centre, instantaneous velocity, and acceleration. Both instantaneous acceleration and velocity were particularly low, indicating a lower speed activity. The main difference between these behaviours was found in angular velocity as behaviour #1 had a value between -100 and -150 while the value of behaviour #3 was between 100 and 150 degree/s. Since only the sign of the value was different, this suggests the only change was in the orientation of the organisms, not the velocity itself. The same characteristics were observed for the meander. Values closer to 0 indicate ample curves and values higher or lower are indicative of sharper turns. Organisms exhibiting these behaviours would thus be expected to show sharper turns with more erratic movements, when compared to the other behaviours. The Correspondence Analysis extracted three significant dimensions, with the first and second dimensions explaining 94.35% of the data dispersion. The first dimension clearly discriminated the control group from the TBTO treatments (**Figure 3.5**). The second dimension further discriminated the intermediate TBTO treatment from the lowest and highest concentrations tested. Control organisms tended to exhibit the behaviour type #6, compared to the remaining treatments (the homogenous subsets are shown in the histograms of **Figure 3.5**). Organisms exposed to the lowest TBTO concentration tended to exhibit significantly higher frequencies of behaviours #2, #5 and #6. Organisms exposed to 0.25µg/L TBTO tended to show behaviours #1, #3 and #4. All three of these behaviours are associated with lower instantaneous velocity and acceleration. Organisms exposed to 1.54µg/L TBTO tended to

exhibit behaviours #2 and #5, which were associated with swimming in the periphery of the arena and high instantaneous velocity and acceleration.

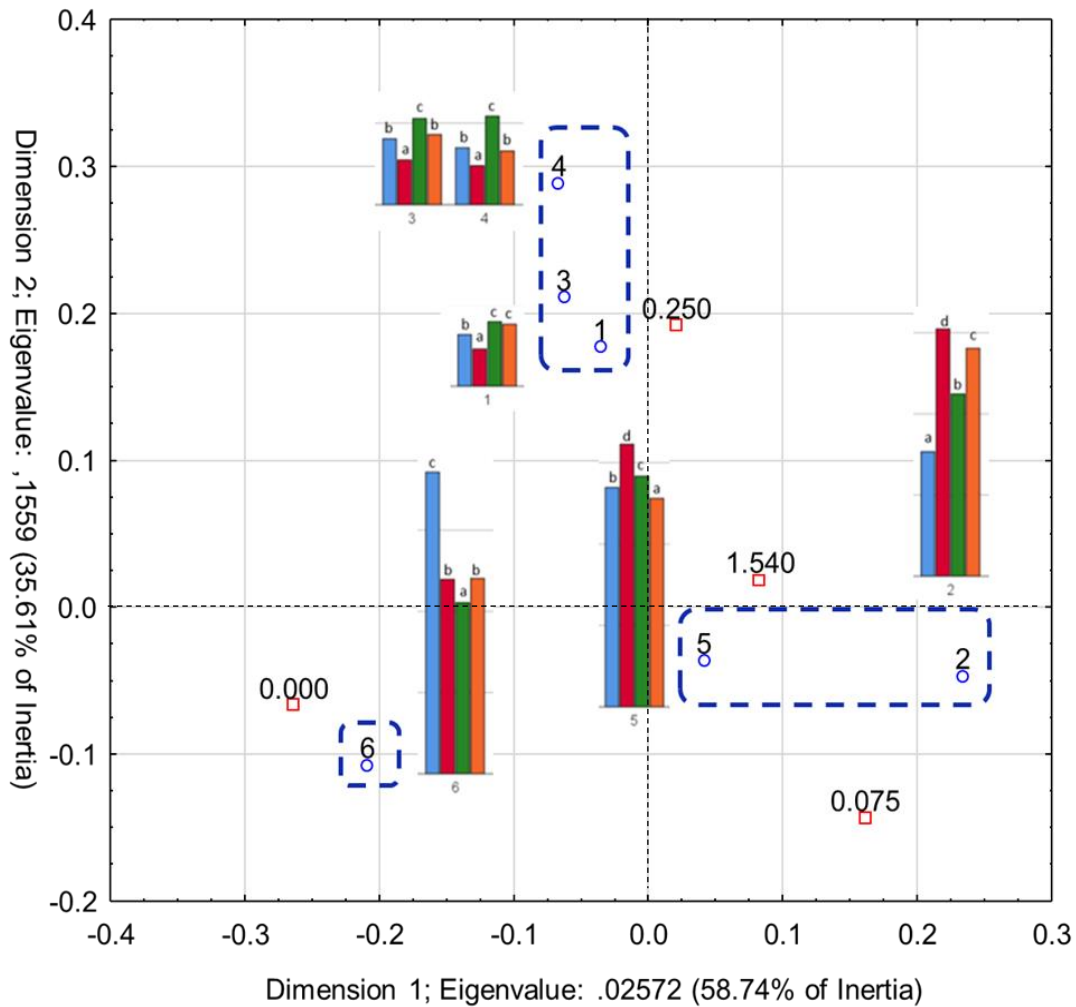


Figure 3.5- Correspondence analysis carried out with the behaviour types identified for the *T. longicaudatus* and the four treatments tested. Three significant dimensions were extracted. The plot shows the discrimination of the treatments, and their associated behaviours, by the first and second dimensions. The blue discontinuous squares indicate the clusters of similar behaviours identified. The histograms show the significant differences in the frequency of the six behaviours found among groups as indicated by the Chi-square test, with pairwise comparisons adjusted with the Bonferroni correction for the family-wise error rate

3.4. Discussion

The absence of mortality observed in the different treatment is most likely related to the reduced length of the exposure which was in total 110 minutes long. This becomes more evident at the highest concentration (1.54µg/L) since it is close to three times the previously calculated 96h-LC₅₀ value.

Only two of the selected movement variables were statistically different among treatments; instantaneous velocity was higher for every treatment compared to the control, and distance to centre was reduced for the 0.25µg/L treatment compared to the control. This increase in swimming activity has been attributed to escaping or avoidance behaviour, where the organism dispends more energy trying to find a non-contaminated space which in this case could be an early response to the TBT contamination (Araújo & Blasco, 2019). Regarding the distance to centre, this tendency of the control group to move closer to the walls of the plastic arena could translate into thigmotaxis behaviour. This well-documented behaviour is usually described as a coping mechanism by organisms placed in novel environments as a way to assess the new conditions and recover from high anxiety levels. Since the same behaviour is found in several species, it is safe to admit that this behaviour is well conserved from an evolutionary point of view (Schnörr *et al.*, 2012). As the distance to centre is higher in the control treatment, it could be linked to a more evident thigmotaxis behaviour. The reduced distance to centre observed in the 0.25µg/L treatment could be indicative of an inhibitory effect where loss of spatial awareness becomes noticeable while also being in line with a more evident escaping behaviour.

Behaviour characterisation

This study identified six behaviour types exhibited by control and exposed *T. longicaudatus* and showed that even at the lowest concentration TBT could affect their behaviour in a short time frame, especially in terms of instantaneous velocity. This was shown by the clear separation of the control group from the TBTO treatments in the Correspondence analysis, which was typically characterised by behaviour #6. This is indicative that TBT clearly impacts what is considered normal behaviour even at the lowest concentrations.

In the study of Egan *et al.* (2009) erratic movements were associated to higher levels of anxiety in zebrafish larvae. In this work three results suggest that TBT exposure can rise the frequency of erratic movements in *T. longicaudatus*. A study by de Paulo *et al.* (2020) also showed that TBT-based compounds in low concentrations could cause a reduction of the swimming speed and lower overall fitness of fish larvae. The slower swimming pattern

combined with the trend to avoid the centre of the arena could possibly be a sign of thigmotaxis. Kohler *et al.*, (2018) observed that wild amphipods, exhibited thigmotaxis tendencies whenever the organisms were under higher stress; in their study the stress source was lighting. Tierney *et al.*, (2016) observed the same tendencies in juvenile crayfish exposed to fluoxetine indicating that thigmotaxis is a behaviour adopted under stressful conditions independently of the nature of the stressor. Behaviour #4 was characterised by low instantaneous velocity and acceleration, though slightly higher than that of behaviours #1 and #3, as well as swimming in the centre of the arena while meander and angular velocity close to 0. Overall, these organisms appeared to be swimmers of intermediate speed and relative straight trajectories. Behaviours #1, #3 and #4 discriminated organisms exposed to 0.25µg/L TBTO from those of the remaining treatments. The Correspondence analysis also indicated a non-monotonic U-shaped response to TBTO exposure which should be taken into consideration, for example, when performing risk assessments.

Little and Finger (1990) suggested in their work that organisms irritated by a chemical may attempt to escape the exposure due to intensification of sensory reactions, and therefore initial changes in speed and movement are prone to occur. After the initial phase settles, more severe effects may start to appear in the locomotory behaviour as the detoxification system becomes overwhelmed with the exposure and neurochemical/metabolic processes start to be critically impacted. In his study, Ribeiro, (2019) exposed *T. longicaudatus* to 0.243µg/L of TBT, here the exposure was associated to three different behaviours, all of them were characterised by having slow velocity and one of them, described as behaviour “B”, had lower angular velocity and lower distance to centre. The variables contributing to this behaviour “B” followed the same trend as behaviour #4 while the two remaining had higher distance to centre and lower instantaneous velocity which are similar to what was observed in this work for behaviours #1 and #3. Ribeiro, (2019) associated the presence of three behaviours to disruptive effects caused by TBT.

TBT in other species

Several studies showed that TBT contamination led to similar effects in different species. Schmidt *et al.* (2005) observed significant decrease in swimming activity during daytime in juvenile carps exposed to 4µg/L. In a similar study Schmidt, Steinberg, *et al.* (2005) acutely exposed daphnids to 6.6µg/L of TBT and again reduction in swimming activity was recorded while also observing that exposed daphnids were closer to the surface which could be associated to spatial unawareness. Fleet (2010) exposed amphipods to 1, 10 and 100µg/L of TBT for six hours and observed an increase in swimming activity in the

lower concentration while in the higher concentration a reduction in swimming activity was recorded. Although the concentrations were higher than the ones used in the present study a similar trend of hyperactivity at a low exposure concentration (1µg/L) and hypoactivity at a higher exposure concentration (10µg/L) was observed. Yu *et al.* (2013) observed reduction in predatory success in sea ruffes exposed chronically to 0.1 and 1µg/L of TBT, in this study they linked these results to cognitive dysregulation due to changes in the quantity of neurotransmitters in the central nervous system of the organisms., similarly we found increase in neurotransmitter receptors which could be associated to a greater number of neurotransmitters such as CCHamide. These results suggest that although the effective concentrations vary from species to species, TBT could cause similar impairments.

Gene expression

In Chapter II, we evaluated the gene expression of this species to the same three concentrations. Although most of the significantly up or down-regulated genes were associated with other biological pathways, we found some pathways that could be associated to disruption of locomotory behaviour. We found upregulation of the myelin-associated glycoprotein (Mag) gene in all treatments, which is responsible for the proper myelination of axons and preventing neuron apoptosis. The sodium channel protein gene (Nach) was also found significantly upregulated, and since this gene encodes a protein crucial for the correct transmission of electric impulses, this suggests that TBT could cause abnormal neuron excitability. Regarding down-regulation, we found that the Down syndrome cell adhesion molecule gene (Dscam) had a similar pattern as our behaviour results, down-regulation started at $\text{Log}_2\text{FC} = -3.835$ for the 0.075µg/L treatment, decreased to -3.973 in the 0.25µg/L and interestingly, recovered to -3.879 in the 1.54µg/L treatment thereby exhibiting the same U-shaped curve response. The proteins encoded by this gene are maintenance proteins that focus on correct neuronal organisation by inducing dendritic self-avoidance and consequently efficient synapses (Millard & Zipursky, 2008). Lastly, as mentioned before, we found an upregulation of CCHamide neuropeptide receptors which could be associated to an increase of the same neuropeptide in the central nervous system of the organisms leading to cognitive impairment (Yu *et al.*, 2013).

To summarise, data regarding swimming activity was easily obtained and showed to be a sensitive early sign of TBTO exposure. The results of this study showed hyperactivity in the 0.075µg/L treatment followed by hypoactivity in the 0.25µg/L treatment, while in the 1.54µg/L treatment hyperactivity was again observed. Swimming activity was, therefore, the most obvious factor impacted. Although it is an endpoint measured at the individual level,

deviant swimming activity could directly and indirectly impair the survival of the organisms by reducing food scavenging, predator evasion or migration. Regarding the neuronal impact, it seems that TBT can affect more than one component of neuronal cells. Although more studies should be performed to pinpoint the impact of TBT, this data suggests that locomotory behaviour could be impacted due to impaired electric transmission that can disrupt movement and explain the erratic behaviour discussed above.

3.5. Conclusion

Here we showed that *T. longicaudatus* is a suitable species for behavioural experiments since even at residual concentrations, it is possible to distinguish changes to the normal locomotory activity pattern. Additionally, being a crustacean allows for experiments even in adult stages without the ethical implications involved in experiments with vertebrate species. This will significantly improve the readiness of results and allow for wider comparison and replicability. This adds a suitable candidate for experiments with invertebrates and, more specifically, crustaceans, which are often overlooked. Here the importance of sub-lethal endpoints as an early warning system for chemical toxicity exposure is highlighted, while providing an efficient way to evaluate it. The study also showed that TBT contamination can impact normal behaviour even at the lowest concentration affecting swimming activity, swimming pattern and spatial awareness. These impacts could partially be associated with neurological toxicity, as suggested by the results of the gene expression study carried out. Future work involving a wider range of concentrations and time frames combined with molecular biomarkers could help to better clarify the specific biological processes impacted by this chemical.

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4. Chapter IV: Toxic relationships: Bioinformatic prediction of TBTs affinity to the RXR- α of *Triops longicaudatus*.

Abstract

Retinoid X receptors (RXR) are nuclear receptors that mediate several endocrine pathways essential for cell differentiation and lipid homeostasis. This protein operates both as a homodimer and heterodimer, complexing with several other nuclear receptors such as ecdysteroid receptors (EcR). Among the known ligands of RXR is tributyltin (TBT), a biocide used mainly in the '60s has a long record of endocrine disruption and mortality amongst aquatic species. This study gathers assembled genetic sequences for this protein for various Metazoan species and compared their three-dimensional structure and phylogenetic distribution, focusing mainly on invertebrate species to simplify the comparison to the sequence of the study species *Triops longicaudatus*. The number of sequences available led to the conclusion that this protein has a crucial function as it is highly conserved across the animal kingdom. Our results showed great conservation across the species, especially regarding the aminoacids that directly interact with TBT and a phylogenetic clustering of species with the same modifications in these aminoacids. A three-dimensional comparison was also performed, which showed little conformational variation even in distant species and substantially different sequences.

With these results, the potential of bioinformatics becomes evident for protein sensitivity and comparison, which could be an alternative for animal or cellular experimentation. These techniques could be adapted for various chemicals and proteins, allowing for observations in a shorter timeframe, which could provide information on a wider spectrum.

Keywords: Comparative genomics, Tributyltin, Proteomics, Phylogeny, Crustacea.

4.1. Introduction

Biocides are a group of chemicals whose main objective, according to European legislation, is to kill or deter any harmful organisms. From this group, tributyltin-based compounds (TBT) were one of the most used in the '60s by improving aquatic transportation through elimination of any organism that tried to colonise the vessel's hull (Kannan *et al.*, 1997). Unfortunately, not much information on the persistence and long-term effects were available, so its unrestricted use led to ubiquitous environmental contamination (UNEP/FAO, 2015; Sousa *et al.*, 2017). TBT was banned in the European Union in 2008, but although several efforts and legislation were created to reduce its usage, measurable concentrations are consistently found in environmental samples, suggesting illegal or unregulated use (Anastasiou *et al.*, 2016b). Environmental concentrations of TBT, such as 1540 ng/L, were found in the United Kingdom, 262 ng/L in Brazil and 26.9 ng/L in South Korea (Sayer *et al.*, 2006; Artifon *et al.*, 2016; Kim *et al.*, 2014). Besides being persistent in sediments, TBT is also known to bioaccumulate in aquatic organisms, potentially leading to bioamplification throughout the food chain (Kannan *et al.*, 1997). Regarding its effects, TBT is known to cause endocrine disruption, mitochondrial toxicity (Nesci *et al.*, 2011), immune system suppression (Jacobson *et al.*, 2011), and developmental/genetic toxicity (Sousa *et al.*, 2017), which were observed both in vertebrates and invertebrates. TBT is known to target specific proteins and heterodimers with homologous and orthologous functions in different taxa. In vertebrates, the retinoid X receptor (RXR) and the heterodimer formed with the peroxisome proliferator-activated receptors (PPAR) are the main target of TBT molecules (Krężel *et al.*, 2019). In invertebrates, this heterodimer is less frequent. Instead, insects and crustaceans have homologous proteins with slightly different or additional functions. For insects, the RXR homologue is the ultraspiracle protein (USP) which forms a heterodimer with ecdysteroid receptor (EcR). As for crustaceans, they retain the RXR protein but combine it with EcR (Durica *et al.*, 2002; Morales *et al.*, 2013). The EcR is a nuclear receptor that belongs to the well-conserved ecdysteroid regulatory circuit, which is of great importance to organisms that moult their exoskeleton during their lifespan. The most emblematic examples being insects and crustaceans. Although several studies have been performed around TBT, its molecular mechanism of action is not yet fully understood. Besides the more evident effects listed above, TBT also severely impact moulting processes in Arthropods through key changes in the hormonal pathways (Batista *et al.*, 2016; Song *et al.*, 2017).

Triops longicaudatus are small freshwater crustaceans that inhabit ephemeral water ponds across regions of low latitude. Their small size, simple maintenance, rapid

development and short life cycle/generation makes this species quite suitable for most ecotoxicological assays (Scholnick, 1995). This species is also known for possessing a simple neurotic system unable to feel pain or distress, thereby becoming an attractive model organism and complying with the 3R's initiative (Reduce, Refine, Replace - EFSA, 2005). This species mainly reproduces asexually, mainly resulting in clonal individuals, thus reducing genetic variations during bioassays. Lastly, *T. longicaudatus* is considered a living fossil; thereby, its genetic information and fundamental morphology have not changed significantly for more than 170 million years, which is a feature of great value for comparative genomics (Sunouchi, 1997). The main focus of comparative genomics and similar approaches is to compare different sequences (whole genomes or specific sections of it) in order to infer the functional annotation of both genes and genomes and assess variation between those sequences which can be interspecific or intraspecific (Miller *et al.*, 2004). These studies can also be combined with predictions of the three-dimensional structure, mainly using the information obtained through the alignment of the sequence of interest to one or more proteins with well-documented structures (Webb & Sali, 2016). This prediction could then be used to quantify the level of affinity between a molecule and a specific binding site of a protein, which in turn would be useful to infer the molecule's effect at higher organisational levels (Öztürk *et al.*, 2018). Predicting protein sensitivity would greatly help the scientific community by saving time and resources and removing specific bioassays. This replacement would avoid the sacrifice of countless organisms and therefore be in line with the 3R's initiative while ensuring satisfactory results and conclusions (Ibrahim, 2006).

In this sense, this study's main objective was to predict the interaction of the TBT molecule to the RXR- α of *T. longicaudatus*. The sequence and conformation were compared with different invertebrate species to infer the inherent toxicity.

4.2. Materials and Methods

Toxicity data

Ecotoxicity data regarding freshwater organisms were collected from the public database USEPA ECOTOX Knowledgebase (cfpub.epa.gov/ecotox/). Ecotox database presents an excellent option to extract raw information regarding toxic effects and values of many chemical compounds on an even greater number of species. The search included freshwater organisms exposed between 48 to 96 hours to TBT and TBT-Oxide (TBTO). After that refinement, additional information such as the life stage of the organism, type of

exposure and the value of LC_{50} was collected for multiple species of multiple genera, including fish, molluscs, insects, crustaceans, and rotifers. Additionally, Google Scholar was utilised to search for complementary information and more recent toxicological studies. The metadata table was constructed in Excel to facilitate comparison.

In order to calculate the species sensitivity distribution curve (SSD), the SSD toolbox software was used (www.epa.gov/chemical-research/species-sensitivity-distribution-ssd-toolbox) This software combines several algorithms which aid users in the interpretation of the SSD, also providing different distribution types for different scenarios (Fox *et al.*, 2021). A total of 40 LC_{50} (lethal concentration that kills 50% of the population) values were found for different taxa ranging from rotifer to vertebrate species.

Protein sequence extraction

NCBI is a collection of databases ideal for gathering genomic information [National Center for Biotechnology Information (US), 2019]. The genetic resources available go from full genomes and transcriptomes to single nucleotide polymorphisms making this platform highly versatile and useful for bioinformatics (Hendrix, 2019). The platform was searched for high-quality sequences of our target proteins RXR- α . With the respective accession number a second search engine inside NCBI was used, the BLAST functionality. From the several options, the tBLASTn option was selected, which requires as input a protein sequence or code, providing the nucleic sequence that originates that same protein. Using this tool, several high-quality sequences were gathered for this species, prioritising Reference RNA sequences (Refseq_rna) and, when those were not available, Transcriptome Shotgun Assemblies (TSA). Main focus was put into the search of invertebrate species and eight sequences belong to Arthropoda phylum were obtained, one to Chordata (Urochordates), eight to Mollusca and one to Rotifera. The Human sequence was also used for comparison. The nucleotide sequence of the RXR- α gene is approximately 2000 bp in length, and the coding region, or open reading frame (ORF), fluctuates around 1200 bp originating proteins consisted of an average of 430 aminoacids (AAs).

After the sequences have been downloaded, in Genbank format, they were imported into Geneious® software v9.1.8 (www.geneious.com). This software is designed to be a user-friendly platform whose main goal is the organisation and analysis of biological data, with a greater focus on molecular sequences (Kearse *et al.*, 2012). From its vast ray of options, the most relevant for this study was the ability to perform protein sequence alignments. In order to obtain the AA sequence of the RXR- α for different species, two searches were performed, one to the Universal Protein Resource (UniProt) database and

a second one to the NCBI database. The UniProt is a database of free access where it is possible to take both protein sequences and annotation data. The UniProt database was consulted to search for relevant accession codes of each species related to the isoform of interest. After selection, the data was uploaded into Geneious® for further analysis (Wu *et al.*, 2017). In the cases where more than one sequence was present for the same species, an alignment was performed to assess which sequence to use forward, based mainly in the sequence length and the quality of the region containing TBT's binding pocket. The selected sequences and accession code are listed in **Table 4.1**.

Table 4.1- Accession number and corresponding database of the sequences of RXR- α obtained for each species of interest.

Phylum	Genus	Species	Database	Accession Number
Chordata	<i>Homo</i>	<i>sapiens</i>	UniProt	P19793
	<i>Ciona</i>	<i>intestinalis</i>	UniProt	F6Y6W3
Mollusca	<i>Nucella</i>	<i>lapillus</i>	UniProt	A7LIS3
	<i>Biomphalaria</i>	<i>glabrata</i>	UniProt	A0A2C9JMM9
	<i>Lymnaea</i>	<i>stagnalis</i>	NCBI	FX194644.1
	<i>Elliptio</i>	<i>complanata</i>	NCBI	GAHW01034005.1
	<i>Crassostrea</i>	<i>gigas</i>	NCBI	XM_022473239.1
	<i>Crassostrea</i>	<i>virginica</i>	NCBI	XM_020062358.2
	<i>Mytilus</i>	<i>edulis</i>	NCBI	GHII01035878.1
	<i>Perna</i>	<i>viridis</i>	NCBI	GEKL01014856.1
Rotifera	<i>Brachionus</i>	<i>calyciflorus</i>	NCBI	GACL01002323.1
Arthropoda	<i>Crangon</i>	<i>crangon</i>	UniProt	C4N541
	<i>Carcinus</i>	<i>maenas</i>	UniProt	Q6RIB4
	<i>Leptuca</i>	<i>pugilator</i>	UniProt	A0A075FC47
	<i>Tigriopus</i>	<i>japonicus</i>	UniProt	D4N3A3
	<i>Daphnia</i>	<i>magna</i>	UniProt	A4F2F2
	<i>Amphibalanus</i>	<i>amphitrite</i>	UniProt	A0A6A4WXE5
	<i>Triops</i>	<i>longicaudatus</i>	Chapter II of the Dissertation	

Sequence alignment and Phylogenetic analysis

With Geneious® it is possible to perform an alignment of the protein sequences. From the variety of algorithms provided, the MUSCLE-alignment was chosen since it uses k-mer contig for faster distance estimations and refinement made possible due to tree-dependent restricted partitioning. These factors combined lead to more accurate results (Edgar, 2004).

The alignment of the AAs was visually inspected, and the residues of interest were annotated and compared among species.

The sequences for the RXR- α were composed of a maximum number of 507 AAs. The alignments were used afterwards to build a phylogenetic tree. The selected genetic distance model was Jukes-cantor since it is the simplest substitution model that assumes the same base frequency for all AAs. Regarding the tree build method the Neighbour-Joining, which is a bottom-up clustering method, was the most appropriated for this case with 100 bootstrap replications to assess confidence in each node. Bootstrap values were also added to quantify the amount of genetic change over time. This information will allow for comparison between the species and similarity between the sequences of the same protein. A table with the Patristic distances of the several species can be visualised in **SD 4**.

Sequence treatment and Protein Modelling

After the different sequences of interest were analysed, they were imported into Swiss-model (<https://swissmodel.expasy.org>). The Swiss-model server was the first automated protein homology server to model homo and heteromeric complexes (Waterhouse *et al.*, 2018). With the AA sequence, it is possible to assess the three-dimensional conformation of the protein. The server provides a variety of templates that can be selected according to several factors, such as Global Model Quality Estimate (GMQE) and Quaternary Structure Quality Estimate (QSQE). After the model was built the Qualitative Model Energy ANalysis (QMEAN) was taken into consideration to assess the model. The QMEAN is a composite estimator which takes into consideration geometrical properties giving absolute quality estimates for the whole structure or for individual residues (Studer *et al.*, 2020). Finally, the structure was downloaded, and the analysis and visualisation of models of interest were performed using PyMOL (version 2.4.0).

PyMOL is a useful graphical tool mainly used for visualising three-dimensional structures of macromolecules. In order to assess the structure of the protein, the model created previously was imported, and hetero molecules such as Zinc were removed. Interaction AAs were highlighted, and different models were superposed to assess key differences in structure and in the binding pocket of the TBT molecule.

4.3. Results

Species Sensitivity Distribution curve (SSD)

A species sensitivity distribution curve (SSD) using aquatic vertebrate and invertebrate species, based on the LC₅₀ values, is presented in **Figure 4.1**. The Normal distribution was selected for the construction of the curve with the Metropolis-Hastings fitting method. Goodness of fit contained a total of 1000 iterations for more conclusive results. Here only data collected from juvenile organisms or larvae stages were included so the values could be effectively compared. It is possible to observe that vertebrate and invertebrate species are distributed throughout the curve with no evident grouping. The species *Elliptio complanata*, a mollusc, was considered the most resistant, while and *Mercenaria mercenaria*, a second mollusc, was considered the most sensitive species. The HC₀₅ value obtained was 0.2069 [0.1897 - 0.2322] µg/L, with a p-value of 0.8824. Three species were found below the HC₀₅ (*M. mercenaria*, *Chironomus plumosus* and *Anadara rhombea*). This indicates that these species are extremely sensitive to TBT since their LC₅₀ value is lower than the HC₀₅ (a concentration that should affect 5% of the species). Although several relevant species are missing (e.g. dragonfly, stonefly nymphs), the low HC₀₅ value, below many of TBT concentrations found in the environment worldwide (e.g. 1.54µg/L - Sayer *et al.*, 2006) highlighted the need to continue to perform research with this contaminant.

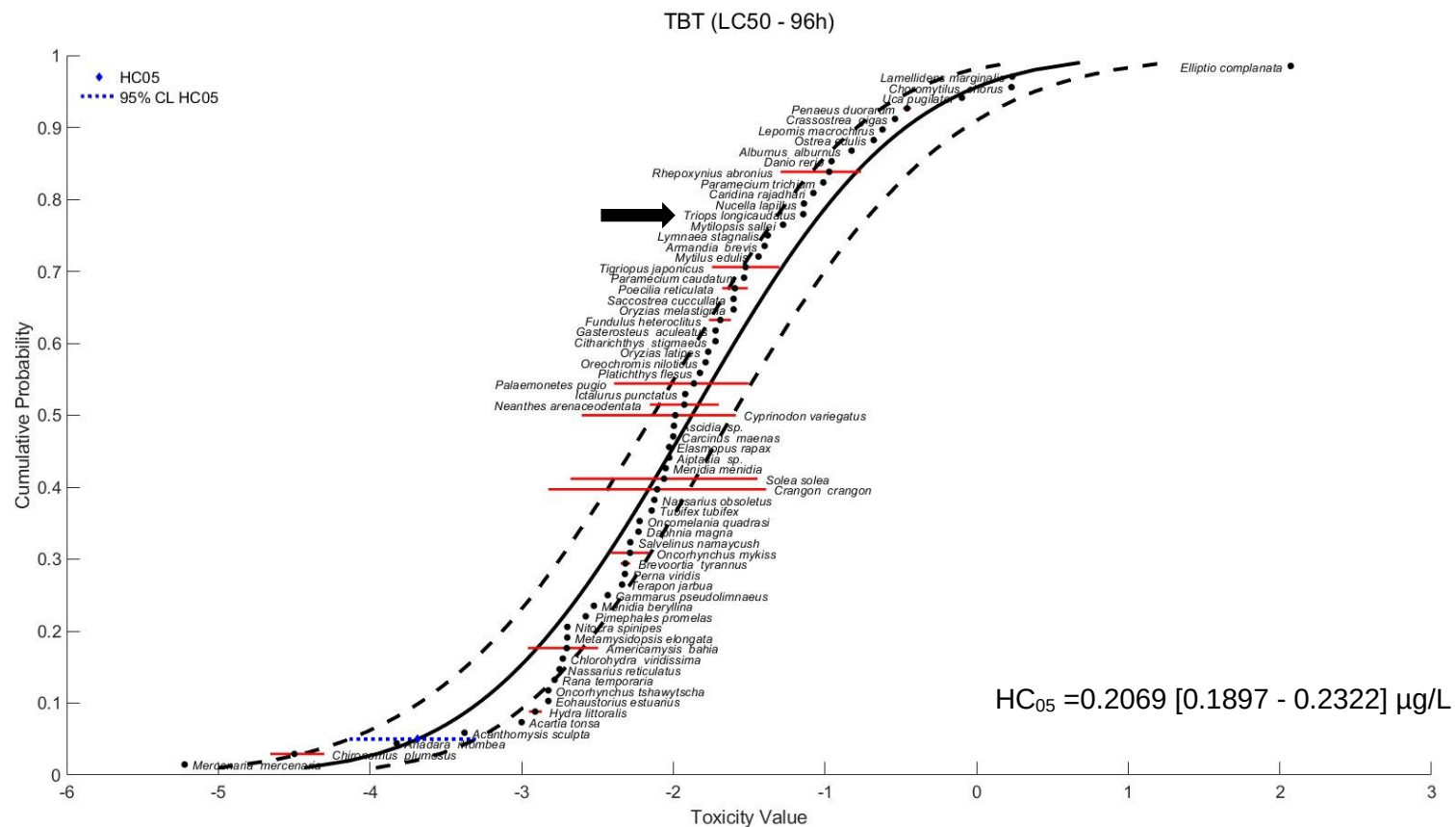


Figure 4.1- Species Sensitivity Distribution curve (SSD) based on the LC50 values for 96h of aquatic vertebrate and invertebrate species to tributyltin-based compounds and correspondent HC₀₅. Black arrow indicates the position of *T. longicaudatus*.

Sequence alignments

To determine the evolutionary relationships between different species from different taxa, a MUSCLE alignment was performed using the sequences presented on **Table 4.1**. The alignments shown in **Figure 4.2** provide insight into the level of conservation of targeted AAs from the RXR- α isoform. Comparing the sequences of different species allows to pinpoint similarities in key regions and the overall completeness of the sequence itself. Alignment of these sequences, in particular the centre section, reveal a strong conservation of several short AA sequences in particular regarding binding sites to the TBT molecule. The majority of the diversity for both sequences is focused on the first section of the sequence.

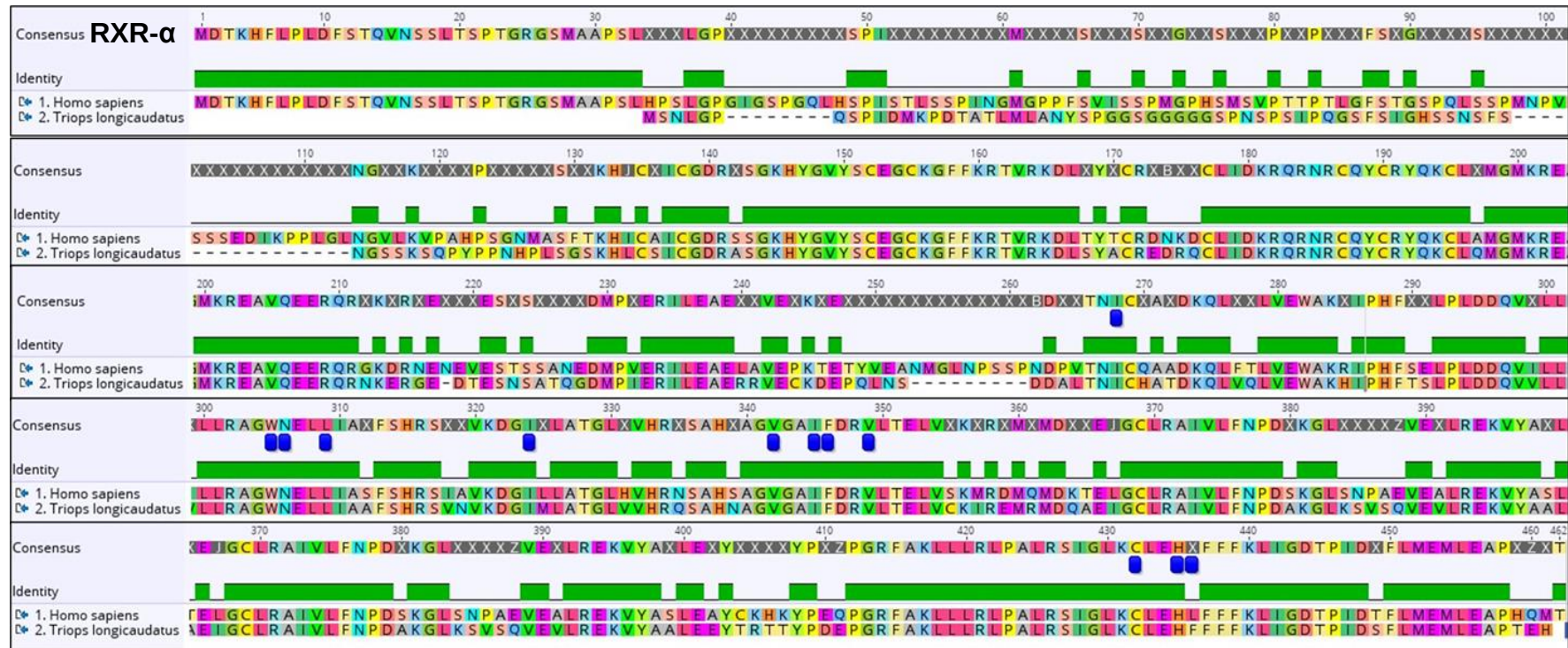


Figure 4.2- MUSCLE sequence alignment of the RXR- α isoform of *H. sapiens* and *T. longicaudatus*. Colour code shows aligned sequences that are possess the same aminoacid indicating conservation in the sequence. Gray coloration demonstrates non-conserved residues. Dark blue labels next to the consensus sequence indicates customized annotation of interaction residues.

The general overview of the MUSCLE alignment provides the sequences separated by a colour code to simplify the comparison. The consensus sequence shows the more probable and frequent AA that appears in the sequence, and the identity graph allows for a quick view of the conservation level between species. The identity label represents the percentage of identity between species, green boxes represent identical bases. Highlighted in blue are annotations to the interaction AAs that are responsible for the affinity of the TBT molecule to the protein. The RXR- α interaction AAs were annotated according to Le Maire *et al.*, 2009 for the *Homo sapiens* sequence. The conservation around these specific AAs is quite high. In the cases where AA replacement occurs, the resulting AA have low values in the Grantham's distance matrix. Examples of which are the Isoleucines 575, 647 and 683, which, when replaced, are usually replaced by Leucines. This replacement has a value of 5 in the Grantham's distance, the lowest number in the table, which translates into AA with very similar chemical properties.

In **Table 4.2** it is possible to see in more detail the conservation of interaction AAs, in particular, Cystein 432 (in red), which is responsible for the covalent bond to the central tin atom of the TBT molecule and is, therefore, the main contributor for the binding strength. The information regarding these AAs was collected from the PDBsum (id 3e94), which is focused on the interaction of RXR and a TBT molecule (*PDBsum- Tributyltin*, 2013). This high level of conservation indicates evolutionary conservation between invertebrates and vertebrates as the conservation level is one of the highest among the alignment. The number of the Cystein refers to its position on the *H. sapiens* sequence, so the number attributed to it does not coincide as some of the sequences for other species have different protein lengths. Mutations in the sequence can alter both the function and quaternary structure of a domain; thus, alterations in the protein conformity may dictate its affinity to the TBT molecule.

Table 4.2- Section of the MUSCLE alignment showing in greater detail the conservation of interaction residues. Cystein responsible for the covalent bind to the tin atom of the TBT molecule is highlighted in red.

<i>H. Sapiens</i>	Isoleucine 268	Tripsine 305	Asparagine 306	Leucine 309	Isoleucine 324	Valine 342	Isoleucine 345	Phenylalanine 346	Valine 349	Cystein 432	Histidine 435	Leucine 436
<i>B. calyciflorus</i>	Phe	*	Arg	*	Leu	Leu	Leu	Leu	Leu	Gly	Asn	*
<i>A. amphitrie</i>	—	*	*	Iso	*	Met	*	*	*	*	Lys	Iso
<i>T. japonicus</i>	Met	*	*	*	*	*	*	*	*	*	*	*
<i>C. intestinalis</i>	Val	*	*	*	*	*	*	*	*	*	*	*
<i>D. magna</i>	*	*	*	*	*	*	*	*	*	*	*	*
<i>T. longicaudatus</i>	*	*	*	*	*	*	*	*	*	*	*	Phe
<i>C. cragon</i>	*	*	*	*	*	*	*	*	*	*	Tyr	*
<i>C. Maenas</i>	*	*	*	*	*	*	*	*	*	*	Tyr	*
<i>L. pugillator</i>	*	*	*	*	*	*	*	*	*	*	Tyr	*
<i>N. Lapillus</i>	*	*	*	*	*	*	*	*	*	*	*	*
<i>B. glabrata</i>	*	*	*	*	*	*	*	*	*	*	*	*
<i>L. stagnalis</i>	*	*	*	*	*	*	*	*	*	*	*	*
<i>C. gigas</i>	*	*	*	*	*	*	*	*	*	*	*	*
<i>C. virginica</i>	*	*	*	*	*	*	*	*	*	*	*	*
<i>E. complanata</i>	*	*	*	*	*	*	*	*	*	*	*	*
<i>M. edulis</i>	*	*	*	*	*	*	*	*	*	*	*	*
<i>P. viridis</i>	*	*	*	*	*	*	*	*	*	*	*	*

Phylogenetic analysis

In order to evaluate the evolutionary relationship of the gathered RXR- α sequences, a Juke-Cantor consensus tree was made, here the quality score obtained through the previous alignment can be transformed into a measure of distance. This calculation method describes the amount of genetic change that is represented by a tree with a resource to the length of the branches themselves (Fourment & Gibbs, 2006). The sequences did not define major phylogenetic branches, but almost all species got clustered according to their taxa. This could be associated with the present minimum sequence conservation. The complete phylogeny for the RXR- α alignment, with respective bootstrap values, is presented **Figure 4.3** as a cladogram. The clusters could also be explained by the existence of an ortholog, which could indicate their existence before the divergence of a common ancestor. The high conservation of this gene could be indicative of its functional significance among species. The patristic distance matrix for this phylogenetic tree is presented in **SD 4**. The phylogenetic tree presents clear clustering of the species, which could indicate a similar evolutionary strategy. The selected root for the tree was *H. sapiens* as all sequences were compared to this sequence, and since it is the only vertebrate in this phylogenetic tree, it became the most valid option to root upon.

Consistent with what is shown in the MUSCLE alignment the RXR- α sequence has little variation across species in the same taxonomic group. This is reflected in the phylogenetic tree since those same species tend to cluster together.

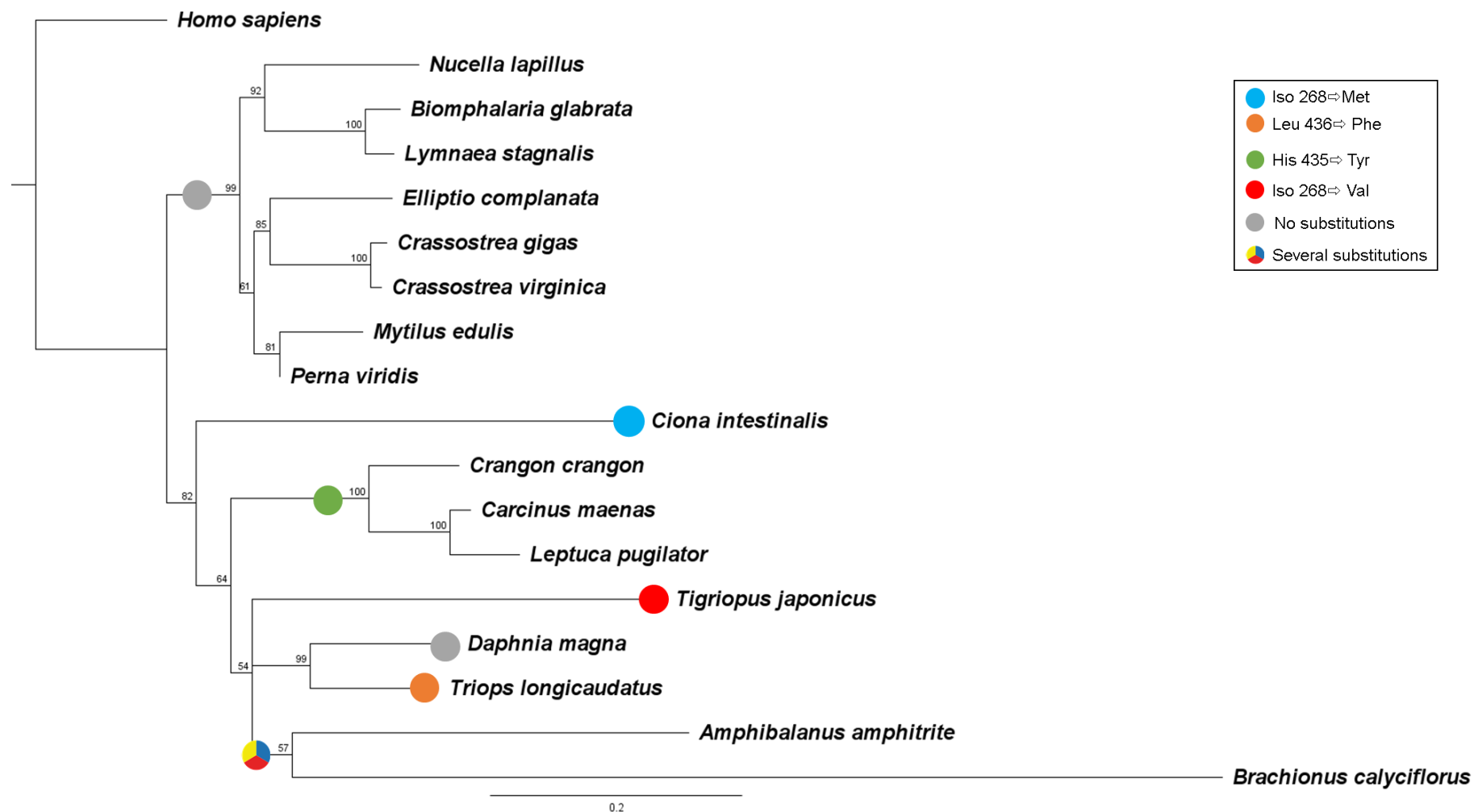


Figure 4.3- Neighbour-joining phylogenetic tree based on Jukes-Cantor model regarding RXR- α protein sequences. Bootstrap values represented at the beginning of each branch. Genetic distance can be assessed through branch lengths. Different colour circles represent changes in the interaction aminoacids when compared to the *H. sapiens* sequence.

Protein Modelling

The RXR- α sequence for *T. longicaudatus*, *H. sapiens* and *Daphnia magna* were imported into Swiss-Model where several three-dimensional models were built. The sequences served as a query to search for related protein structures available in the SWISS-MODEL template library. After the appropriate template was selected, based on general quality and coverage, it is used to build the three-dimensional protein structure - an example of which is showed in **Figure 4.4**. Regarding coverage and identity, both parameters were similar to the target sequence identity 75-77% and coverage 80-85%. Regarding the oligomeric state, stoichiometry and topology, no significant differences were found and therefore not taken into consideration for the selection of the template. The 4nqa.1 template was selected due to a higher global model quality estimation (GMQE, ~ 0.7).

Despite the availability of other templates on the platform (e.g. 3e94.1), the 4nqa.1 template presented a higher GMQE and QMEAN while the identity percentage remained essentially the same and thus being selected for the remaining work. The resulting structure can be visualised in Figure 4.4A, where the colour gradient reflects the quality assessment. Blue colouration reflects higher quality while red reflects the opposite evaluated by the all-atom local distance difference test (IDDT) as expected central regions of the protein scored higher than the peripheral regions. These scores are automatically attributed by Swiss-Model, which takes into consideration the templates it has available. Regions located on the surface of the protein as well as smaller sections like terminal tails and loops present a lower quality estimation when compared to the rest of the structure. In Figure 4.4B is presented a Local Quality Estimate plot, this plot associates a score to each residue of the sequence. This score reflects the theoretical similarity of the residue (X-axis) to the original sequence (Y-axis), values over 0.6 are considered to be of higher quality. The model had a GMQE of 0.61 and a QMEAN of -2.47.

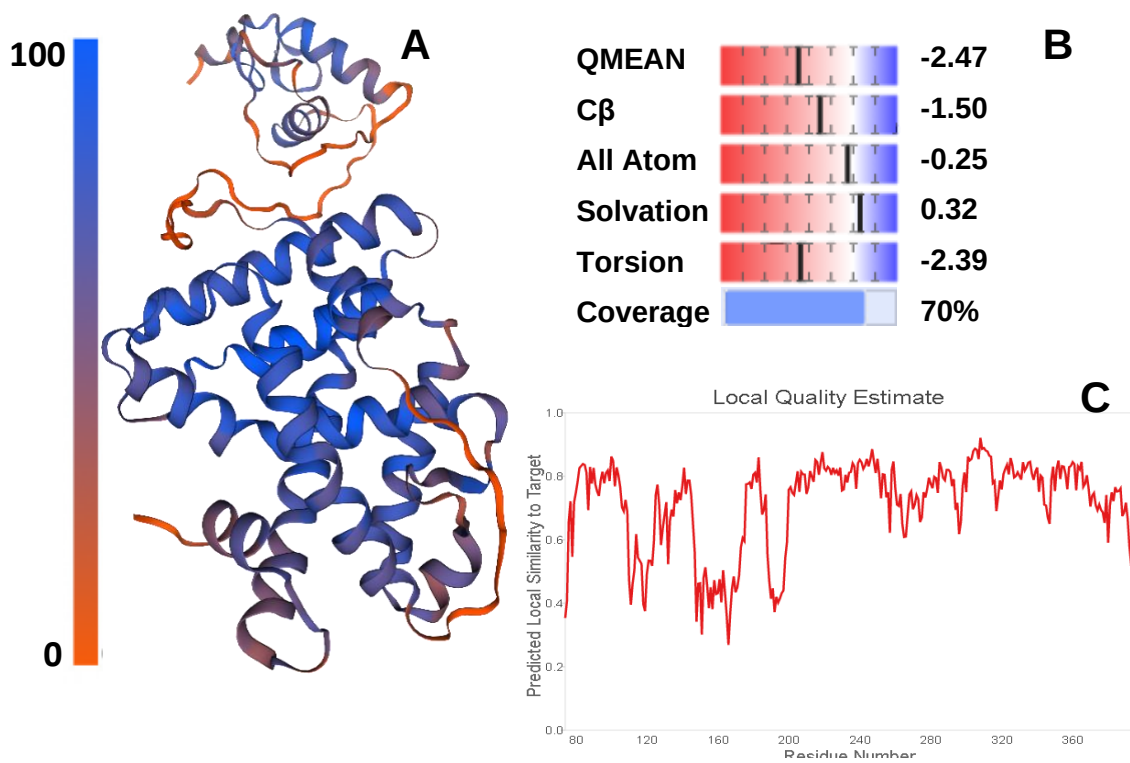


Figure 4.4- Predicted 3D structure of the RXR protein and related information regarding its quality. **A-** Predicted 3D structure of the RXR protein. **B-** Global quality of the RXR protein model. **C-** Local quality estimate of the RXR protein model.

Protein structure comparison

After creating the model, it was imported into PyMOL, here the structure was treated and any heteromolecules removed, remaining only the proteins. The ligand-binding domain of the protein was prioritised, whereas other sections (e.g. DNA binding domain), were purged as they have no direct impact on our analysis. Afterwards, a second model was imported so the comparison may begin. In **Figure 4.5** it is possible to see the results of the superposition of the ligand-binding domain, in **Figure 4.5A** is presented the *T. longicaudatus* protein (Red) superposed to the *H. sapiens* protein (Green), and in **Figure 4.5B** the *T. longicaudatus* protein (Red) is superposed to the *D. magna* protein (Blue). It is possible to see that, although the sequences contained several differences, the essential conformation remains conserved. To assess the binding pocket of the proteins more effectively, the interaction AAs were extracted from the main sequence and displayed in **Figure 4.6** for easier comparison. Here both the AAs and the polar interaction are displayed as well as different colouration for differences to the well documented *H. sapiens* sequence. The AAs were labelled, and the main colouration reflects the molecular composition of the AA.

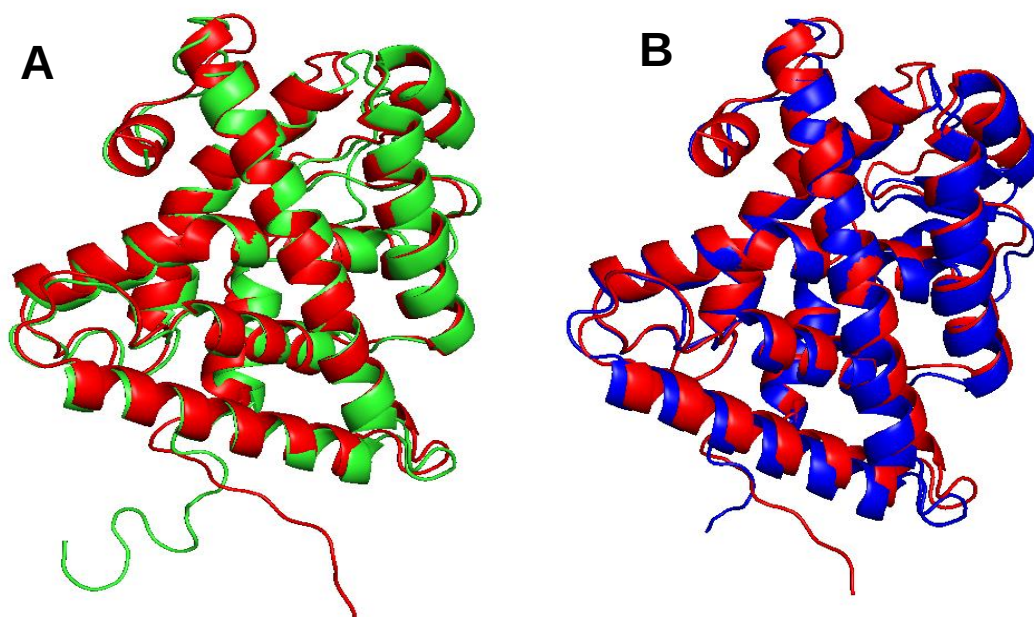


Figure 4.5- RXR- α ligand binding domain visualization. **A-** *T. longicaudatus* sequence (Red) superposed to *H. sapiens* sequence (Green). **B-** *T. longicaudatus* sequence (Red) superposed to *D. magna* sequence (Blue).

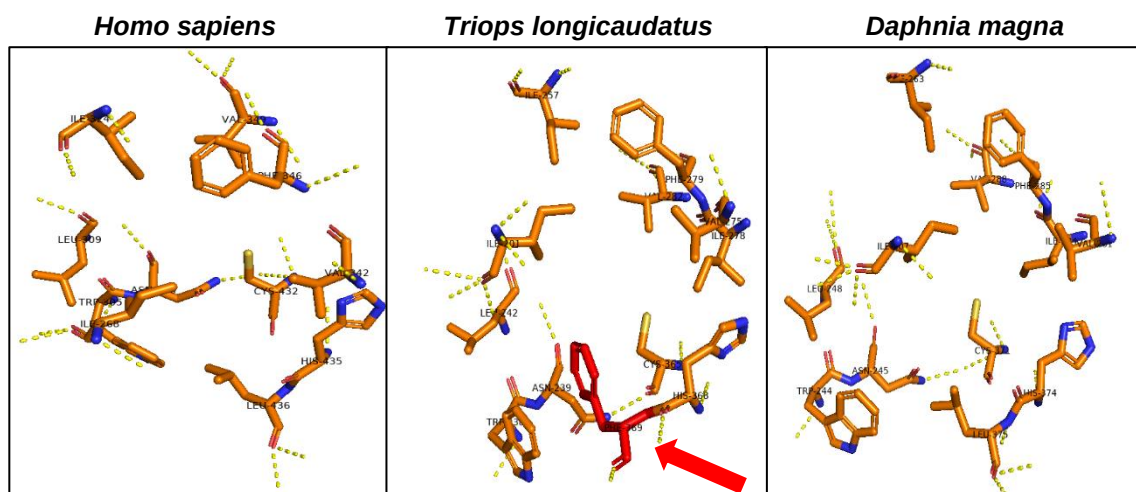


Figure 4.6- Representation of interaction AAs for each of the sequences of interest. AA in red is indicative of a substitution when compared to the *H. sapiens* structure.

4.4. Discussion

Our results show several full-length RXR gene sequences found throughout different databases. Special attention was given to sequences belonging to invertebrate species as it became easier to compare to our study species *T. longicaudatus*. The sequences found belonged to a variety of phyla which is indicative that the function performed by this nuclear receptor is of high evolutionary importance. The insect class was not included in our analysis since they possess a homologous protein to RXR, the ultraspiracle protein (USP). Alignment of the RXR- α isoform revealed a high level of conservation across different species. The cysteine responsible for the covalent bond to the central Sn atom of the TBT molecule remained largely conserved except for one species, *Brachionus calyciflorus*, that presented a non-conservative mutation presenting a glycine. This AA substitution was classified with a value of 159 in the Grantham's distance matrix, indicating that those two AAs have substantially different chemical properties. With this overall level of conservation, it is safe to assume that sensitivity of the protein to TBT is probably similar across species which in turn will lead to the agonistic effect that is characteristic of this compound as shown by Morales *et al.*, (2013) in insects, Lima *et al.*, (2011) in molluscs, Hernando *et al.*, (2005) in crustaceans and Zhang *et al.*, (2017) in fish. The endocrine disruption typically associated with this chemical is mainly linked to the direct activation of both proteins of the RXR- α /PPAR- γ heterodimer. Although the PPAR- γ protein is missing in the majority of our test species, the partial activation of the heterodimer through the binding of TBT to RXR- α , and the requirement of a natural steroid to activate the EcR - thus activating the heterodimer - does not necessarily mean that endocrine disruption will not occur.

Evolutionary relationship

RXR- α sequences have been found in a variety of species across the animal kingdom. This could indicate these proteins existence before the split between Protostomia and Deuterostomia based on its existence in the *B. calyciflorus* species. The most conserved section of the RXR- α sequence is, as expected, the domains related to DNA binding and ligand binding (Mangelsdorf *et al.*, 1992). Our comparison of the TBT's interaction AAs showed great conservation among them, with some species showing little differences despite of the species evolutionary distance. This is the case of *H. sapiens* and all the present mollusc species and, more interestingly, between *H. sapiens* and the crustacean *D. magna* as shown in **Table 4.2** and **Figure 4.3**. The sequence responsible for encoding these domains are in the central section or interface of the protein sequence, which is also known to be less prone to variation when compared to the peripheral region (Caffrey, 2004). The sequence from *Amphibalanus amphitrite* is missing the first interaction AA (Isoleucine 268). Still, it is impossible to pinpoint if there is an incomplete sequence of the protein in the databases or if it shows a more distinctive conformation, since the alignment shows an overall high level of conservation. The cysteine 432, which is crucial for TBT's affinity to RXR, was conserved in all species with the exception to *B. calyciflorus*, which, as mentioned before, contained a non-conservative mutation. This could indicate that this species has a significantly different protein modulation which consequently could make the RXR unresponsive to TBT molecules. Unfortunately, no LC₅₀ value for 96h was found for this species.

The conservation of this specific binding pocket is most likely related to the fact that it is the same binding site as the most abundant RXR natural ligand, the 9-cis stereoisomers, which in fact is a site conserved across all three known RXR isoforms (Egea *et al.*, 2000).

Protein superposition

In this last section the obtained models were compared among them, focusing on *H. sapiens*, *D. magna* and our study species *T. longicaudatus*. The *D. magna* protein was selected as it served as a good intermediate species since it is the species more closely related to *T. longicaudatus* and showed no variation when compared to the interaction AAs present in *H. sapiens*. The superposition performed on PyMOL revealed that although the sequences contained variations the conformation remained mostly unchanged, with the most obvious changes spotted near the hinge of the protein which is more susceptible to mutations. Even so, our analysis of the ligand-binding domain showed that even small AAs changes could lead to a different conformation of this section which in turn could lead to different affinity levels

shown both by the position of the interaction AA and the number change, indicative of addition or removal of AAs elsewhere. The substitution present in the *T. longicaudatus* sequence trades the Leucine for a Phenylalanine (**Table 4.2**). These two AAs have a low value of 22 in Grantham's distance matrix, indicating that although different, the chemical properties are similar, which in turn should not significantly impact its affinity level.

Since no evident clusters formed in the SSD analysis, it becomes apparent that just the protein structure is not sufficient to infer the sensitivity of the organisms as a whole. The calculated 96h-LC₅₀ value for *T. longicaudatus* was 0.072 [0.046-0.139] µg/L which is a value that positions our species in the sensitive part of the spectrum. Clustering of organisms in the same taxonomic group or with the same interaction AAs for the TBT molecule would be expected. Unfortunately, this is not the case. This could possibly be a consequence of the different sizes of the organisms used in the independent mortality tests and the differences in methodology and quantification adapted by the different research groups. From here, it is possible to say that although RXR-α is one of the main targets for TBT, endocrine disruption alone is probably not responsible for the high mortality consequent of its contamination. Otherwise, organisms with a high level of affinity would be on the sensitive part of the spectrum, and the SSD curve does not support that claim. Species belonging to the Mollusca phylum, for example, retained all the interaction AAs in the sequence but in spite of that *E. complanata* was the most resistant species while other species such as *Perna viridis* and *Lymnaea stagnalis* were placed in the intermediate part of the spectrum. This is also suggestive that endocrine disruption operates mainly as a sub-lethal effect, so mortality might be achieved by impairment of other processes or by impacting several, leading to a cascade of events that ultimately results in the death of the organism. In Chapter II, it became evident that one of the most impacted set of genes was directly related to the ecdysone pathway, one of the most over-expressed genes, reaching a Log2FC value of 9.826, which was the highest observed in the experiment. Since these genes can be linked to the ecdysone pathway and thereby to the RXR/EcR heterodimer, it is safe to assume that the activation of this transcription factor is one of the main consequences of TBT contamination. This, in turn, could severely affect the organisms as growth and development get impaired.

4.5. Conclusion

This study successfully approached the molecular properties of the RXR-α protein from several species belonging to the metazoan. The sequence comparison allowed us to find key differences across species that may dictate the sensitivity to TBT molecules. The analysis also allowed us to conclude that the function of the RXR protein is of high value due to its high

conservation across distinct metazoan phyla. A correlation between the three-dimensional conformation and the LC_{50} values obtained throughout the literature was attempted. This proved to be difficult, which led to the conclusion that the main target for endocrine disruption probably translates into a sublethal effect, while other impaired processes could lead more directly to the expected mortality. More analysis remains to be done particularly, calculating the protein's affinity for TBT molecules which would help determine the sensitivity more accurately.

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5. Chapter V: Discussion and conclusion

This progressive work provided novel information on the invisible effects of biocides, in this case, Tributyltin (TBT). When dealing with pollutants, sub-lethal effects are of utmost importance as they could lead to the death of the organism as an indirect consequence of the chemical exposure or due to cumulative negative effects. This in turn, could put in risk the survival of the population and, in more severe scenarios, the survival of the species.

Our results shed light on the several biological processes impacted by TBT and the changes in the transcription pattern as a response taken by the organisms to cope with the contamination. It is safe to assume that TBT negatively impacted *Triops longicaudatus*, promoting cytotoxicity, endocrine disruption, neurotoxicity and even DNA damage. The majority of the cases had concentration dependant responses and were corroborated by other studies performed on other species (e.g. the crustacean *Daphnia magna*). Nonetheless, other biological processes may also be impacted. Still due to the lack of a genome and the low level of annotation of some transcripts their function remains elusive. In this study is presented a comprehensive linkage between lower organisational levels (gene transcription) and an individual level (behaviour), nonetheless, the inclusion of other endpoints such as the quantification of energetic budgets would greatly improve the comprehension of the data. For example, lipid and sugars content could be usefully implemented as a complement to our analysis as literature already shows an impact in these pathways.

Part of our study is dedicated to behavioural changes resulting from TBT providing exciting and clear evidence of its negative impact. From the array of behaviour types that were defined, organisms under the control group showed a clear and separate behaviour than the treatments with TBT, showing that even the lowest concentration led to deviant behaviour and consequently a future impact at the populational level. Several of the observed behavioural changes can be linked with the impact of TBT in the nervous system, energy allocation and even at a hormonal level (transcriptomic data – Chapter II), as motor discoordination and spatial unawareness were the most probable drivers to the observed erratic behaviour. This work also provided proof of the usefulness of *T. longicaudatus* in behavioural analysis improving its potential as a model- organisms in ecotoxicological assays.

The bioinformatic analysis focused on the main target of TBT's endocrine disruption, the retinoid X receptors (RXR), and its activation both as homodimer and heterodimer with ecdysteroid receptor. The phylogenetic analysis revealed a great level of conservation across several invertebrate species, which indicates that the binding site is well conserved, and the

proteins should have a similar affinity to this contaminant. Still, the SSD curve analysis using 96h-LC₅₀ values revealed that sensitivity to TBT hugely differ even among species from the same taxonomic group, being extremely difficult to establish any relationship between the RXR amino acid sequence, the size of the organisms or even their taxonomic distribution. The main conclusion obtained from the collected data, is that the magnitude of the influence of endocrine disruption as a consequence of TBT exposure may vary depending on the species. This conclusion is supported in the initial part of our study related to gene expression, where several genes from different pathways were pinpointed for being highly up- or down-regulated.

In order to better understand and integrated all of the previous results, **Figure 5.1** was built to elucidate the impact of TBT in pathways and the consequences at higher organisational levels, based on the work of Lagadic *et al.*, (2017), which was based primarily in molluscs. Here is possible to observe that TBT impacts several pathways associated with endocrine disruption, genotoxicity, cytotoxicity and neurotoxicity through direct and indirect connections. Some pathways contribute to the impairment of more than one biological process, further elucidating the complexity of TBT's mode of action.

In conclusion, this work provided information on a pollutant extensively used in the past, but due to its high persistence in the environment, still impacts aquatic organisms and endangers ecosystems. This work also highlighted the potential of *T. longicaudatus* as a model organism both in behavioural assays as well as in gene expression. The bioinformatics project ended up being a good alternative to transactivation assays as similar conclusions were possible to achieve with bioinformatic tools this way saving both time and resources. This dissertation is the first step for developing future work under different conditions and with different endpoints, such as tissue-specific sequencing, enzymatic biomarkers or energy reserves quantification to understand the full extension of the consequences of this biocide.

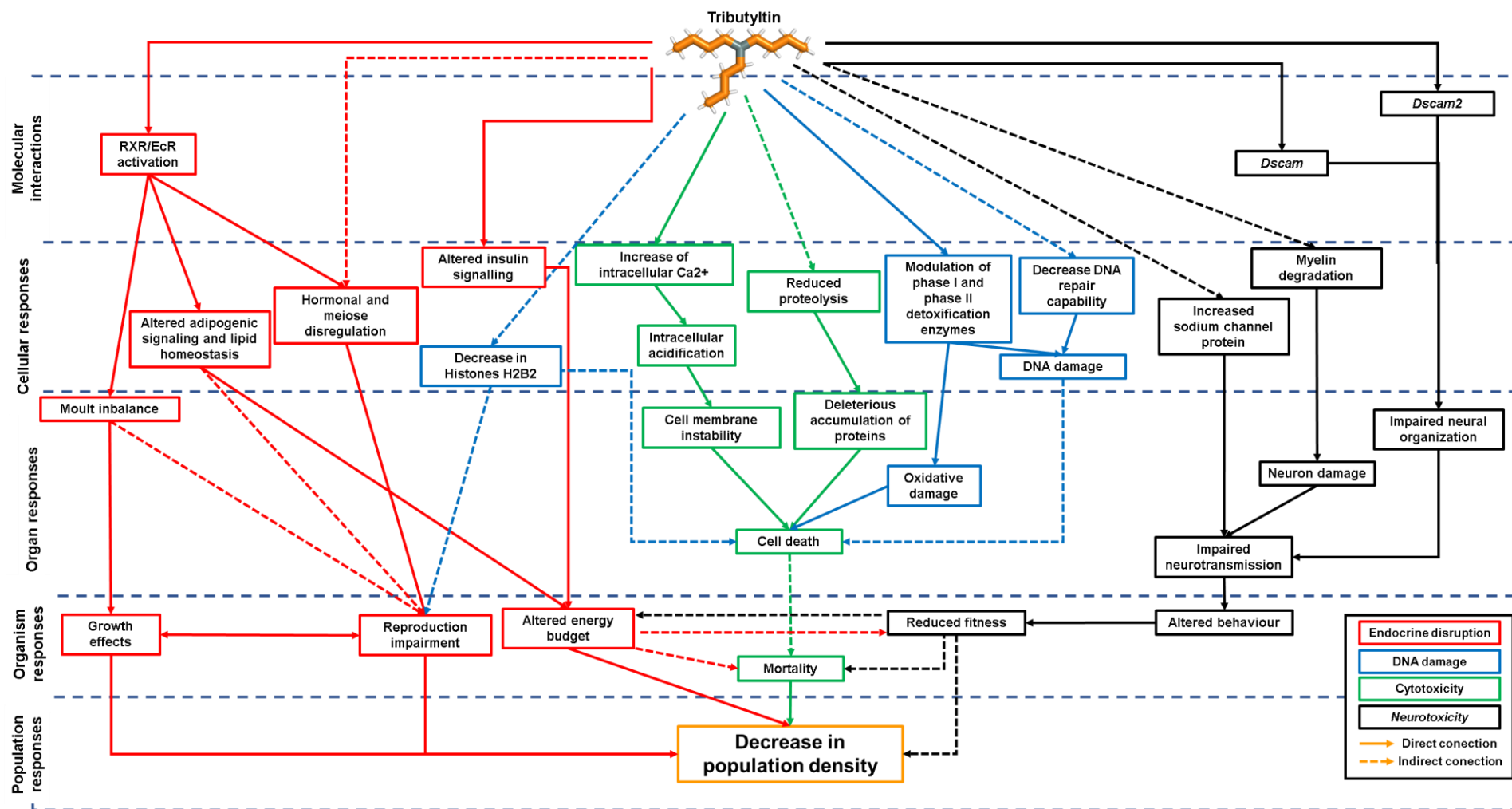


Figure 5.1- Theoretical adverse outcome pathway for TBT toxicity based on the obtained results.

5.1. References

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6. Supplementary data (SD)

SD 1- *Triops longicaudatus* rearing protocol

Day 0 – Hatching phase

To hatch test organisms, approximately 12.5 g of sand containing cysts are homogeneously placed into a plastic vessel (with 8 cm diameter) with 250mL of dechlorinated tap water (DTW). The sand should be wet with a small amount of water before filling the whole pot, this will reduce the amount of air pockets in the sand, ensuring that every cyst has been hydrated. The vessels are maintained at $25.0 \pm 1.0^{\circ}\text{C}$ with a photoperiod of 12h:12h (light:dark), the process should be started early in the morning to maximise the amount of light the eggs receive.

Day 1-2

At the first day the hatchlings will be too small to be spotted with bare eye and since their feeding behaviour is mainly of filtering nature a specific procedure is necessary. The necessary diet for each pot consists of 25 mg of grounded granulate of algae mix dissolved in 10mL of DTW and should be gently distributed through the pot. No need to change the water at this point since the disturbance may affect the hatchlings.

Day 3

On the third day, although the feeding process remains the same, approximately 50% of the medium (DTW) should be renewed to replenish oxygen levels and remove metabolite by-products such as ammonia. If an Alpha has already established himself in the pot, consider adding 1-3 food pellets to avoid cannibalism.

Day 4

On the fourth day, since the organisms feeding behaviour changes from filtering to detritus the feeding procedure should change as well. The algae food should be reduced to 50%, for the animals with a slower development, and 1 pellet per animal of fish granulate (Tropical Vitality & Color Granulat). The DTW should be renewed as much as possible, using a thin mesh to avoid the loss of animals, without putting the well-being of the animals at risk.

Day 5-7

On the fifth day no more algae food is required. The number of given pellets should increase by one per animal each day until the 10th day (adulthood): 2 pellets on the 5th day, 3 pellets on the 6th day, etc. Water should be changed daily to avoid mortality.

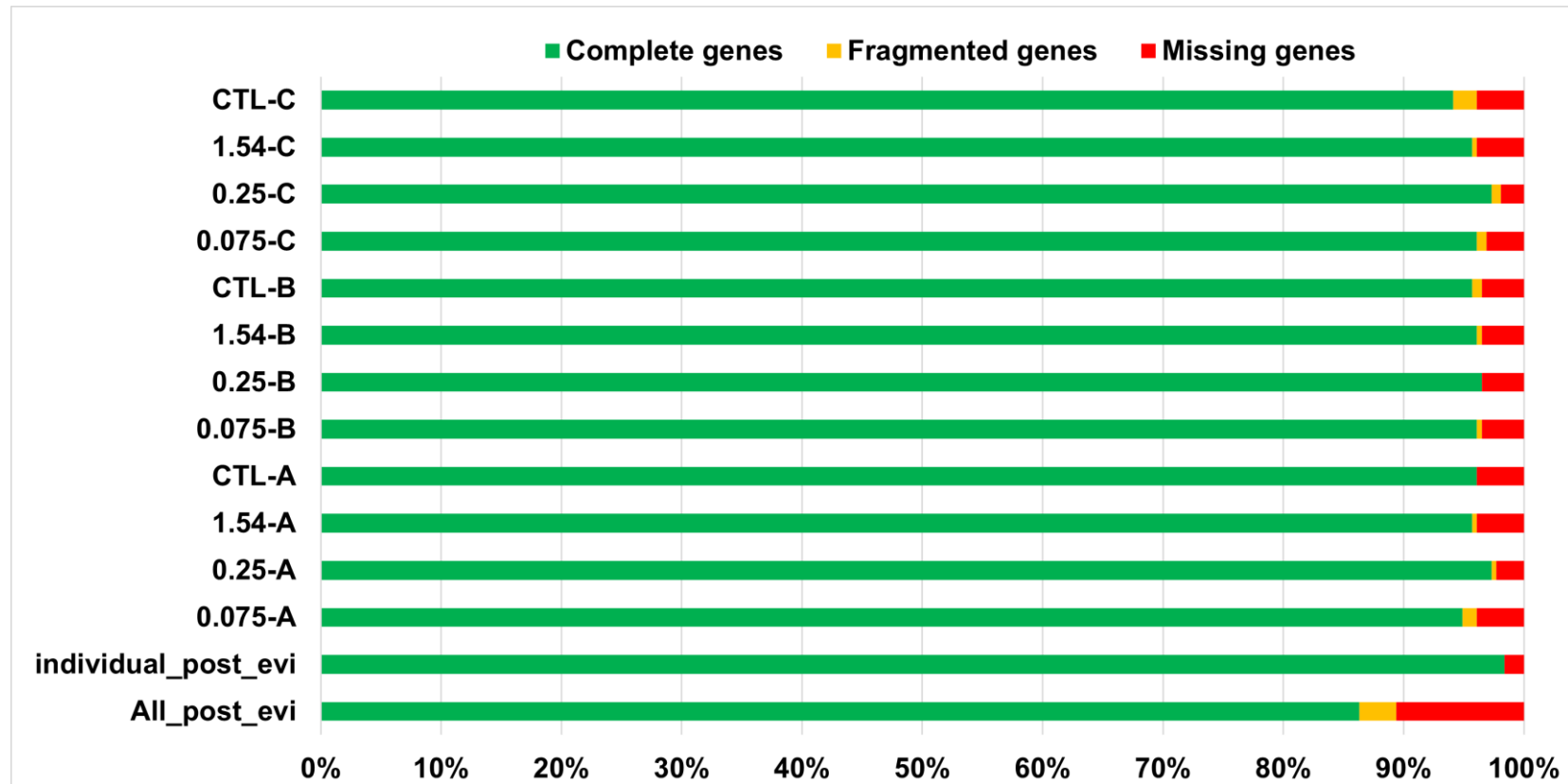
Adulthood

From day 10, organisms are moved to full sized aquariums with air enrichment with attention not to mix different sized organisms to avoid cannibalism. Avoid adding smaller animals to the main culture, as they should be kept in the pots for a few more days to give them the opportunity to grow. This allows for an increase in growth if the population density is not too high.

The organisms are fed *ad libitum* in order to avoid predation.

SD 2- *Triops longicaudatus* assembly with Trinity v2.6.6 before (pre) and after (post) Evigene analysis. Contig length are given in bp. All nomenclature refers to all the samples assembled as one and Ind nomenclature refers to compilation of previously assembled samples.

Treatment (µg/ L)	Filename	Max contig length	Number of contigs >200	Total bases in contigs >200	N50 for contigs >200	Contigs >200 in N50	GC contigs >200	nonATGC in contigs >200	Mean length for contigs >200
0.075	T3_pre_evi	19292	51821	106120551	3635	9636	44.7	0	2047.8
	T3_post_evi	12453	14375	17556999	1890	2750	47.7	0	1221.4
0.25	T4_pre_evi	19123	58415	133057133	4091	10625	44.5	0	2277.8
	T4_post_evi	14739	14950	18141522	1908	2771	47.6	0	1213.5
1.54	T5_pre_evi	21717	58139	130609355	4084	10450	44.5	0	2246.5
	T5_post_evi	15027	14940	18036639	1899	2761	47.6	0	1207.3
CTR	T6_pre_evi	21117	56600	123408496	3961	10268	44.6	0	2180.4
	T6_post_evi	15027	14817	18024813	1905	2757	47.7	0	1216.5
0.075	T8_pre_evi	20170	57434	126015167	3995	10264	44.6	0	2194.1
	T8_post_evi	15027	14914	18112665	1920	2734	47.7	0	1214.5
0.25	T11_pre_evi	20007	56925	124693964	3944	10383	44.6	0	2190.5
	T11_post_evi	14739	14841	18009291	1893	2761	47.7	0	1213.5
1.54	T12_pre_evi	18894	50863	107887220	3751	9642	44.6	0	2121.1
	T12_post_evi	14361	14179	17493654	1902	2715	47.7	0	1233.8
CTR	T13_pre_evi	21163	59763	138143053	4256	10619	44.4	0	2311.5
	T13_post_evi	11127	15242	18294816	1911	2774	47.6	0	1200.3
0.25	T16_pre_evi	21846	63303	144842563	4266	11153	44.4	0	2288.1
	T16_post_evi	14739	15293	18269562	1893	2790	47.6	0	1194.6
CTR	T18_pre_evi	16576	50834	103233248	3626	9535	44.6	0	2030.8
	T18_post_evi	11112	14231	17182647	1872	2786	47.7	0	1207.4
0.075	T20_pre_evi	15769	53792	115135280	3893	9940	44.5	0	2140.4
	T20_post_evi	10773	14604	17670897	1881	2793	47.7	0	1210
1.54	T22_pre_evi	17898	55604	120408082	3887	10243	44.5	0	2165.5
	T22_post_evi	10110	14670	17706132	1884	2785	47.6	0	1207
	All_pre_evi	35984	101272	338517247	7103	15135	43.6	0	3342.7
	All_post_evi	26253	20716	20462157	1788	3191	47.4	0	987.7
	Ind_post_evi	15027	28411	22800804	1629	3807	46.7	0	802.5



SD 3- Busco assessment results for 12 different samples. Individual refers to the individual assemblies merged only for the Evigene analysis and All refers to All the samples assembled as one. Colours refer to percentage of complete single-copy genes (green), fragmented genes (yellow) and missing genes (red).

SD 4- Patristic distances matrix of RXR sequences using the Jukes-Cantor building model. The table includes 26 species and approximately 500 AA's for each sequence obtained from the GenBank database.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. <i>Homo sapiens</i>																		
2. <i>Nucella lapillus</i>	0.409																	
3. <i>Biomphalaria glabrata</i>	0.393	0.231																
4. <i>Lymnaea stagnalis</i>	0.388	0.226	0.05															
5. <i>Elliptio complanata</i>	0.387	0.263	0.247	0.242														
6. <i>Crassostrea gigas</i>	0.383	0.258	0.243	0.238	0.189													
7. <i>Crassostrea virginica</i>	0.379	0.254	0.239	0.234	0.185	0.021												
8. <i>Mytilus edulis</i>	0.364	0.239	0.224	0.219	0.196	0.192	0.188											
9. <i>Perna viridis</i>	0.298	0.174	0.159	0.153	0.131	0.127	0.123	0.065										
10. <i>Ciona intestinalis</i>	0.585	0.577	0.562	0.557	0.556	0.551	0.547	0.532	0.467									
11. <i>Crangon crangon</i>	0.44	0.432	0.417	0.412	0.411	0.406	0.402	0.387	0.322	0.561								
12. <i>Carcinus maenas</i>	0.449	0.442	0.426	0.421	0.42	0.416	0.412	0.397	0.331	0.57	0.152							
13. <i>Leptuca pugilator</i>	0.488	0.481	0.466	0.46	0.459	0.455	0.451	0.436	0.37	0.61	0.191	0.071						
14. <i>Tigriopus japonicus</i>	0.603	0.596	0.581	0.575	0.574	0.57	0.566	0.551	0.485	0.724	0.525	0.534	0.573					
15. <i>Daphnia magna</i>	0.439	0.432	0.416	0.411	0.41	0.406	0.402	0.387	0.321	0.56	0.361	0.37	0.409	0.49				
16. <i>Triops longicaudatus</i>	0.424	0.417	0.401	0.396	0.395	0.391	0.387	0.372	0.306	0.545	0.346	0.355	0.394	0.475	0.219			
17. <i>Amphibalanus amphitrite</i>	0.623	0.615	0.6	0.595	0.594	0.589	0.585	0.57	0.505	0.744	0.544	0.553	0.593	0.673	0.509	0.494		
18. <i>Brachionus calyciflorus</i>	1.046	1.038	1.023	1.018	1.017	1.012	1.008	0.994	0.928	1.167	0.967	0.977	1.016	1.097	0.932	0.917	1.053	